ORIGINAL ARTICLE

Dynamics of microbial community composition and soil organic carbon mineralization in soil following addition of pyrogenic and fresh organic matter

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Pyrogenic organic matter (PyOM) additions to soils can have large impacts on soil organic carbon (SOC) cycling. As the soil microbial community drives SOC fluxes, understanding how PyOM additions affect soil microbes is essential to understanding how PyOM affects SOC. We studied SOC dynamics and surveyed soil bacterial communities after OM additions in a field experiment. We produced and mixed in either 350 °C corn stover PyOM or an equivalent initial amount of dried corn stover to a Typic Fragiudept soil. Stover increased SOC-derived and total CO₂ fluxes (up to 6x), and caused rapid and persistent changes in bacterial community composition over 82 days. In contrast, PyOM only temporarily increased total soil CO₂ fluxes (up to 2x) and caused fewer changes in bacterial community composition. Of the operational taxonomic units (OTUs) that increased in response to PyOM additions, 70% also responded to stover additions. These OTUs likely thrive on easily mineralizable carbon (C) that is found both in stover and, to a lesser extent, in PyOM. In contrast, we also identified unique PyOM responders, which may respond to substrates such as polyaromatic C. In particular, members of *Gemmatimonadetes* tended to increase in relative abundance in response to PyOM but not to fresh organic matter. We identify taxa to target for future investigations of the mechanistic underpinnings of ecological phenomena associated with PyOM additions to soil.

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Introduction

Large inputs of pyrogenic organic matter (PyOM) in fire-affected ecosystems can constitute up to 80% of total soil organic carbon (SOC) (Lehmann *et al.*, 2008). Whether PyOM is produced naturally in fires (Czimczik and Masiello, 2007), intentionally for carbon (C) management and/or as an agricultural amendment (Lehmann, 2007; Laird, 2008), it is important to understand how it affects the C cycle (Whitman *et al.*, 2010). PyOM additions to soil can significantly affect plant growth and crop yields (Jeffery *et al.*, 2011) and SOC dynamics (Maestrini *et al.*, 2014; Watzinger *et al.*, 2014; Whitman *et al.*, 2015). For example, among other effects, PyOM can change soil pH (Gul *et al.*, 2015), add nutrients to soils (Enders *et al.*, 2012) and alter soil water-holding capacity (Abel *et al.*, 2013). Although PyOM

additions to soils can alter inorganic C dynamics through changes to soil pH, a key mechanism for changes in SOC mineralization is altered microbial mineralization of SOC in response to PyOM (Maestrini *et al.*, 2014; Whitman *et al.*, 2015). Hence, an understanding of the effects of PyOM on the soil microbial community composition and function is required to understand the effects of PyOM on SOC stocks.

There are many ways PyOM could affect soil microorganisms (Kuzakov and Bol, 2004; Lehmann *et al.*, 2011; Ameloot *et al.*, 2013), including, but not limited to, microbial use of PyOM as a source of energy or nutrients, changes in soil physical or chemical characteristics, impacts on plant growth and interference with microbial signaling (Masiello *et al.*, 2013). Recent research has only begun to identify PyOM effects on soil microbial communities, and it is clear that PyOM additions to soil can induce changes in soil microbial community composition. Most current evidence has been gathered using fingerprinting approaches, such as terminal restriction fragment length polymorphism (Bingeman *et al.*, 1953;

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Jin, 2010; Kolton *et al.*, 2011) or denaturing gradient gel electrophoresis (Kolton *et al.*, 2011; Chen *et al.*, 2013), or by surveying phospholipid fatty acids to assess microbial diversity at low phylogenetic resolution (Dunavin, 1969; Jindo *et al.*, 2012; Gomez *et al.*, 2014; Watzinger *et al.*, 2014; Mitchell *et al.*, 2015). In addition, clone libraries targeting functional genes (Song *et al.*, 2014) and some high-throughput DNA sequencing approaches have been applied to survey microorganisms in PyOM systems. For example, significant differences were found between soil bacterial communities in Amazonian Dark Earth soils (amended with PyOM thousands of years ago), PyOM isolated from the Amazonian Dark Earth soils and adjacent unamended Acrisols, by surveying small subunit ribosomal RNA (SSU rRNA) genes (Taketani *et al.*, 2013). A handful of other studies have used high-throughput sequencing approaches to characterize PyOM effects on soil bacterial community composition (for example, Nielsen *et al.*, 2014; Xu *et al.*, 2014). However, owing to the low number of studies and the wide diversity of PyOM materials, addition rates, soils and environmental conditions, it is difficult to draw generalizable conclusions about the effects of PyOM on soil microbial community composition, particularly at the level of individual taxa.

In this study, we investigated the effects of PyOM additions on SOC mineralization and soil bacterial community composition in a field setting over 12 weeks. We also included a treatment where plots received a mass of fresh corn stover equivalent to the mass required to produce the PyOM we added. This treatment serves as a system-level control that addresses the question, 'What if a given amount of biomass were not used to produce PyOM, but were applied directly to the soil?' We predicted that there would be significant differences in C dynamics between the two systems, with fresh biomass decomposing faster and having a greater effect on SOC mineralization. In addition, we predicted that the addition of fresh biomass would induce the greatest changes in the bacterial community composition, with organisms that access easily mineralizable C sources proliferating initially, and the organisms that are able to decompose aromatic or insoluble substrates, such as lignin or cellulose, emerging later. We expected that the majority of the microorganisms that increase in relative abundance in response to PyOM additions will also increase in response to fresh organic matter additions, but they will respond to PyOM to a smaller degree, as a smaller fraction of the PyOM-C is easily mineralizable.

Materials and methods

Experimental design

We conducted a field trial, with soil left unamended, soil amended with ^{13}C -labeled 350 °C corn stover (*Zea mays* (L.)) PyOM or soil amended with fresh

corn stover (Supplementary Tables 1 and 2). The ^{13}C label allowed for the C sources to be partitioned between SOC and PyOM-C or corn stover-derived C (Whitman and Lehmann, 2015). The corn stover addition was designed so that the dried original corn biomass was equivalent to that which would have been required to produce the mass of corn PyOM that was applied to each plot (that is, 4.1 Mg ha^{-1} corn-derived PyOM were applied, which, with 0.365 mass fraction conserved during PyOM production, translates into 11.2 Mg ha^{-1} corn stover, which is representative of a productive corn crop in the United States (Shinners and Binversie, 2007)).

The field site is located in Cornell's research fields in Mt Pleasant, NY, USA, and is a Mardin soil (coarse-loamy, mixed, active, mesic Typic Fragiudept) (Supplementary Table 2). The soil has been historically planted to a potato, rye, clover rotation, for the past >30 years, but was kept in rye-clover rotation for the past 5 years, with one planting of sudangrass 3 years ago. The plot was sprayed with Roundup (glyphosate) herbicide in the fall of 2012 (1.8 l ha^{-1}), plowed on 3 May 2013, and kept weed-free by hand-weeding and water-permeable landscape fabric through the summer until trial initiation.

The trial initiation date was 16 August 2013 (day 0). Square plots ($0.7 \text{ m} \times 0.7 \text{ m}$ for the soil-only and PyOM, $0.45 \text{ m} \times 0.45 \text{ m}$ for the stover) were surrounded by 0.7-m wide weed-free borders, maintained by hand weeding. Treatments were organized using a spatially balanced complete block design with 16 replicates of the unamended and PyOM plots, and 8 replicates of the stover plots (van Es *et al.*, 2007). Soil (6.1 kg or $\sim 1\text{--}2 \text{ cm}$) was removed from the surface of each plot, combined with stover or PyOM additions, if needed, and mixed in a V-mixer (Twin shell dry blender, Patterson-Kelley, East Stroudsburg, PA, USA). Amended plots received 4.1 Mg ha^{-1} of PyOM or 11.2 Mg ha^{-1} of dried original corn stover. After mixing, soils were returned to their respective plots and evenly spread at the surface. Soil was gently tamped down using a flat piece of plywood. Two soil respiration collars made from 194 mm diameter white polyvinylchloride pipes were installed directly adjacent to each other at the center of each plot with the collar protruding 30 mm and reaching 30 mm into the ground. Plots, including soil collars, were covered with water-permeable landscape fabric except during measurement for the first 2 weeks, after which fabric was removed and plots were kept weed-free by hand-weeding multiple times a week. The plots were not fertilized or watered during the trial, but were exposed to natural rainfall (temperature and precipitation are plotted in Supplementary Figure 1).

Biomass production

Two sets of corn plants (*Zea mays* (L.)) were grown, one in an enriched $^{13}\text{CO}_2$ atmosphere growth

chamber and the other in an ambient $^{13}\text{CO}_2$ greenhouse. The labeled plants were grown in potting mix in a Percival AR-100L3 CO_2 -controlled growth chamber (Percival, Perry, IA, USA). The plants were exposed to cycles of 18-h light/6-h darkness. During light cycles, the atmosphere was maintained at 400 p.p.m. CO_2 . During the dark cycle, CO_2 was allowed to accumulate through respiration, and was then drawn down by photosynthesis during the next light cycle. This was done in order to reduce net respiratory losses of labeled $^{13}\text{CO}_2$. Plants were pulse labeled with 13 l of 99% $^{13}\text{CO}_2$ at regular intervals over the course of their growth in order to produce an even label. Pulse labels were delivered by opening the $^{13}\text{CO}_2$ cylinder to fill a balloon with ~500 ml $^{13}\text{CO}_2$. The balloon remained attached to the cylinder so that the $^{13}\text{CO}_2$ slowly diffused out of the balloon, delivering the pulse at a rate so that the total atmospheric concentration of CO_2 was not affected. Plants in the growth chambers and the greenhouse were harvested just before they reached reproductive maturity and were oven-dried at 70 °C.

PyOM production and amendment mixing

Oven-dried corn plants were ground in a hammer mill (Viking Manufacturing Company, Manhattan, KS, USA) to <2 mm. The milled corn was pyrolyzed in a modified Fisher Scientific Isotemp programmable muffle furnace (Thermo Fisher Scientific, Waltham, MA, USA) (described in detail in Guereña *et al.* 2015) by ramping at 5 °C min⁻¹ to 350 °C, then holding at 350 °C for 45 min, under Ar (Supplementary Table 1). The ^{13}C -labeled and natural abundance corn PyOM materials were mixed together to produce a $\delta^{13}\text{C}$ value of +37.5‰. For the corn biomass-only plots, mixtures of ^{13}C -labeled and natural abundance corn with a $\delta^{13}\text{C}$ value of +1.7‰ were created. Mixing was done in plot-level batches to ensure that each plot received exactly these proportions of labeled and unlabeled materials.

CO_2 flux and $^{13}\text{CO}_2$ measurements

Soil CO_2 flux was measured using a LI-6400XT infrared gas analyzer with a 6400-09 soil CO_2 flux chamber attachment (LI-COR, Lincoln, NE, USA). Three flux measurements were taken in succession for each plot and averaged. Measurements were taken on days 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 14, 16, 18, 26, 30, 34, 38, 41, 45, 49, 53, 57, 62, 66, 74 and 81. Additional gas samples were taken for $^{13}\text{CO}_2$ analysis and emissions partitioning on day 12 and on day 66 using modified static Iso-FD chambers (Nickerson *et al.*, 2013; Whitman and Lehmann, 2015) (Supplementary Figure 2).

Soil sampling

Soil samples for bacterial community analyses were taken on days 1, 12 (chosen because it was after the

first major rainfall; Supplementary Figure 1) and 82. Samples were sieved to <2 mm, and immediately frozen in Whirl-Paks in liquid N_2 and stored at -80 °C. On days 1 and 12, two 25-mm deep soil probe samples were pooled, while on day 82, soil within the entire collar was destructively sampled.

Isotopic partitioning of CO_2 samples

We partitioned the total CO_2 fluxes between SOC and stover or PyOM-C on days 12 and 66. To determine the relative contributions of SOC and added C (either PyOM or corn stover) to soil CO_2 fluxes, a standard isotope partitioning approach was applied (Balesdent and Mariotti, 1996). For example, for the plots with PyOM additions, the isotopic signature of the total emissions will be:

$$\delta_T = f_S * \delta_S + f_{\text{PyOM}} * \delta_{\text{PyOM}}, \quad (1)$$

where δ represents the ^{13}C signature of CO_2 from total CO_2 (δ_T), soil alone (δ_S), or PyOM (δ_{PyOM}) and f_S and f_{PyOM} represent the fraction of total emissions made up by soil and PyOM, respectively (Werth and Kuzyakov, 2010). As δ_T , δ_S , and δ_{PyOM} were all measured, and we know

$$f_S + f_{\text{PyOM}} = 1, \quad (2)$$

we can solve this system of two equations for the two unknown values, f_S and f_{PyOM} .

Data processing and statistical analyses for CO_2 fluxes

On rare occasions toward the end of the trial, we recorded negative CO_2 fluxes, which we interpret as experimental error because of low flux rates, and have excluded these values from analyses. In addition, we excluded two data points where recorded fluxes were 56 and 16 s.d. away from the mean of the remaining plots. All statistical analyses were performed in R (R Core Team, 2015). CO_2 fluxes were evaluated using a linear mixed effects model, with amendment, day, interaction between amendment and day, and plot ID (a repeated-measures approach) as factors, using the R package 'lme4' (Bates *et al.*, 2015). To make *post-hoc* comparisons, we performed pairwise comparisons between the different soil amendments for a given day with a Tukey's adjustment of *P*-values, using the 'lsmeans' R package (Lenth, 2014).

Bacterial community analyses

DNA was extracted from 0.25 g moist soil samples using the MoBio PowerLyzer PowerSoil kit (Carlsbad, CA, USA), following the kit's directions. The DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Madison, WI, USA) with a multimode microplate reader (Molecular Devices, Sunnyvale, CA, USA). DNA yields were normalized on the basis of grams of dry soil extracted. SSU rRNA genes were PCR amplified

in triplicate for each sample. PCR was conducted with 12.5 µl Q5 Hot Start High Fidelity 2X mastermix (New England Biolabs, Ipswich, MA, USA), 5 µl of DNA template diluted with water at a ratio of 1:50, 5 µl water and 2.5 µl primer mixtures to a total volume of 25 µl. Each PCR consisted of a 98 °C hold for 30 s, followed by 30 cycles of (5 s at 98 °C, 20 s at 20 °C and 10 s at 72 °C), with a final extension for 2 min at 72 °C. Modified 515F and 907R primers (Supplementary Tables 3 and 4) were used to target the V4/V5 regions of the 16S ribosomal RNA gene. Unique barcodes were added to the primers used for each sample so that the SSU rRNA sequences from each sample could be demultiplexed post sequencing (Supplementary Tables 3 and 4). Each PCR product was run on a 0.5% agarose gel, along with the negative control, to determine whether amplification was successful. Replicate PCR products were pooled, and DNA concentrations were normalized across all samples using SequelPrep normalization plates (Applied Biosystems, Foster City, CA, USA). The pooled sample was purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA). This sample was submitted with sequencing primers (Supplementary Table 5) for paired ends 2 × 300-bp sequencing on the Illumina MiSeq v3 platform (Illumina, Inc., San Diego, CA, USA) at Cornell's Biotech Core Facility.

Bacterial community bioinformatics

Paired read merging, demultiplexing and quality control. We merged the forward and reverse reads using the paired end read merger (Zhang *et al.*, 2014) and demultiplexed them by barcode. DNA sequences were managed using screed databases (Nolley and Brown, 2012).

We removed merged reads with more than one expected error using USEARCH (Edgar, 2013). We also removed any sequences that had ambiguous base calls. We further identified erroneous sequences with alignment based quality control (Schloss *et al.*, 2009), by aligning our sequences to the SILVA Reference Alignment as provided by the Mothur developers and removing reads that did not align to the expected region of the SSU rRNA gene. We then removed any sequences that were < 370-bp or > 376-bp long or had homopolymers (runs of the same base in a row) > 8 nucleotides in length. Quality control removed 6 265 604 reads, leaving 10 237 689 high-quality sequences. With 3 treatments, 2 with 16 replicates and 1 with 8 replicates, and 3 time-points, we had a total of 120 samples. Individual samples contained from 8830 to 194 356 total sequences, with a mean of $69\,419 \pm 44\,070$.

Operational taxonomic unit (OTU) picking. Reads were clustered into OTUs using the UPARSE methodology (Edgar, 2013) with an OTU sequence identity cutoff of 97%. In the UPARSE workflow,

chimeras are detected and discarded when OTU centroids are selected. Of the quality-controlled reads, 81% could be mapped to OTU centroids. We taxonomically annotated OTU centroids using the 'uclust' based taxonomic annotation framework in QIIME (v1.8) (Caporaso *et al.*, 2010) with default parameters (Caporaso *et al.*, 2010; Edgar, 2013). OTUs annotated as 'Archaea', 'mitochondria' or 'Eukarya' were removed in downstream analyses. One sample (an unamended plot from day 82) was left with only eight sequences, so it was excluded from further analyses.

Community analysis. OTU centroids were taxonomically annotated within the Greengenes taxonomic nomenclature using the 'uclust' taxonomic annotation framework in QIIME with default parameters (Caporaso *et al.*, 2010). Centroids from 97% sequence identity clusters of Greengenes database SSU rRNA gene sequences (version 13_8) and corresponding annotations were used as reference for taxonomic annotation. We used the R package 'vegan' (Oksanen *et al.*, 2015) to perform a nonmetric multidimensional scaling analysis on the weighted UniFrac distance between communities across all time points and amendments (Lozupone *et al.*, 2011). Weighted UniFrac accounts for the relative abundance of each OTU, not just considering its presence/absence. We then tested whether there were significant effects on the bacterial community because of amendment types and day of sampling with a nonparametric multivariate analysis of variance (NPMANOVA) on weighted UniFrac distances, using the 'adonis' function from the R package 'vegan' (Oksanen *et al.*, 2015). As we found day and amendment both had significant effects ($P < 0.001$), we performed a separate weighted UniFrac analysis and NPMANOVA for each day and amendment type (comparing each amendment with either each other or with no additions, for each day), adjusting P -values using a Bonferroni correction for six comparisons ($P_{\text{adj}} = P \times 6$).

We used the R package 'DESeq2' (Love *et al.*, 2014) to calculate the differential abundance ($\log_2$ -fold change in relative abundance of each OTU) for each amendment type as compared with the unamended plots for both sampling days (McMurdie and Holmes, 2014). We independently filtered out OTUs that were sparsely represented across samples (that is, those OTUs for which the DESeq2-normalized count across samples ('baseMean') was < 0.6). Sparse OTUs will not contain sufficient sequence counts to provide statistically significant results and their removal reduces the number of multiple comparisons performed, thereby mitigating problems associated with multiple comparisons with some extent. We adjusted the P -values with the Benjamini and Hochberg correction method and selected a study-wide false discovery rate of 10% to denote statistical significance (Love *et al.*, 2014). We defined 'responding OTUs' as OTUs with a differential abundance > 1

and an adjusted P -value of <0.1 . We performed a BLAST (nucleotide blast, version 2.2.29+, default parameters) search with OTU centroid sequences of responding OTUs, against the Living Tree Project database (version 115) (Yarza *et al.*, 2008). The Living Tree Project database contains 16S rRNA gene sequences for all sequenced archaeal and bacterial type strains.

It is important to note that the detection of bacterial DNA in the soil does not indicate that these microbes are active, let alone necessarily alive (Blazewicz *et al.*, 2013). In addition, although the detection of a significant increase in relative abundance against the diverse and abundant background soil community likely does indicate growth and cell replication, some organisms may increase their activity in response to OM inputs, but not grow or divide, thereby evading detection via this technique.

Results

Soil C dynamics

Total CO_2 fluxes from plots that received uncharred stover additions were significantly (mixed model repeated-measures design, Tukey-adjusted *post-hoc* comparisons, $P < 0.05$) higher than all other plots for the first 26 days and for 19 of the 27 days for which fluxes were determined (Figure 1). Stover additions had the greatest effects on CO_2 fluxes (CO_2 fluxes six times those of unamended soils) on days 7 and 11, after strong rain events (Supplementary Figure 1). Plots with PyOM additions experienced significantly

Figure 1 Mean CO_2 flux rates over time. Error bars ± 1 s.e., $n=8-16$. Dotted line indicates plots that received fresh stover additions. Dashed orange line indicates plots that received PyOM additions. Solid yellow line indicates plots that had no additions. *Indicates significant differences between plots with PyOM additions and stover or no-addition plots, whereas # indicates significant differences between plots that received fresh stover additions and PyOM or no-addition plots (mixed model repeated measures design, Tukey-adjusted *post-hoc* comparisons, $P < 0.05$). X indicates days where microbial community was sampled. • indicates days where $^{13}\text{CO}_2$ flux was partitioned between SOC and amendments. The color reproduction of this figure is available at the *The ISME Journal* online.

higher CO_2 fluxes than plots with no additions for the first 12 days, after which there were no significant differences (Figure 1). The increases in fluxes with PyOM additions were much less marked than those in the stover-amended plots, and were never >2.1 times the CO_2 emissions from unamended soils.

On day 12, ^{13}C partitioning revealed that corn stover additions significantly increased SOC-derived CO_2 fluxes as compared with soils with no additions, but PyOM additions did not (Supplementary Figure 3). On day 66, there were no significant differences in SOC-derived CO_2 fluxes between all soils, and overall fluxes were much lower on this date (Figure 1 and Supplementary Figure 3).

Bacterial community analyses

We did not detect significant differences in DNA yield because of the addition of PyOM or stover directly after amendment additions (day 1), but DNA yield was significantly higher in plots with stover additions on days 12 and 82 (Supplementary Table 6; analysis of variance and Tukey's honest significant difference, $P < 0.05$). There were significant changes in the bacterial community composition over time (NPMANOVA, $P < 0.006$, $R^2 = 0.19$) and with amendment type (NPMANOVA, $P < 0.006$, $R^2 = 0.20$) (Figure 2). Bacterial community composition in unamended plots remained relatively consistent over time (NPMANOVAs comparing day 1 with day 12 ($P < 0.06$, $R^2 = 0.18$) and with day 82 ($P < 0.29$, $R^2 = 0.11$)), whereas PyOM-amended plots varied slightly and corn stover-amended plots varied greatly over time (Figure 2). The different treatments were not observed to alter bacterial community composition on day 1 (only 24 h after OM additions). By day 12, the stover treatment caused a significant change in community composition (NPMANOVA, $P < 0.006$, $R^2 = 0.75$), although no effect of PyOM was observed. By day 82, the bacterial communities in the PyOM-amended plots (NPMANOVA, $P < 0.02$, $R^2 = 0.19$) and corn stover-amended plots (NPMANOVA, $P < 0.02$, $R^2 = 0.56$) were both distinct from the unamended plots and from each other (NPMANOVA, $P < 0.01$, $R^2 = 0.36$).

The stover treatment caused multiple phyla to change significantly in relative abundance as compared with control plots on days 12 and 82 (Figure 3). In contrast, PyOM additions only led to a significant change in the relative abundance of the phyla *Armatimonadetes* (decreased), and *Bacteroidetes* (increased) and only on day 82 (Figure 3). However, we caution that decreases in relative abundance do not necessarily correspond to decreases in absolute abundance—rather, they could result from an increase in the absolute abundance of other phyla. Hence, we transformed the relative abundance data from stover treatments by scaling by the mass of extracted DNA per soil sample, but found that the only changes were that declines in relative

Figure 2 Nonmetric multidimensional scaling (NMDS) ordination ($k=2$, stress = 0.09) of weighted UniFrac distances between bacterial communities, showing differences across amendments for a given day (top row) and across days for a given amendment (bottom row).

abundance for *Acidobacteria* and *Gemmatimonadetes* with stover additions on day 82 were no longer statistically significant (Supplementary Note 1).

Many OTUs, from several phyla, responded significantly to stover and/or PyOM additions (Figure 4; Supplementary Tables 7 and 8; Supplementary Figures 6–14 and 20). We use the term ‘responders’ to refer specifically to those OTUs that increase significantly in relative abundance by more than doubling in response to stover and/or PyOM additions as compared with corresponding plots that did not receive amendments. We identified 806 responders to either stover and/or PyOM from among the 7770 total OTUs observed across all soil samples. There were more responders to stover (677 OTUs) than to PyOM (264 OTUs) (Figures 2, 4 and 5). A total of 8% of all OTUs responded specifically to stover (Figure 5, blue region), 2% responded specifically to PyOM (Figure 5, pink region), and 2% responded to both PyOM and stover (Figure 5, purple region and Supplementary Figures 4 and 5). The total number of responders to both stover and PyOM increased over time and nearly all phyla had more OTUs respond at day 82 than day 12. The only notable exception is *Firmicutes*, which had more stover responders at day 12 (36 OTUs) than day 82 (11 OTUs), and which only had 1 OTU that responded to PyOM (Supplementary Figure 6).

Many OTUs from *Firmicutes*, *Proteobacteria* and *Bacteroidetes* responded to stover additions (Figures 4 and 5; Supplementary Figures 6). Among *Proteobacteria*, 125 OTUs responded to stover additions on day 12, increasing to 235 responders on day 82 (primarily OTUs from the orders *Rhizobiales*, *Burkholderiales*, *Sphingomonadales*, *Xanthomonadales* and *Pseudomonadales* on both days, and *Rhodospirillales* and *Myxococcales* on day 82) (Supplementary Figure 7). Among *Bacteroidetes*, 60 OTUs responded to stover additions on day 12, increasing to 128 on day 82 (primarily orders *Saprospirales*, *Cytophagales* and *Sphingobacteriales*, on both days and *Flavobacteriales* on day 12) (Supplementary Figure 8). In *Firmicutes*, 36 OTUs responded to stover on day 12, declining to 11 OTUs by day 82 (orders *Bacillales* and *Clostridiales* at both times) (Supplementary Figure 6).

Only 19 OTUs were observed to respond to both stover and PyOM at day 12, but this increased to 113 OTUs by day 82. The OTUs that responded to both stover and PyOM included *Proteobacteria* (66 OTUs; Supplementary Figure 7), *Bacteroidetes* (24 OTUs; Supplementary Figure 8) and *Verrucomicrobia* (11 OTUs; Supplementary Figure 9). In particular, a number of OTUs from *Verrucomicrobia* were observed to increase greatly in relative abundance in response to both stover and PyOM as compared

Figure 3 Relative abundance (unscaled by total microbial DNA) of top 12 phyla observed in the unamended, stover-amended and PyOM-amended soils ($n=8-16$). Values beyond 1.5 times the interquartile range are indicated (x), as are values that differ significantly from unamended (*, t -test, $P<0.05$, Bonferroni-corrected for 72 comparisons).

with untreated control plots (Figure 6 and Supplementary Figures 9 and 15).

Only 12 OTUs increased in relative abundance specifically in response to PyOM at day 12, but this increased to 138 OTUs by day 82. The OTUs that responded specifically to PyOM included *Proteobacteria* (47 OTUs; Supplementary Figure 7), *Bacteroidetes* (30 OTUs; Supplementary Figure 8), *Planctomycetes* (13 OTUs; Supplementary Figure 10), *Gemmatimonadetes* (12 OTUs; Supplementary Figure 11) and *Verrucomicrobia* (6 OTUs; Supplementary Figure 9). The strongest specific response to PyOM and not stover, by far, was observed for OTUs from *Gemmatimonadetes* (Figure 5). These included OTUs from the orders

Gemmatimonadales and ‘Ellin5290’ (Figure 6 and Supplementary Figure 16). Two of these *Gemmatimonadetes* OTUs were also among the top 10 most abundant PyOM responders. The most abundant PyOM responders also included two OTUs from *Bacteroidetes* (from the *Oxalobacteraceae*), and two OTUs from *Proteobacteria* (*Rhizobiales* and *Erythrobacteraceae*).

Discussion

Soil bacterial community dynamics

The soils that received corn stover amendments showed marked increases in CO_2 emissions almost

Figure 4 Log₂-fold change in relative abundance of OTUs as compared with unamended plots. Each circle represents a single OTU and dashed and dotted lines represent increases or decreases of 2x and 10x, respectively. Colors are scaled from yellow to red in decreasing *P*-value, with gray points indicating OTUs with Benjamini and Hochberg (BH)-adjusted *P*-values > 0.10. The color reproduction of this figure is available at the *The ISME Journal* online.

Figure 5 Log₂-fold change in relative abundance of OTUs in response to stover or PyOM as compared with unamended plots. Data are the same as those depicted in Figure 4. Note different scales on axes. Each point represents the response for a single OTU across replicates, colored by phylum. Dashed oval overlays indicate response groupings, where blue indicates strong stover responders, pink indicates strong PyOM responders and purple indicates common responders. Gray histograms at the sides of the plot indicate density of OTU points. The color reproduction of this figure is available at the *The ISME Journal* online.

Figure 6 Log₂-fold change of the relative abundance of OTUs in PyOM or stover plots vs soil-only plots on days 12 and 82, for *Firmicutes*, *Gemmatimonadetes* and *Verrucomicrobia* phyla. Each point represents the response for a single OTU across replicates, colored by order. Circles represent day 12 and triangles represent day 82. Square brackets indicate candidate order in Greengenes taxonomic nomenclature.

immediately (Figure 1), and bacterial community composition in those plots had changed significantly by day 12 (Figures 2 and 3). Stover additions resulted in significant increases in the relative abundance of OTUs from the phyla *Proteobacteria* and *Bacteroidetes*, and the orders *Actinomycetales*, *Bacillales* and *Clostridiales* (Figures 3 and 4), which is consistent with previous studies (Pascault *et al.*, 2013). For example, wheat residue additions to a calcareous silty clay farm soil stimulated *Firmicutes* OTUs, whereas alfalfa additions stimulated *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Pascault *et al.*, 2013). It is possible that these microorganisms are adapted to grow rapidly in response to inputs of easily mineralizable organic matter (for example, aliphatic C structures). These early-responding OTUs are likely responsible for the strong increase in total soil CO₂ emissions during the first weeks after stover was applied (Figure 1). As corn stover is more easily mineralizable than pre-existing SOC, its addition may be predicted to stimulate a broad spectrum of microorganisms. However, corn stover additions stimulated only a narrow subset of microorganisms (~10% of OTUs).

Although the greatest effect of amendments on CO₂ emissions took place within the first 2 weeks (Figure 1), the bacterial community response to organic amendments grew stronger over 82 days. The number of PyOM responders increased 11.5-fold from days 12 to 82, whereas the number of stover responders only increased 1.6-fold during this

timeframe. A total of 70% of the PyOM responders that were observed by day 12 also responded to stover (Figure 5 and Supplementary Figure 4). These early PyOM responders likely represent those microorganisms responsible for short-term mineralization of PyOM-C and for the CO₂ emissions we observed within the first 2 weeks (although we note that this abundance-based approach would not detect possible changes to the contributions to CO₂ efflux from microbes that did not actively grow/divide during the study (Blazewicz *et al.*, 2013)). The fact that 70% of these rapidly responding PyOM responders also responded to stover suggests that they are likely metabolizing easily mineralizable, possibly aliphatic components of PyOM, which are also present within fresh corn stover (Cheng *et al.*, 2008). In contrast, the OTUs that responded to both PyOM and stover on day 82 may represent microbes that were accessing the polyaromatic bulk of the PyOM-C and the remaining, less easily mineralizable stover-C compounds (Whitman *et al.*, 2013). In addition, late-responding OTUs could include microbes that are responding to other effects of the amendments, such as changes in soil physical or chemical properties, or the ecology of the system (for example, changes to the soil food web, competition or mutualisms).

PyOM effects on the soil bacterial community

The OTUs that responded uniquely to PyOM include representatives from 14 phyla (Figure 5, pink region and Supplementary Figures 4). Although the short-term effects of PyOM on SOC dynamics may be driven to a large extent by a relatively small, but easily mineralizable fraction of PyOM-C (Whitman *et al.*, 2014), it is essential to also understand the long-term effects driven by chemical or physical changes to the soil environment, such as pH, soil moisture, nutrient status or the mineralization of more chemically complex PyOM-C sources. For this, the *Gemmatimonadetes*, particularly those of classes *Gemm-5*, *Gemm-3* and *Gemmatimonadales*, are a prime target (Figure 6 and Supplementary Figures 11 and 16). The first isolates of the *Gemmatimonadetes* phylum were described in 2003 (Zhang *et al.*, 2003), and although little is yet known about them ecologically and physiologically, our findings seem consistent with previous observations. For example, *Gemmatimonadetes* have been found to decrease in relative abundance with the addition of wheat residues (Bernard *et al.*, 2007), were more active in soil microcosms that did not receive leaf litter (Pfeiffer *et al.*, 2013), and were more likely to be decomposing existing SOM than fresh OM (Pascault *et al.*, 2013). Of particular relevance, *Gemmatimonadetes* increased with the addition of rice straw PyOM made at 500 °C to a farmed Acrisol in a pot trial (Xu *et al.*, 2014). This increase was driven by increases from the class *Gemmatimonadetes*, with some *Gemm-1* and *Gemm-3* increasing in relative abundance as well. These studies and our own

findings suggest they may be adapted to a lifestyle associated with OM sources that are challenging to mineralize. Increased pH with PyOM additions may also have had a positive effect on *Gemmatimonadetes*, which have been reported to be more abundant in neutral pH soils (Lauber *et al.*, 2009; Vishnivetskaya *et al.*, 2011), although this effect was not significant for a much larger pH range than that observed in this study (2.6 vs 0.75) (DeBruyn *et al.*, 2011). *Gemmatimonadetes* may also be adapted for low soil moisture (DeBruyn *et al.*, 2011), but this is not a likely explanation in our study, as we did not measure significant differences in soil moisture in the PyOM-amended soils on any sampling day (data not shown). The top five most abundant OTUs that responded uniquely to PyOM, including members of the *Oxalobacteraceae*, *Rhizobiales* and *Erythrobacteraceae*, could also be good targets for future investigations into microbial interactions with PyOM.

PyOM produced from different materials under different conditions can result in a wide range of pH values (Enders *et al.*, 2012), and in this study, PyOM additions significantly increased soil pH (Supplementary Figure 17), albeit by less than a full pH unit. Soil pH is strongly correlated with community composition (Lauber *et al.*, 2009; Rousk *et al.*, 2010). In particular, the aptly named phylum *Acidobacteria* has been shown to be especially sensitive to pH shifts, although its subgroups show variable responses to acidity: subgroups 1, 2 and 3 have been shown to increase at lower pHs, whereas subgroups 4, 5, 6, 7 and 17 have been shown to increase at higher pHs (Rousk *et al.*, 2010; Bartram *et al.*, 2013). Both Bartram *et al.* (2013) and Rousk *et al.* (2010) characterized soils from long-term (50+ and 100+ years, respectively) liming trials, so it is not possible to predict from those studies the expected timescale of a soil bacterial community response to pH changes. However, we may ask whether the PyOM-specific response in this study is driven by pH shifts. We found that OTUs from subgroup 4 did generally increase with PyOM additions (Supplementary Figure 18). However, we also found that members of subgroup 6 decreased in relative abundance, whereas subgroup 3 increased with PyOM additions (Supplementary Figure 18), which is counter to what trends in previous studies would predict if these changes were being driven purely by the pH increase with PyOM additions. This does not necessarily contrast with the previous studies—rather, it may show that factors other than pH are likely important for driving the observed changes in subgroups 3 and 6 in this system. Explanations besides pH are particularly likely, as the pH shift by +0.75 units was small in comparison with the potential magnitude of natural pH gradients common in soils (for example, owing to biological activity, rhizosphere effects or wet–dry cycles (Husson, 2012)). *Acidobacteria* have sometimes been characterized as being poorly equipped to compete in high nutrient conditions (Fierer *et al.*, 2007). If some

Acidobacteria are able to mineralize challenging C forms, such as fused aromatic C ring structures, but are poorly adapted for metabolizing easily mineralizable C, for example, this could explain the positive response of OTUs in *Acidobacteria* subgroup 3 to PyOM additions on day 82, despite the accompanying small pH increase.

Although our study focused on soil bacterial communities, soil fungi have an essential role in SOC cycling (Schimel and Schaeffer, 2012). In addition, soil fungi and their oxidative enzymes have an important role in the degradation of organic matter compounds with condensed aromatic ring structures, such as lignin (Talbot *et al.*, 2011; Burns *et al.*, 2013). This makes fungi an excellent target for future investigations of the effects of PyOM on soil microbial communities.

Soil C dynamics

Stover amendments had a larger effect than PyOM on both soil bacterial community composition (Figures 2–6) and soil CO₂ emissions (Figure 1), and also resulted in significantly greater SOC-derived CO₂ emissions on day 12 (Supplementary Figure 3). However, this increase in mineralization of existing SOC disappeared by day 66 (Supplementary Figure 3). These dynamics likely reflect the relative microbial accessibility or solubility of the two amendments (Lehmann and Kleber, 2015).

As the PyOM was produced at a relatively low temperature (350 °C), there was likely a substantial fraction of relatively easily mineralizable C (Zimmerman, 2010; Whitman *et al.*, 2013) that contributed to the increases in total CO₂ during the first week after application (Figure 1 and Supplementary Figure 19). This fraction could include aliphatic C compounds, carboxylic acids, cellulose and hemicellulose (Whitman *et al.*, 2013). (It is also possible that a small portion of the PyOM-C losses were due to the dissolution of carbonates (Supplementary Figure 17)). However, unlike with stover, we did not find evidence that increased microbial activity because of PyOM additions affected SOC-derived CO₂ emissions (Supplementary Figure 3). This is likely because the PyOM-C was more challenging for microorganisms to mineralize than the stover-C, resulting in slower mineralization of PyOM as compared with the stover. Although there was 1.8 times as much total stover-C added as PyOM-C, CO₂ emissions increased with stover additions much more than 1.8 times the amount they increased with PyOM additions (Figure 1 and Supplementary Figure 19). Only the stover provided a large easily mineralizable C subsidy to the microbial community, which stimulated the microbial community, increased microbial biomass and may have increased general enzymatic activity. This, in turn, increased mineralization of existing SOC (Blagodatskaya and Kuzyakov, 2008). The lack of any detectable net effect on SOC mineralization from

PyOM additions (often termed a 'priming effect' (Bingeman *et al.*, 1953; Woolf and Lehmann, 2012; Whitman *et al.*, 2014)) is not unexpected. Specific combinations of PyOM materials and soils have been found to produce a wide range of effects on SOC mineralization (Maestrini *et al.*, 2014; Whitman *et al.*, 2015). The finding that PyOM application may result in less SOC loss than the application of an equivalent amount of fresh stover has implications for residue and C management at the farm scale. In systems where organic matter sources will rapidly mineralize under baseline conditions, PyOM production from this material and its return to the soil may result in greater net C gains than adding the fresh organic matter directly to the soil, because of the strong impact of fresh organic matter on the soil bacterial community and their C mineralization activity.

Conflict of Interest

The authors declare no conflict of interest.

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Accession codes

Sequence data are deposited in the NCBI Sequence Read Archive under accession SRP072444.

Author contributions

TW, DHB and JL designed the experiment; TW and AE grew the biomass and produced the PyOM; AE designed and built the CO₂ chambers; TW conducted the field trial and performed the biogeochemical lab work; AC, CK and TW performed the molecular lab work; AC, CK and CP-R developed the bioinformatics analysis pipeline; TW and CP-R performed the bioinformatics analyses; TW, CP-R, DHB and JL interpreted the data; and TW wrote the manuscript, and all authors commented on the paper.

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