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

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Abstract

Pyrogenic organic matter (PyOM) additions to soils can have large impacts on soil organic C (SOC) cycling. Because 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 microbial communities after OM additions in a field experiment. We produced and applied either 350°C corn stover PyOM or an equivalent amount of dried corn stover to a Typic Fragiudept soil. Stover increased SOC-derived and total CO_2 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 CO_2 fluxes (up to 2x) and caused fewer changes in bacterial community composition. 70% of the OTUs that increased in response to PyOM additions also responded to stover additions. These OTUs likely thrive on easily-mineralizable C that is found both in stover and, to a lesser extent, in PyOM. In contrast,

22 we also identified unique PyOM-responders, which may respond to substrates such as
23 polyaromatic C. In particular, members of *Gemmatimonadetes* tended to increase in relative
24 abundance in response to PyOM but not to fresh organic matter. We identify taxa to target for
25 future investigations of the mechanistic underpinnings of ecological phenomena associated with
26 PyOM additions to soil.

27

28 **1 Introduction**

29

30 Large inputs of pyrogenic organic matter (PyOM) in fire-affected ecosystems can constitute up
31 to 80% of total soil organic carbon (SOC) (Lehmann *et al.*, 2008). Whether PyOM is produced
32 naturally in fires (Czimczik and Masiello, 2007), intentionally for carbon (C) management,
33 and/or as an agricultural amendment (Lehmann, 2007; Laird, 2008), it is important to understand
34 how it affects the C cycle (Whitman *et al*., 2010). PyOM additions to soil can significantly affect
35 plant growth and crop yields (*e.g.*, Jeffery *et al.*, 2011) and SOC dynamics (Watzinger *et al.*,
36 2014; Maestrini *et al.*, 2014; Whitman *et al.*, 2015). For example, among other effects, PyOM
37 can change soil pH (Gul *et al.*, 2015), add nutrients to soils (Enders *et al.*, 2012), and alter soil
38 water-holding capacity (Abel *et al.*, 2013). While PyOM additions to soils can alter inorganic C
39 dynamics through changes to soil pH, a key mechanism for changes in SOC mineralization is
40 altered microbial mineralization of SOC in response to PyOM (Maestrini *et al*., 2014; Whitman
41 *et al.*, 2015). Hence, an understanding of the effects of PyOM on the soil microbial community is
42 required to understand the effects of PyOM on SOC stocks.

43

There are many ways PyOM could affect soil microorganisms (Kuzyakov 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 (*e.g.*, pH), 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 T-RFLP (Bingeman *et al.*, 1953; Jin, 2010; Kolton *et al.*, 2011) or DGGE (Kolton *et al.*, 2011; Chen *et al.*, 2013), or by surveying PLFAs 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, 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 SSU rRNA genes (Taketani *et al.*, 2013). A handful of other studies have used high-throughput sequencing approaches to characterize PyOM effects on soil microbial communities (*e.g.*, Nielsen *et al.*, 2014; Xu *et al.*, 2014). However, due 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 communities, particularly at the level of individual taxa.

In this study, we investigated the effects of PyOM additions on SOC mineralization and soil microbial 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. Additionally, we predicted that the addition of fresh biomass would induce the greatest changes in the microbial community, 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 micro-organisms 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, since a smaller fraction of the PyOM-C is easily-mineralizable.

2 Materials and methods

2.1 Experimental design

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

88 designed so that the dried original corn biomass was equivalent to that which would have been
89 required to produce the mass of corn PyOM that was applied to each plot. (*I.e.*, 4.1 Mg ha^{-1} corn-
90 derived PyOM were applied, which, with 0.365 mass fraction conserved during PyOM
91 production, translates into 11.2 Mg ha^{-1} corn stover, which is representative of a productive corn
92 crop in the US (Shinners and Binversie, 2007)).

93

94 The field site is located in Cornell's research fields in Mt. Pleasant, N.Y., and is a Mardin soil
95 (Coarse-loamy, mixed, active, mesic Typic Fragiudept) (Supplementary Table 2). The soil has
96 been historically planted to a potato, rye, clover rotation, for the past > 30 years, but was kept in
97 rye-clover rotation for the past 5 years, with one planting of sudangrass 3 years ago. The plot
98 was sprayed with Roundup (glyphosate) herbicide in the fall of 2012, ploughed on May 3, 2013,
99 and kept weed-free by hand-weeding and water-permeable landscape fabric through the summer
100 until trial initiation.

101

102 The trial initiation date was August 16, 2013 (Day 0). Square plots (0.7 m x 0.7 m for the soil-
103 only and PyOM, 0.45 m x 0.45 m for the stover) were surrounded by 0.7-m wide weed-free
104 borders, maintained by hand weeding. Treatments were organized using a spatially balanced
105 complete block design with 16 replicates of the unamended and PyOM plots, and 8 replicates of
106 the stover plots (van Es *et al*., 2007). Soil (6.1 kg) was removed from the surface of each plot,
107 combined with stover or PyOM additions, if needed, and mixed in a V-mixer (Twin shell dry
108 blender, Patterson-Kelley, East Stroudsburg, PA, USA). Amended plots received 4.1 Mg ha^{-1} of
109 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 centre 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).

2.2 Biomass production

Two sets of corn plants (*Zea mays* (L.)) were grown, one in an enriched $^{13}CO_2$ atmosphere growth chamber and the other in an ambient $^{13}CO_2$ greenhouse. The labeled plants were grown in potting mix in a Percival AR-100L3 CO_2-controlled growth chamber (Percival, Perry, IA). The plants were exposed to cycles of 18 h light / 6 h darkness. During light cycles, the atmosphere was maintained at 400 ppm 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 $^{13}CO_2$. Plants were pulse-labeled with 13 L of 99% $^{13}CO_2$ at regular intervals over the course of their growth in order to produce an even label. Pulse labels were delivered by opening the $^{13}CO_2$ cylinder to fill a balloon with ~500 mL $^{13}CO_2$. The balloon remained attached to the cylinder so that the $^{13}CO_2$ 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.

2.3 PyOM production and amendment mixing

Oven-dried corn plants were ground in a hammer mill (Viking) 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 Güereña *et al*. 2015) by ramping at 5°C min^{-1} to 350°C, then holding at 350°C for 45 minutes, under Ar (Supplementary Table 1). The ^{13}C-labeled and natural abundance corn PyOM materials were mixed together to produce a $\delta^{13}C$ value of +37.5‰. For the corn biomass-only plots, mixtures of ^{13}C-labeled and natural abundance corn with a $\delta^{13}C$ 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.

2.4 CO_2 flux and $^{13}CO_2$ measurements

Soil CO_2 flux was measured using a LI-6400XT infra-red gas analyser with a 6400-09 soil CO_2 flux chamber attachment (LI-COR, Lincoln, Nebraska). 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 $^{13}CO_2$ analysis and emissions partitioning on day 12 and on day 66 using

153 modified static Iso-FD chambers (Nickerson *et al*., 2013; Whitman and Lehmann, 2015)
154 (Supplementary Figure 2).

155

156 *2.5 Soil sampling*

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158 Soil samples for microbial community analyses were taken on days 1, 12 (chosen because it was
159 after the first major rainfall; Supplementary Figure 1), and 82. Samples were sieved to < 2 mm,
160 and immediately frozen in Whirl-Paks in liquid N_2 and stored at -80°C. On days 1 and 12, two
161 25-mm deep soil probe samples were pooled, while on day 82, soil within the entire collar was
162 destructively sampled.

163

164 *2.6 Isotopic partitioning of CO_2 samples*

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

171 $$(1)\ \delta_T = f_S * \delta_S + f_{PyOM} * \delta_{PyOM},$$

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). Since δ_T, δ_S, and δ_{PyOM} were all measured, and we know

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

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

2.7 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 due to low flux rates, and have excluded these values from analyses. In addition, we excluded two data points where recorded fluxes were 56 and 16 SD 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*., 2014). To make post-hoc comparisons, we performed pairwise comparisons between the different soil amendments for a given day with a Tukey adjustment of *p*-values, using the "lsmeans" R package (Lenth, 2014).

2.8 Microbial community analyses

DNA was extracted from 0.25 g moist soil samples using the MoBio PowerLyzer PowerSoil kit, following the kit's directions. The DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) with a multimode microplate reader (Molecular Devices, Sunnyvale, CA). 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), 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 30s, followed by 30 cycles of [5s at 98°C, 20s at 20°C, and 10s at 72°C], with a final extension for 2min 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 SequalPrep normalization plates (Applied Biosystems). The pooled sample was purified using a Wizard SV Gel and PCR Clean-Up System (Promega). This sample was submitted with sequencing primers (Supplementary Table 5) for paired ends 2 x 300 bp sequencing on the Illumina MiSeq v3 platform at Cornell's Biotech Core Facility.

2.9 Microbial community bioinformatics

2.9.1 Paired read merging, demultiplexing, and quality control

We merged the forward and reverse reads using the Paired End reAd mergeR (PEAR) (Flouri and Zhang, 2013) 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 less than 370 bp or more than 376 bp long or had homopolymers (runs of the same base in a row) more than 8 nucleotides in length. Quality control removed 6 265 604 reads, leaving 10 237 689 high quality sequences. With 3 treatments, two with 16 replicates and one with 8 replicates, and 3 time-points, we had a total of 120 samples. Individual samples contained from 8 830 to 194 356 total sequences, with a mean of 69 419 ± 44 070.

2.9.2 Operational taxonomic unit (OTU) picking

Reads were clustered into OTUs using the UPARSE methodology (Edgar, 2013) with an OTU sequence identity cut-off 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) 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 8 sequences, so it was excluded from further analyses.

2.9.3 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 (NMDS) analysis on the weighted UniFrac distance between communities across all timepoints 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 microbial community due to 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

256 "vegan" (Oksanen *et al.*, 2015). Because we found day and amendment both had significant
257 effects ($p < 0.001$), we performed a separate weighted UniFrac analysis and NPMANOVA for
258 each day and amendment type (comparing each amendment to either each other or to no
259 additions, for each day), adjusting *p*-values using a Bonferroni correction for 6 comparisons (p_{adj}
260 $= p * 6$).

261

262 We used the R package "DESeq2" (Love *et al.*, 2014) to calculate the differential abundance
263 ($\log_2$-fold change in relative abundance of each OTU) for each amendment type as compared to
264 the unamended plots for both sampling days (McMurdie and Holmes, 2014). We independently
265 filtered out OTUs that were sparsely represented across samples (*i.e.*, those OTUs for which the
266 DESeq2-normalized count across samples ("baseMean") was less than 0.6). Sparse OTUs will
267 not contain sufficient sequence counts to provide statistically significant results and their
268 removal reduces the number of multiple comparisons performed, thereby mitigating problems
269 associated with multiple comparisons to some extent. We adjusted the *p*-values with the
270 Benjamini and Hochberg (BH) correction method and selected a study-wide false discovery rate
271 (FDR) of 10% to denote statistical significance (Love *et al.*, 2014). We defined "responding
272 OTUs" as OTUs with a differential abundance greater than 1 and an adjusted *p*-value of <0.1.
273 We performed a BLAST (nucleotide blast, version 2.2.29+, default parameters) search with OTU
274 centroid sequences of responding OTUs, against the Living Tree Project (LTP) database (version
275 115) (Yarza 2008). The LTP database contains 16S rRNA gene sequences for all sequenced
276 archaeal and bacterial type strains.

277

278 **3 Results**

279

280 *3.1 Soil C dynamics*

281

282 Total CO_2 fluxes from plots that received uncharred stover additions were significantly (mixed
283 model repeated measures design, Tukey-adjusted post-hoc comparisons, $p < 0.05$) higher than all
284 other plots for the first 26 days and for 19 of the 27 days for which fluxes were determined
285 (Figure 1). Stover additions had the greatest effects on CO_2 fluxes (CO_2 fluxes 6 times those of
286 unamended soils) on days 7 and 11, after strong rain events (Supplementary Figure 1). Plots with
287 PyOM additions experienced significantly higher CO_2 fluxes than plots with no additions for the
288 first 12 days, after which there were no significant differences (Figure 1). The increases in fluxes
289 with PyOM additions were much less dramatic than those in the stover-amended plots, and were
290 never greater than 2.1 times the CO_2 emissions from unamended soils.

291

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

297

298 *3.2 Microbial Community Analyses*

299

300 There were significant changes in the microbial community composition over time
301 (NPMANOVA, $p < 0.006$, R^2=0.19) and with amendment type (NPMANOVA, p<0.006,
302 R^2=0.20) (Figure 2). Microbial community composition in unamended plots remained relatively
303 consistent over time (NPMANOVAs comparing Day 1 to Day 12 [$p < 0.06$, $R^2 = 0.18$] and to
304 Day 82 [$p < 0.29$, $R^2 = 0.11$]), while PyOM-amended plots varied slightly and corn stover-
305 amended plots varied greatly over time (Figure 2). The different treatments were not observed to
306 alter microbial community composition on day 1 (only 24 hours after OM additions). By day 12,
307 the stover treatment caused a significant change in community composition (NPMANOVA, $p <$
308 0.006, R^2=0.75), though no effect of PyOM was observed. By day 82, the microbial communities
309 in the PyOM-amended plots (NPMANOVA, $p < 0.02$, R^2=0.19) and corn stover-amended plots
310 (NPMANOVA, $p < 0.02$, R^2=0.56) were both distinct from the unamended plots and from each
311 other (NPMANOVA, $p < 0.01$, $R^2 = 0.36$). We did not detect significant differences in DNA
312 yield due to the addition of PyOM or stover directly after amendment additions (day 1), but
313 DNA yield was significantly higher in plots with stover additions on days 12 and 82
314 (Supplementary Table 6).

315

316 The stover treatment caused multiple phyla to change significantly in relative abundance as
317 compared to control plots on days 12 and 82 (Figure 3). In contrast, PyOM additions only led to
318 a significant change in the relative abundance of the phyla *Armatimonadetes* (decreased), and
319 *Bacteroidetes* (increased) and only on day 82 (Figure 3). However, we caution that decreases in
320 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. It is notable that extracted DNA increased significantly in response to stover additions on Days 12 and 82 (Supplementary Table 6; ANOVA and Tukey's HSD, $p < 0.05$). Since total DNA is correlated with microbial biomass (Anderson and Martens, 2013; Fornasier *et al*., 2014; Gagneux *et al*., 2011, but see Leckie *et al*., 2004), this result likely indicates an increase in microbial biomass in association with stover additions. Hence, we transformed the relative abundance data from stover treatments by scaling by the mass of extracted DNA per soil sample and performing the t-tests on those scaled values (Supplementary Note 1). The results obtained with scaled data matched those obtained with unscaled data, except declines in relative abundance for *Acidobacteria* and *Gemmatimonadetes* on day 82 were no longer statistically significant when scaled for the change in absolute DNA abundance in the stover treatment.

Many OTUs, from several phyla, responded significantly to stover and/or PyOM additions (Figure 4; Supplementary Tables 7 and 8; Supplementary Figures 6-14). 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 to corresponding plots that did not receive amendments. We identified 806 responders to either stover and/or PyOM from among the 7 770 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, 7, and 8). 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 to 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*).

4 Discussion

4.1 Soil microbial community dynamics

The soils that received corn stover amendments showed dramatic increases in CO_2 emissions almost immediately (Figure 1), and microbial 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, while 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 (*e.g.*, aliphatic C structures). These early-responding OTUs are likely responsible for the strong increase in total soil CO_2 emissions during the first weeks after stover was applied (Figure 1). Since corn stover is more easily-mineralizable than pre-existing SOC, its addition might 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 CO_2 emissions took place within the first two weeks (Figure 1), the microbial community response to organic amendments grew stronger over 82 days. The number of PyOM responders increased 11.5-fold from day 12 to day 82, while 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, Supplementary Figure 4). These early PyOM responders likely represent those microorganisms responsible for short-term mineralization of PyOM-C and for the CO_2 emissions we observed within the first two weeks (although we note that this abundance-based approach would not detect possible changes to the contributions to CO_2 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 (*e.g.*, changes to the soil food web, competition, or mutualisms).

4.2 PyOM effects on the soil microbial community

The OTUs that responded uniquely to PyOM include representatives from 14 phyla (Figure 5, pink region and Supplementary Figures 4-14). While 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 longer-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 Figure 11 and 16). The first isolates of the *Gemmatimonadetes* phylum were described in 2003 (Zhang *et al.*, 2003), and while 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 *Gemm1* and *Gemm3* 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, since we did not measure significant differences in soil moisture in the PyOM-amended soils on any sampling day (data not shown). The top 5 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, while 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 microbial 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, while 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 to the potential magnitude of natural pH gradients common in soils (*e.g.*, due 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.

4.3 Soil C dynamics

Stover amendments had a larger effect than PyOM on both soil microbial community composition and also the mineralization of existing SOC. Stover additions significantly increased plot-level CO_2 emissions over a longer period of time (Figure 1), and also resulted in significantly greater SOC-derived CO_2 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).

Because the PyOM was produced at a relatively low temperature (350°C), there was likely a substantial fraction of relatively easily-mineralizable C (Whitman *et al*., 2013; Zimmerman, 2010) that contributed to the increases in total CO_2 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, we did not find evidence that increased microbial activity due to PyOM additions affected SOC-derived CO_2 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 to the stover. While there was 1.8 times as much total stover-C added as PyOM-C, CO_2 emissions increased with stover additions much more than 1.8 times the amount they increased with PyOM additions (Figure 1; 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; Whitman *et al*., 2014; Woolf and Lehmann, 2012)) is not unexpected. Specific combinations of PyOM materials and soils have been found to produce a wide range of effects on SOC mineralization (Whitman *et al.*, 2015; Maestrini *et al.*, 2014). 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, due to the strong impact of fresh organic matter on the soil microbial community and their C mineralization activity.

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Author contributions

T.W., D.H.B., and J.L. designed the experiment, T.W. and A.E. grew the biomass and produced the PyOM, A.E. designed and built the CO_2 chambers, T.W. conducted the field trial, T.W. performed the biogeochemical lab work, A.C., C.K., and T.W. performed the molecular lab work, A.C., C.K., and C.P.-R. developed the bioinformatics analysis pipeline, T.W. and C.P.R. performed the bioinformatics analyses, T.W., C.P.-R., D.H.B., and J.L. interpreted the data, and T.W. wrote the manuscript, and all authors commented on the paper.

Supplementary information

Supplementary information is available at ISME J's website. All scripts used for microbial sequence data processing and analysis are openly available at https://github.com/TheaWhitman/PyOM. Sequence data are deposited in the NCBI Sequence Read Archive under accession number XXX.

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716

Figure legends

Figure 1 Mean CO_2 flux rates over time. Error bars ±1 SE, 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, while ‡ 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 $^{13}CO_2$ flux was partitioned between SOC and amendments.

Figure 2 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).

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 inter-quartile range are indicated (x), as are values that differ significantly from unamended (*, *t*-test, $p < 0.05$, Bonferroni-corrected for 72 comparisons).

737 **Figure 4** Log_2-fold change in relative abundance of OTUs as compared to unamended plots.
738 Each circle represents a single OTU and dashed and dotted lines represent increases or decreases
739 of 2x and 10x, respectively. Colours are scaled from yellow to red in decreasing *p*-value, with
740 grey points indicating OTUs with BH-adjusted *p*-values > 0.10).

741

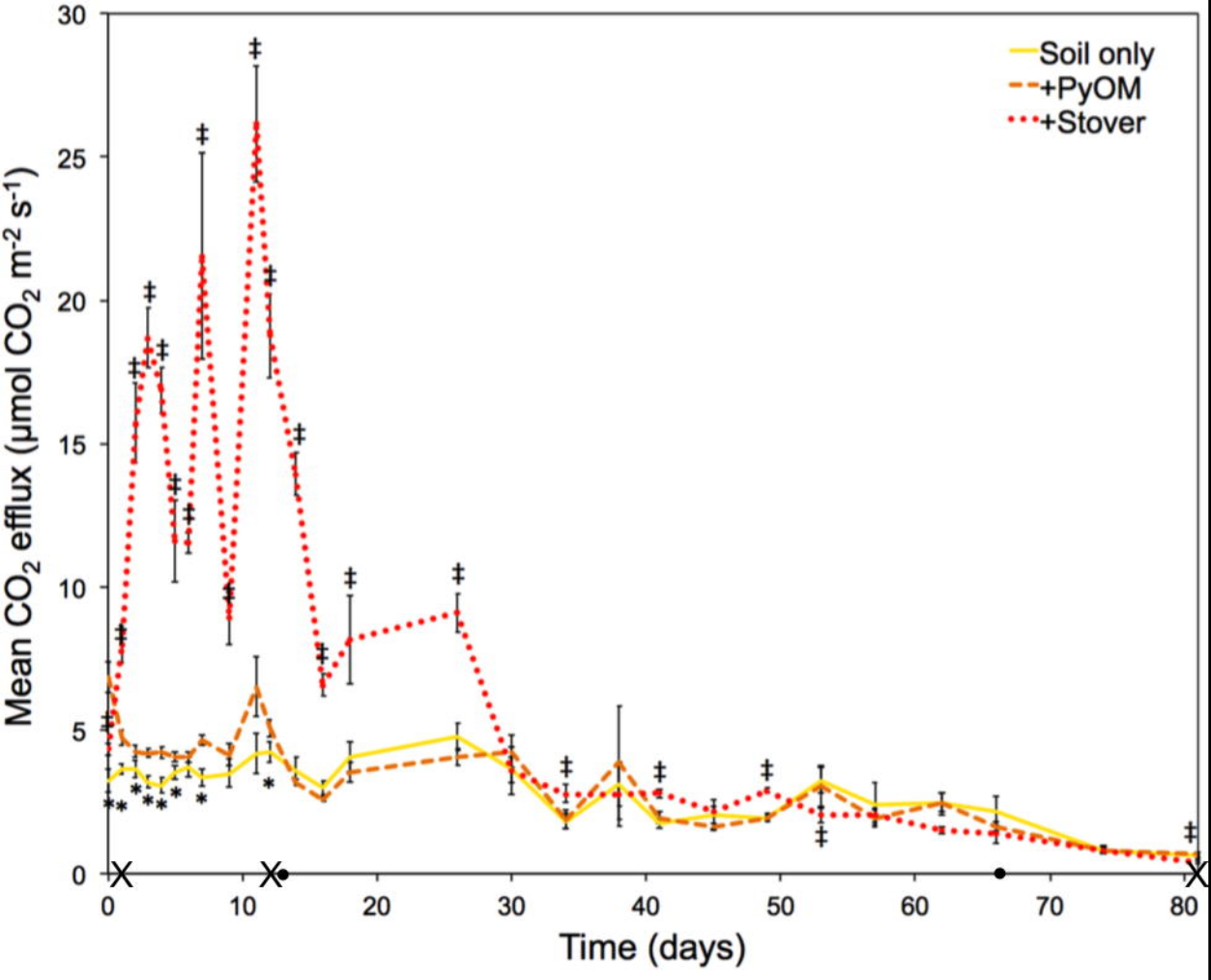
742 **Figure 5** Log_2-fold change in relative abundance of OTUs in response to stover or PyOM as
743 compared to unamended plots. Data are the same as those depicted in Figure 4. Note different
744 scales on axes. Each point represents the response for a single OTU across replicates, colored by
745 phylum. Dashed oval overlays indicate response groupings, where blue indicates strong stover
746 responders, pink indicates strong PyOM responders, and purple indicates common responders.
747 Grey histograms at the sides of the plot indicate density of OTU points.

748

749 **Figure 6** Log_2-fold change of the relative abundance of OTUs in PyOM or stover plots *vs*. soil-
750 only plots on days 12 and 82, for *Firmicutes*, *Gemmatimonadetes*, and *Verrucomicrobia* phyla.
751 Each point represents the response for a single OTU across replicates, coloured by order. Circles
752 represent day 12 and triangles represent day 82. Square brackets indicate candidate order in
753 Greengenes taxonomic nomenclature.

754

755


Soil only
+PyOM
+Stover
Mean CO_2 efflux (µmol CO_2 m^{-2} s^{-1})
Time (days)
0
5
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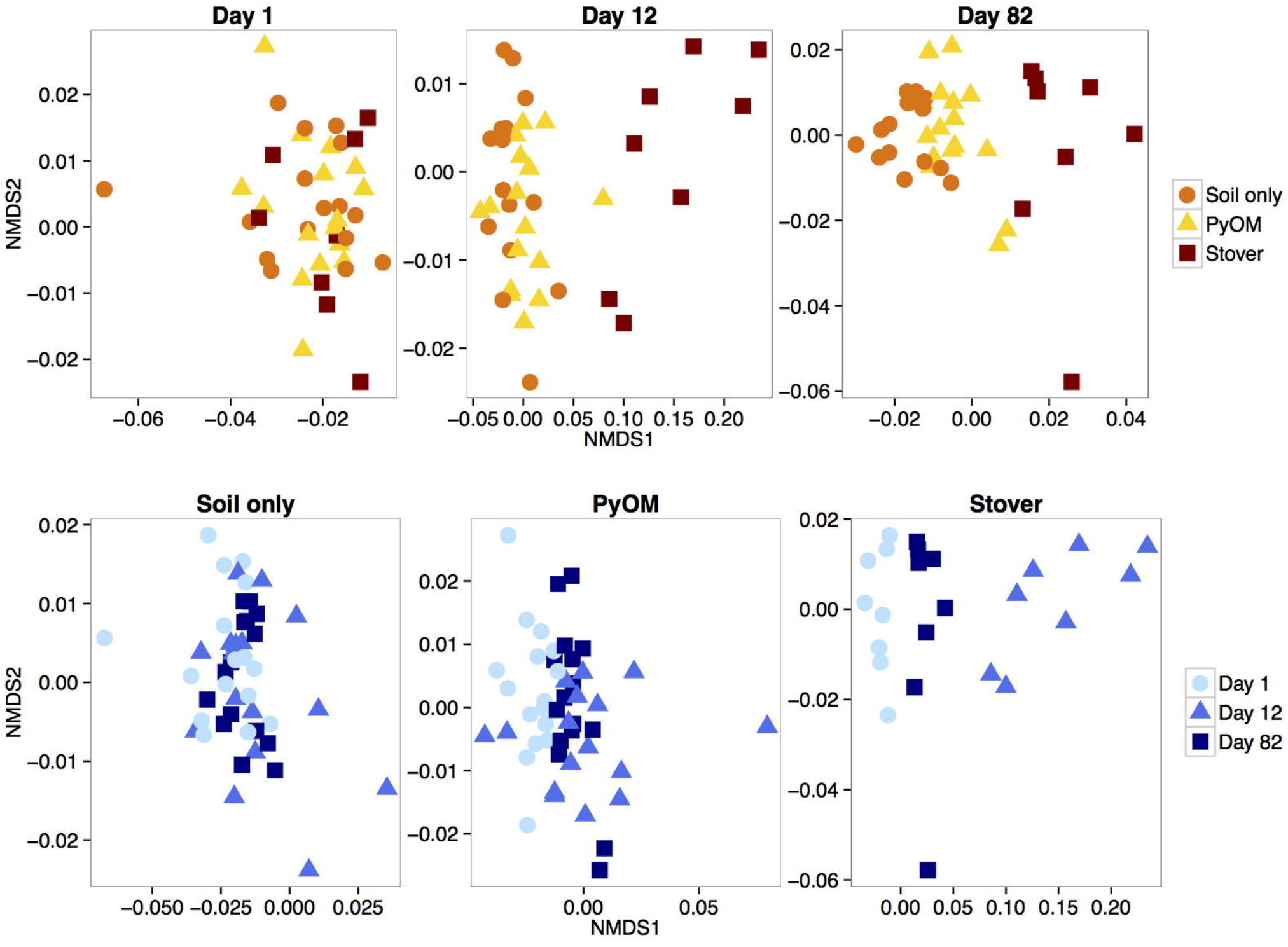
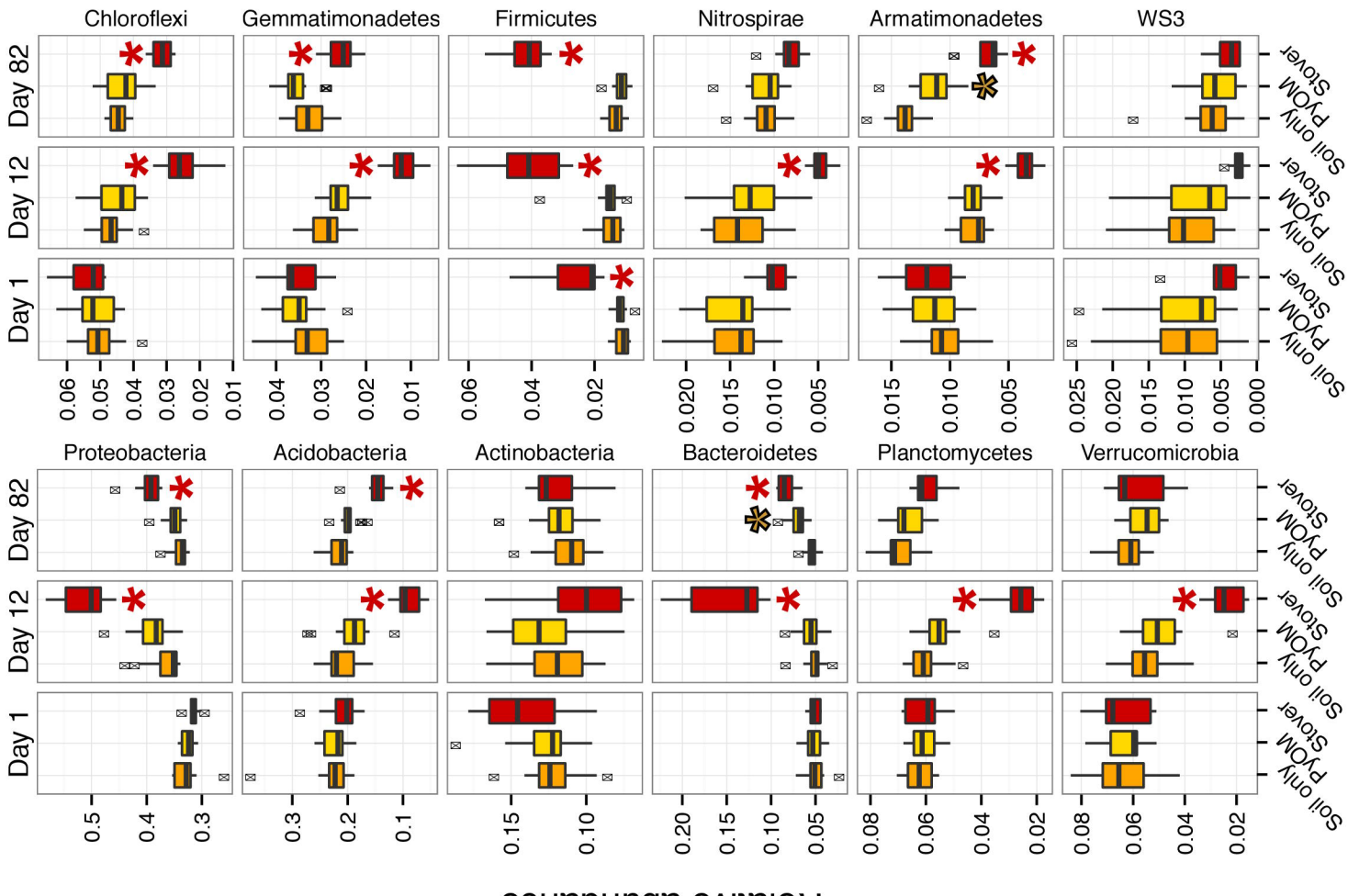
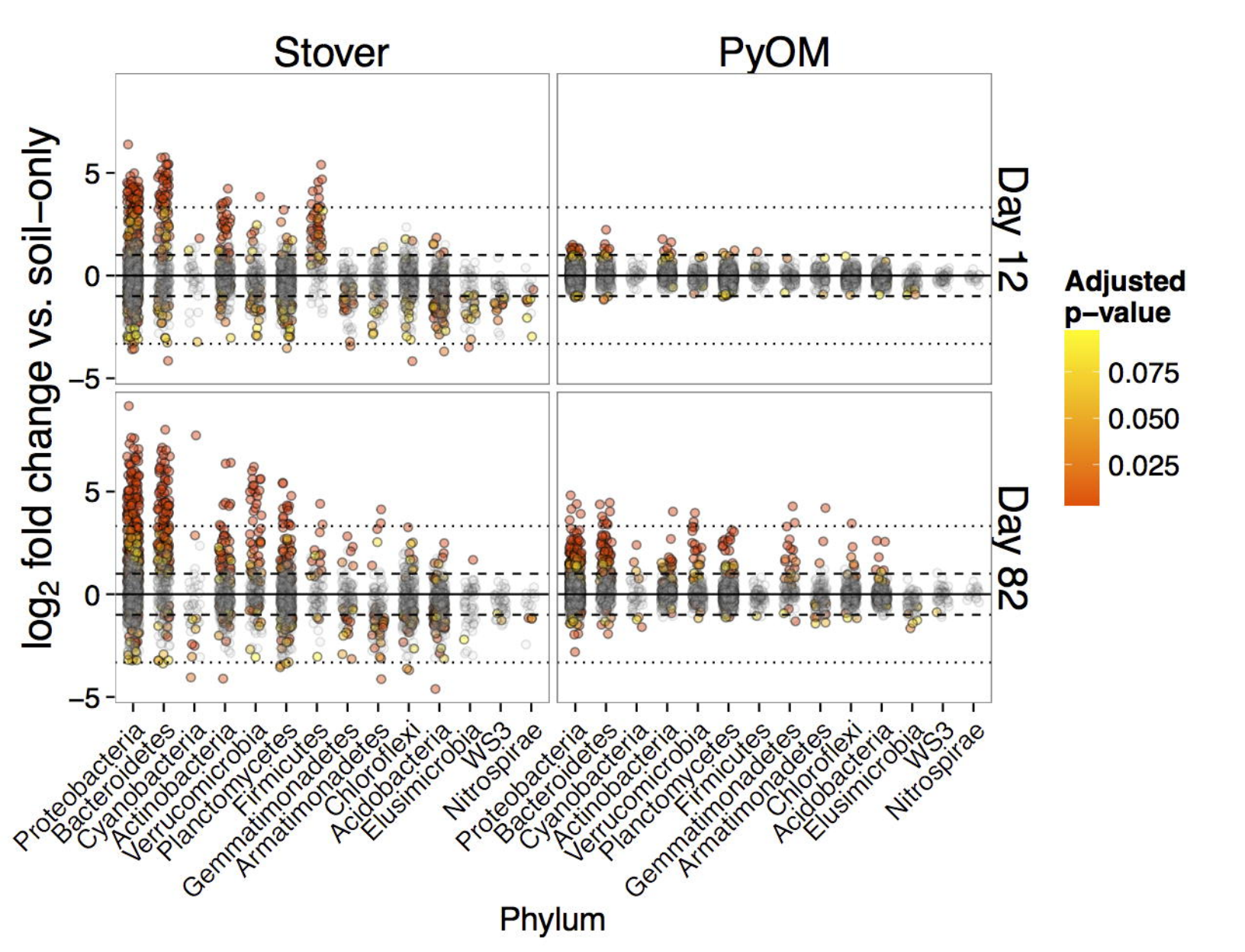
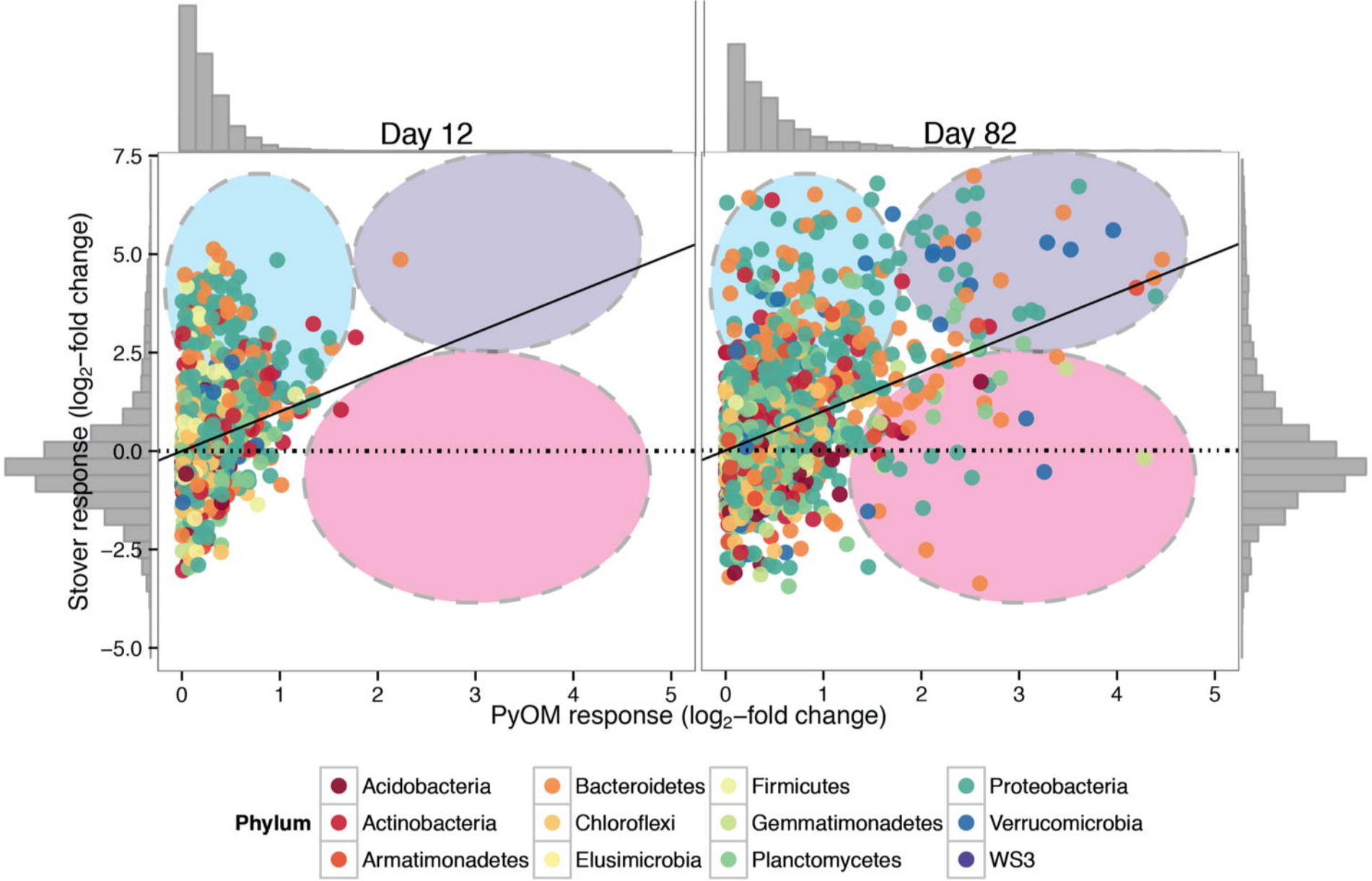
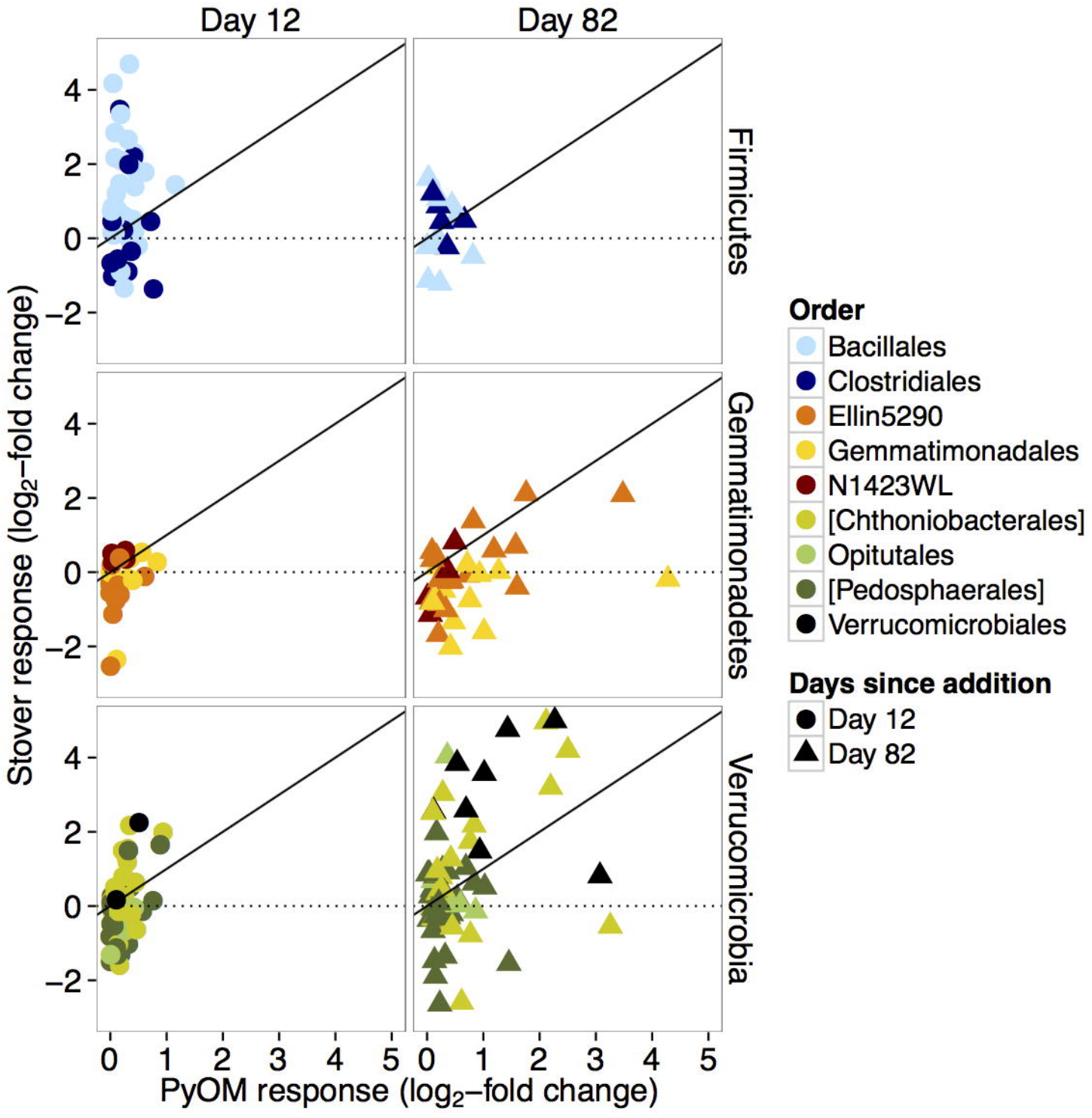

Day 1
Day 12
Day 82
NMDS2
NMDS1
Soil only
PyOM
Stover
Soil only
PyOM
Stover
Day 1
Day 12
Day 82


Chloroflexi
Gemmatimonadetes
Firmicutes
Nitrospirae
Armatimonadetes
WS3
Proteobacteria
Acidobacteria
Actinobacteria
Bacteroidetes
Planctomycetes
Verrucomicrobia
Day 82
Day 12
Day 1
Soil only
pYOM
Stover
Relative abundance


Stover
PyOM
Day 12
Day 82
log2 fold change vs. soil−only
5
0
−5
Adjusted p−value
0.075
0.050
0.025
Proteobacteria
Bacteroidetes
Cyanobacteria
Actinobacteria
Verrucomicrobia
Planctomycetes
Firmicutes
Gemmatimonadetes
Armatimonadetes
Chloroflexi
Acidobacteria
Elusimicrobia
WS3
Nitrospirae
Phylum


Day 12
Day 82
Stover response (log2−fold change)
PyOM response (log2−fold change)
7.5
5.0
2.5
0.0
−2.5
−5.0
0
1
2
3
4
5
Phylum
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chloroflexi
Elusimicrobia
Firmicutes
Gemmatimonadetes
Planctomycetes
Proteobacteria
Verrucomicrobia
WS3


Day 12
Day 82
Firmicutes
Gemmatimonadetes
Verrucomicrobia
Stover response (log2–fold change)
PyOM response (log2–fold change)
Order
Bacillales
Clostridiales
Ellin5290
Gemmatimonadales
N1423WL
[Chthoniobacterales]
Opitutales
[Pedosphaerales]
Verrucomicrobiales
Days since addition
Day 12
Day 82