

April 15th, 2011

Colorado Department of Agriculture
700 Kipling Street, Suite 4000
Lakewood, Colorado 80215-8000

Final report for: *Carbon negative bioenergy through the soil sequestration of pyrolysis biochar on Colorado pastureland: measuring the effects on forage yield, soil chemical properties, and microbial activity.*

CLIN# 09BAA00162

Dear ACRE Board:

Flux Farm Foundation is happy to offer this final report to the Colorado Department of Agriculture for the western Colorado biochar demonstration project. Three of our proposed application techniques have been tested, and soil and forage analyses are completed.

Biochar has received a great deal of attention over the course of our project, and we are confident that biochar will play a roll in improving soil conditions moving forward.

Sever discoveries have indirectly come out of our research including a peer reviewed journal article: *Williams M., and Arnott J. A Comparison of Variable Economic Costs Associated with Two Proposed Biochar Application Methods. Annals of Environmental Science, Volume 4, August 2010, Pages 23-30.*

Additional advancements in the understanding of how biochar impacts soil microbial communities along with the dynamics of lateral movement of biochar through the soil profile were also made.

Summary and Conclusions

- Trenching with high rates of CQuest biochar (50 or 75 tons/acre) may create areas in the field with enhanced water holding capacity, as evidenced by the greater moisture content of trenched biochar compared to soil outside the trench.

- High rates of CQuest biochar(50 or 75 tons/acre) increased microbial respiration soon after trenching, but the effect did not persist through July 2010.
- CQuest biochar trenched at 12.5 tons/acre or more resulted in greater soil peroxidase activity. Therefore, it is possible that biochar trenching could enhance the decomposition of chemically stabilized soil organic matter (humus). However, this effect was short-lived and did not persist through July 2010.
- CQuest biochar contained less extractable FAME mass, diversity and numbers compared to soil.
- There are methodological issues to measuring microbial biomass in biochar. High respiration activity indicates that relative to soil, microbial biomass was high in biochar trenched at 50 or 75 tons/acre. However, low FAME mass in biochar indicates low microbial biomass based on the measurement of actual cellular constituents, although these results may be inaccurate if FAME extraction efficiency is lower in biochar than in soil.
- CQuest biochar did not immediately impact arbuscular mycorrhizal fungi (AMF). One year after trenching, however, the relative abundance of AMF increased as the biochar trench rate increased, up to 50 tons/acre.
- One year after trenching, the 50 tons/acre treatment significantly altered microbial community structure in soils outside the trench, mainly by increasing the relative abundance of AMF.
- In the longer-term, biochar amendments of up to 50 tons/acre would be a positive management tool for increasing soil moisture holding capacity and relative abundance of AMF without resulting in persistent changes in decomposition activity.
- We did not find that CQuest Biochar applied in trenches significantly impacted plant growth positively at rates up to 50T per acre, but did find insignificant negative responses at rates of 75 T per acre.

Given the lack of a significant yield increase, we believe that the cost of applying CQuest Biochar in trenches in an agricultural setting far outweighs the monetary or environmental benefits gained after two years of data collection.

Our results should not deter further research in the field of biochar use in an agricultural setting, however in the near term, we believe that costs must decrease by at least 70% and additional application methods developed, for biochar to become a viable option for farmers and ranchers growing grasses in the region.

Furthermore, we believe that biochar application at higher value sites on deteriorated or contaminated soils is a likely market stepping-stone in the near term. The agricultural application of biochar at current costs, and government commodity subsidiary structures, simply does not warrant serious consideration for broad acre agricultural use in the region.

STATUS OF TASKS TO COMPLETE:

The following is an overview of the tasks outlined in the “Statement of Work,” and a brief narrative of the status to date.

- a) **Review of biochar literature:** Flux Farm Foundation has completed a thorough review of the biochar literature. Some of our findings can be found on our website: <http://www.fluxfarm.com/biochar.html>. Much has been documented about the agronomic benefits to biochar application on many soils throughout the world. Currently fourteen USDA-ARS locations are directly involved in biochar studies, and most major agricultural colleges have at least one faculty member or post-doctoral student involved in biochar research. Numerous conferences relating to biochar have been held, and many more are currently planned. However, a critical review of potential biochar application methods remains elusive. Our research has contributed considerably to this void.
- b) **Sieving biochar by size:** We initially believed that the particle size of biochar could have a great effect on the agronomic properties of the product and proposed to separate our biochar treatments into various sizes. After receiving our biochar samples from Dynamotive Energy and Best Energies, and attempting to sieve biochar into size classes, we quickly realized that the practice was not viable. Excessive dust was produced during sieving trials and concerns over air pollution forced us to re-think our presumptions. The existing particle size of Dynamotive’s CQuest biochar approaches a low of 5 μm in size and a high of roughly 50 μm , while Best Energies Agrichar has a greater particle distribution ranging from 20 μm to 1.5 cm.
- c) **Defining research plots:**
Research plots were defined in the spring of 2008, and have been maintained ever since.
- d) **Preparing biochar for application:** Various wetting techniques were preformed to minimize the dust produced during application. We found that both CQuest and Agrichar do not like to remain suspended in water, and separate into three distinct layers over time. More thought into surfactants is warranted.
- e) **Applying biochar to test plots (biochar application methods of interest):**

1) Backfilling of line trench: Caterpillar T9B Trenchers for CAT Skid Steer Loaders are designed for cutting narrow straight trenches in the soil prior to laying electrical, telephone and cable lines, or water and gas pipe. At a cutting width of 6 inches and a maximum depth of 54 inches, the CAT T9B trencher has the ability to excavate a great deal of soil while minimizing surface disturbance. For this reason, Flux Farm decided to adapt the method for biochar application. Trenches 24 inches deep by 6 inches wide by 20 feet long were excavated in an existing stand of pasture grass. To calculate application rate, trench spacing was assumed at 6 feet. Using this assumption, a total of 7,350 feet could be trenched in one acre requiring 35 rows. In knowing the volume of space, and the bulk density of the product (31.5 g per 100 mL) biochar was backfilled into trenches at 12.5 ton/ac, 25 ton/ac, 50 ton/ac, and 75 ton/ac rates.

Note: We selected a sampling method that will assess soil conditions along a gradient perpendicular to the trench to investigate if biochar physically migrates through the soil profile, and if agronomic benefits are observed at a distance from the site of application.

2) Slurry (biochar/water) injection by root feeder: A Rittenhouse 100 US Gallon Skid Mount Sprayer with Honda 5.5 hp gas engine, Hypro D30 pump, and soil injector with flow meter was used to inject a mixture of biochar and water into soils.

Note: After our initial investigation, it was determined that an agricultural surfactant will be needed to lower the surface tension of water thereby allowing biochar to remain evenly suspended in solution and suitable for pumping. The concentration of biochar in solution, by volume, is also likely to be somewhat low (10 – 20%) and a significant amount of water will be required to apply a relatively small amount of biochar. We are not yet fully convinced that biochar/water soil injection would be a wise use of limited water.

3) Slurry (biochar/water) injection by modified hydraulic injector: Not yet attempted. We are currently designing and building a hydraulic injector and experimenting with suitable surfactants, biochar:water ratios, and other potential mixing agents.

4) Direct slurry top-dressing (biochar/soil/water) with cement sprayer: Not yet attempted. The technique appears intriguing since the sprayer would accommodate a material with lower viscosity and higher particulate size than the Rittenhouse soil injector.

5) Direct top-dressing of solid powder: Top dressing of solid powder was performed at rates of 12.5 tons/ac and 25 tons/ac. Concerns over air quality were significant given the small particle size of biochar (as noted above). A 3M 6000 series full-face respirator fitted with N100 particulate filters was used to protect against harmful exposure and inhalation. We recommend that a fine

water mister be used to reduce air particulate pollution if commercial scale topical broadcasting of biochar powder is attempted in the future.

Note: Given that biochar was not mixed into the soil, significant erosion of material could be experienced over time. Concerns over biochar flammability are also significant and must be addressed.

6) Direct top-dressing of pelletized biochar: Not yet attempted.

f) **Physiochemical analysis of biochar:** BestEnergies and Dynamotive Energy Corporation have conducted such tests and have made this information available to interested parties.

g) **Soil chemical, physical, and microbial analysis:**

Conducted by Dr. Mary Stromberger, Faculty Soil and Crop Sciences, Colorado State University and Dr. James Ippolito, USDA-ARS, Kimberly Idaho.

Methods

Soil sampling and processing

Microbial analyses were performed on soil and biochar samples collected from the trenched experiment, which consisted of five CQuest biochar trenching rates (0, 12.5, 25, 50, and 75 tons/acre) replicated three times in a completely randomized design. Soil samples were collected to twelve inches depth along a gradient starting from inside the trench (0 inches), a second set 6 inches to either side of the trench, a third sample set 12 inches to either side of the trench, and a fourth set taken 18 inches to either side of the trench. Each sampling set consisted of at least six individual soil samples, which were composited into polyethylene bags to generate four samples per plot. Samples were collected in July 2009, immediately after installation of the biochar trenches, and again in July 2010. For each year, there were a total of 60 samples (5 biochar rates × 4 sampling positions × 3 replicate plots = 60). Samples were shipped overnight to Dr. Stromberger's laboratory (Colorado State University, Fort Collins, CO) for microbial analyses. Subsamples were shipped overnight on ice from to Dr. James Ippolito's laboratory (USDA-ARS, Kimberly, ID) for substrate induced respiration (SIR) assays. The remaining samples were stored at -80°C prior to analyses.

Microbial analyses

Samples were homogenized by hand, and visible pieces of roots and organic residues were removed. Subsamples (10 g) were oven dried at 105°C for 24 h for gravimetric determination of water content. Substrate induced respiration was determined according to the procedure described by Horwath and Paul (1994). In brief, 25 g subsamples (dry

weight) were adjusted to water holding capacity incubated in the presence of glucose for 24 h at 22°C. Samples were incubated in mason jars along with a vial containing 5 ml of 2 M NaOH, and the amount of CO₂ respired was determined according to the CO₂ trapped by the NaOH solution. Microbial biomass C (MBC) was determined based on the SIR results according to the following equation:

$$\text{mg MBC g}^{-1} = (\text{ml CO}_2 \text{ g}^{-1} \text{ d}^{-1} \times 40.04) + 0.37$$

Enzyme assays were performed according to the methods described by Sinsabaugh et al. (2003). Activities of β -D-cellubiosidase and β -glucosidase activity (related to cellulose decomposition) were determined fluorimetrically, whereas peroxidase activity (related to lignin decomposition) was determined colorimetrically. Soil or biochar (5 g) was suspended in 95 mL of 50 mM, pH 5.0 acetate buffer. The buffer was homogenized at high speed for one minute on a Waring blender. The resulting suspensions were continuously stirred using a magnetic stir plate as 200 μ L aliquots were distributed into 96-well microtiter plates. Aliquots of samples, substrates (4-methylumbelliferone- β -D-cellobioside, 4-methylumbelliferone- β -D-glucopyranoside or L-3,4-dihydroxyphenylalanine (L-DOPA)), controls, and blanks were pipetted appropriate wells of 96-well microtiter plates as detailed by Sinsabaugh et al. (2003). β -D-cellubiosidase and β -glucosidase microplates were incubated in the dark at 25°C for 1 h. Fluorescence from these plates was measured with a microplate fluorometer with 365 nm excitation and 450 nm emission filters. Samples were corrected for blank, control, and quenching activities, and sample activity was expressed in units of $\mu\text{mol g}^{-1} \text{ soil h}^{-1}$. Absorbance of wells from peroxidase microplates was read at 450 nm with a microplate spectrophotometer, and after correcting sample absorbances for the blank and control, activity was expressed in units of $\mu\text{mol g}^{-1} \text{ soil h}^{-1}$.

Microbial community structure was characterized by ester-linked fatty acid methyl ester (FAME) analysis as described by Stromberger et al. (2007). In brief, fatty acids were extracted and methylated by the addition of 15 ml of 0.2 M KOH to 3 g soil or 1 g biochar subsamples and a 1 hour incubation at 37°C. The pH of the suspension was neutralized with 3 ml of 1 M acetic acid, and FAMES were partitioned into an organic phase by adding 10 ml of hexane, followed by centrifugation at 480 \times g for 10 min. The hexane layer was transferred to a clean tube, and an internal standard (20 μ g of 19:0) was added to each tube. The hexane solvent was completely evaporated off under a stream of N₂ gas, and samples were shipped overnight and on ice to the University of Delaware for analysis. Samples were redissolved in hexane and analyzed by gas chromatography (GC) analysis with an Agilent 6890 gas chromatograph (Agilent Technologies, Inc., Palo Alto, CA) by the University of Delaware. The GC capillary column was an Ultra 2 Agilent #1909 1B-102 crosslinked 5% phenyl methyl silicone, 25 m long with an internal diameter of 0.2 mm and film thickness of 0.33 μ m. Flame ionization detection (FID) was achieved at a temperature of 250°C using a carrier gas of hydrogen at a flow rate of 0.8 ml min⁻¹. Samples were run using the Microbial ID (Newark, DE) Eukary methods and peak naming table; all functions of the GC were under the control of the computer and this method. To clean the column between samples, oven temperature ramped from 170°C and to 300°C at a rate of 5°C min⁻¹.

¹, with a hold at the maximum temperature for 12 min. Biomarkers of specific functional groups were assigned according to Stromberger et al. (2007). Bacterial biomarkers were the sum of *i15:0*, *a15:0*, *15:0*, *i16:0*, *16:1 ω 9c*, *16:1 ω 7c*, *i17:0*, *a17:0*, *17:0 cy*, *17:0*, and *19:0 cy*. The FAMES *18:2 ω 6c* and *16:1 ω 5c* were used as the indicators for fungi and arbuscular mycorrhizal (AM) fungi, respectively.

Statistical analyses

Analysis of variance tests were conducted in SAS (version 9.1, SAS Institute, Cary, NC) to determine effects of biochar amendment rate, distance from trench, sampling year, and their interactions on microbial properties. Moisture content, MBC, SIR, enzyme activities, total FAME biomass, FAME diversity and richness, and the relative abundance of AMF FAME biomarker were analyzed by a split split plot design with Proc GLM in SAS, with biochar trench rate as the whole plot factor (completely randomized), distance from the trench as the sub plot factor, and sampling year as the sub-sub plot factor. When significant differences among treatments were identified ($p < .05$), the least significance difference (LSD) test was conducted to separate significantly different means.

Microbial community FAME data were expressed on a relative percent basis rather and then analyzed by Principal Components Analysis (PCA) in PC-ORD (MjM Software Design, Gleneden Beach, OR) to determine community patterns among the treatments. A multi-response permutation procedure (MRPP) was performed to determine if community structure, based on microbial FAME patterns, differed significantly among the treatments ($p < 0.05$).

Results

Tables 1 and 2 are a summary of the moisture content and microbial properties that were measured in trenched biochar or soil, and in soils sampled at different distances away from the trench. The values shown are the mean of three replicate plots for 2009 (Table 1), immediately after the trench experiment was implemented, and for 2010 (Table 2), one year after the experiment began. Specific effects of biochar trench rate, distance from trench, and sampling year are described in detail in subsequent tables and figures only when these effects were statistically significant.

Table 1. Moisture content and microbial properties of trenched soil or biochar, and soil sampled 6, 12, or 18 inches from the trench. Samples were collected in July 2009, immediately after trenching. Values are the mean of three replicate field plots

Biochar	Distance (inches)	Soil moisture (%)	MBC ($\mu\text{g g}^{-1}$)	SIR ($\text{mg CO}_2 \text{ g}^{-1} \text{ d}^{-1}$)	Peroxidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	Cellobioside ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	β -glucosidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	FAME mass (nmoles g^{-1})	FAME richness (#)	FAME diversity (Shannon's index, H)	FAME AMF (relative mole %)
0	0	5.1	423	2.08	0.72	0.54	2.35	331	30	3.03	2.36
	6	10.4	420	1.96	0.76	2.04	7.40	361	33	3.06	2.64
	12	11.7	420	1.93	0.59	1.16	5.04	488	32	3.02	2.63
	18	10.3	418	1.90	0.62	1.40	4.78	523	32	3.03	3.20
12.5	0	8.5	426	2.19	3.84	2.44	0.19	125	13	2.26	1.08
	6	9.1	419	1.93	0.60	Nd	3.12	335	33	3.07	3.61
	12	9.6	438	2.69	0.55	1.76	7.32	517	33	3.04	3.54
	18	11.2	417	1.87	0.36	1.29	4.39	397	30	3.00	3.89
25	0	14.9	436	2.61	3.90	1.86	0.00	143	14	2.39	2.92
	6	10.7	416	1.81	0.41	1.15	5.12	362	31	3.01	3.27
	12	11.8	411	1.64	0.44	1.63	4.74	351	31	3.02	3.53
	18	13.7	412	1.67	0.69	1.58	6.45	363	29	2.97	3.49
50	0	13.5	483	4.47	5.40	0.61	0.31	117	12	2.21	1.75
	6	11.1	437	2.66	0.60	1.82	5.15	419	33	3.06	3.42
	12	10.6	437	2.63	0.89	2.32	7.98	499	33	3.05	3.68
	18	10.1	432	2.46	0.96	0.61	2.87	460	32	3.03	4.01
75	0	24.3	417	1.84	2.39	1.03	0.72	115	8	1.81	0.00
	6	11.5	419	1.93	0.88	0.76	3.89	349	33	3.06	5.23
	12	11.4	418	1.90	1.01	1.02	3.94	481	33	3.07	5.48
	18	11.1	420	1.96	0.88	1.27	3.44	501	33	3.05	3.77

MBC = microbial biomass C, SIR = substrate induced respiration, FAME = fatty acid methyl ester, AMF = arbuscular mycorrhizal FAME biomarker (16:1 ω 5c).

Where applicable, units are in per g (g^{-1}) dry soil or biochar.

Nd = no data.

Table 2. Moisture content and microbial properties of trenched soil or biochar, and soil sampled 6, 12, or 18 inches from the trench. Samples were collected in July 2010, one year after trenching. Values are the mean of three replicate field plots

Biochar	Distance (inches)	Soil moisture (%)	MBC ($\mu\text{g g}^{-1}$)	SIR ($\text{mg CO}_2 \text{ g}^{-1} \text{ d}^{-1}$)	Peroxidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	Cellobioside ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	β -glucosidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	FAME mass (nmoles g^{-1})	FAME richness (#)	FAME diversity (Shannon's index, H)	FAME AMF (relative mole %)
0	0	9.9	406	1.42	0.90	0.84	5.62	358	30	2.98	4.59
	6	10.1	402	1.28	1.12	1.37	7.78	256	29	2.93	6.25
	12	13.2	396	1.04	0.87	1.61	5.26	216	27	2.92	3.28
	18	9.8	402	1.28	0.85	1.46	7.15	260	29	2.97	6.64
12.5	0	11.8	415	1.78	0.89	1.25	7.64	163	23	2.77	7.12
	6	9.6	411	1.63	1.02	0.97	6.39	252	28	2.92	7.94
	12	8.4	394	0.95	1.37	1.84	9.29	239	28	2.91	8.90
	18	9.2	398	1.10	1.11	0.84	5.17	162	28	2.91	6.98
25	0	13.3	416	1.80	0.89	1.16	4.66	272	22	2.70	6.76
	6	9.9	400	1.19	0.87	1.46	6.52	244	29	2.92	8.76
	12	13.3	401	1.22	1.09	1.69	6.93	177	28	2.88	7.44
	18	13.9	396	1.04	0.94	1.44	8.34	160	24	2.79	8.31
50	0	22.6	403	1.30	0.86	2.29	14.8	150	18	2.46	2.46
	6	10.8	408	1.48	0.88	1.29	7.18	254	30	2.89	13.9
	12	9.7	407	1.48	0.92	1.18	6.69	198	27	2.80	14.1
	18	10.6	405	1.36	0.93	1.57	11.8	232	28	2.84	13.9
75	0	31.4	405	1.39	0.70	4.48	27.9	123	11	2.11	2.60
	6	11.0	403	1.31	0.87	1.57	5.49	317	27	2.83	7.37
	12	11.1	408	1.48	1.18	1.18	7.60	165	26	2.89	7.97
	18	10.4	403	1.31	1.02	1.45	7.03	179	28	2.94	7.39

MBC = microbial biomass C, SIR = substrate induced respiration, FAME = fatty acid methyl ester, AMF = arbuscular mycorrhizal FAME biomarker (16:1 ω 5c).

Where applicable, units are in per g (g^{-1}) dry soil or biochar.

Nd = no data.

Moisture content

Moisture content of biochar or soil was significantly affected by a biochar × distance interaction, as well as distance × year interaction. For the former, the effect of sampling distance depended on the biochar trench rate, which was consistent for both sampling years. In control plots (0 tons biochar/acre), soil within the trench was drier than soil away from the trench, particularly 12 inches away (Table 3). The opposite pattern occurred for biochar applied at the highest rates (50 or 75 tons/acre), where the biochar in the trench was significantly wetter than soil next to the trench.

Table 3. Moisture content of soils sampled at increasing distance away from trenched soil (control) or biochar. Values are means of three replicate plots sampled twice, in July 2009 and 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Biochar rate tons acre ⁻¹	Sampling distance from trench (inches)			
	0	6	12	18
	-----Moisture content (%)-----			

	(LSD = 4.1)			
0	7.5 b	10.3 ab	12.5 a	10.0 ab
12.5	10.2 a	9.3 a	9.0 a	10.2 a
25	14.1 a	10.3 a	12.6 a	13.8 a
50	18.0 a	10.9 b	10.2 b	10.3 b
75	27.8 a	11.3 b	11.3 b	10.8 b

There was also an effect of sampling distance, although it differed slightly between the two years (Table 4). In 2009, material in the trench (averaged across all biochar rates) was significantly wetter than soil 6 inches away, whereas in 2010, the material in the trench was significantly wetter than soils measured at all distances from the trench.

Table 4. Moisture content (%) of soils sampled at increasing distance away from trenched soil (control) or biochar. Values are means of three replicate plots and five biochar rates, sampled in either July 2009 or 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Year	Sampling distance from trench (inches)			
	0	6	12	18
	-----Moisture content (%)-----			

	(LSD = 2.6)			
2009	13.2 a	10.5 b	11.0 ab	11.3 ab
2010	17.8 a	10.3 b	11.1 b	10.8 b

Microbial biomass C and SIR

There was a significant interaction between biochar trench rate and sampling year on MBC and SIR, with MBC and SIR responding to biochar trench rate in 2009 but not in 2010. Regardless of sampling distance from the trench, trenching with 50 or 75 tons biochar/acre increased soil MBC and respiration activity compared to levels found in the 0, 12.5 and 25 tons/acre plots (Table 5). The effect was not permanent, however, as MBC and respiration activity were similar among all plots in 2010.

MBC and SIR were also affected by sampling distance from the trench. Regardless of biochar trench rate or sampling year, MBC and SIR were greater within the trench than in the nearby soil (Table 6). In the control plots, respiration activity may have been stimulated by the disturbance that occurred during soil trenching; physical disturbance may have destroyed some of the soil aggregates, thereby increasing the bioavailability of once physically-protected organic matter. The results also provide evidence that biochar can support relatively high levels of microbial respiration activity. Because MBC was determined based on the SIR values, MBC would follow the same trends. However, an independent measurement of microbial biomass, based on the biomass of microbial fatty acids, indicates that microbial biomass may actually be lower in biochar than in nearby soil (see below).

Table 5. Microbial biomass carbon (MBC) and substrate-induced respiration (SIR) of field plots receiving increasing rates of biochar amendment. Values are means of three replicate plots and four sampling distances from trenches, sampled in either July 2009 or 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Year	Biochar rate (tons acre ⁻¹)				
	0	12.5	25	50	75
-----MBC (μg g ⁻¹ soil or biochar)-----					
----- (LSD = 9)					
2009	420 b	425 b	419 b	447 a	418 b
2010	402 a	405 a	403 a	405 a	404 a
----- SIR (mg CO ₂ g ⁻¹ soil or biochar d ⁻¹)-----					
----- (LSD = 0.53)					
2009	1.97 b	2.17 b	1.93 b	3.06 a	1.91 b
2010	1.25 a	1.36 a	1.31 a	1.41 a	1.37 a

Table 6. Microbial biomass carbon (MBC) and substrate-induced respiration (SIR) of soils sampled at increasing distance away from trenched soil (control) or biochar. Values are means of three replicate plots, five biochar rates, and two sampling years. Within a column, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Sampling distance from trench (inches)	MBC ($\mu\text{g g}^{-1}$ soil or biochar)	SIR ($\text{mg CO}_2 \text{ g}^{-1}$ soil or biochar d^{-1})
0	423 a	2.09 a
6	414 b	1.72 b
12	413 b	1.70 b
18	410 b	1.59 b
LSD	8	0.33

Enzyme activities

Three enzymes related to C cycling were selected so that a range of C substrate lability were represented: 1) peroxidase degrades recalcitrant and aromatic forms of C, including lignin and soil humus, 2) β -D-cellubioside degrades cellulose (intermediate lability) into cellobiose, a dimer of two glucose units, and 3) β -glucosidase degrades cellobiose (highly labile) into individual glucose units.

Peroxidase enzyme activity was significantly affected by a biochar \times sampling distance \times sampling year interaction. In 2009, peroxidase enzyme activity was greater in the trenched biochar than in soil outside the trench, whereas peroxidase enzyme activity was similar among soils sampled at 0, 6, 12, and 18 inches in the control plots. The effect was not permanent, however, peroxidase enzyme activity in 2010 was similar between trenched material and nearby soil for all biochar trench rates. During the processing of soil samples in 2010, it was noted that biochar collected from the trenches often contained soil, indicating that mixing of soil into the trench had occurred during the study year. The input of soil into the trench may have diluted the peroxidase activity so that by 2010, activities became similar across the sampling distances.

Due to high variability among replicate samples, β -D-cellubioside activity was not affected by biochar trench rate, sampling distance from the trench, sampling year, or any of their interactions. β -D-cellubioside activities ranged from 0.54 to 2.44 $\mu\text{mol product g}^{-1} \text{ h}^{-1}$ in 2009 and from 0.84 to 4.48 $\mu\text{mol product g}^{-1} \text{ h}^{-1}$ in 2010 (Tables 1 and 2).

The only factor that significantly affected β -glucosidase activity was that of sampling year. When averaged across all plots, the activity of this enzyme class was 3.93 $\mu\text{mol product g}^{-1} \text{ h}^{-1}$ in 2009 and 8.46 $\mu\text{mol product g}^{-1} \text{ h}^{-1}$ in 2010.

Table 7. Peroxidase enzyme activity of trenched soil or biochar, and soil sampled 6, 12, or 18 inches from the trench. Samples were collected either in July 2009 or July 2010. Values are the mean of three replicate field plots. Within a year and biochar application rate combination, means followed by different lowercase letters are significantly different ($p < 0.05$, LSD mean separation test).

Biochar (ton acre ⁻¹)	Distance (inches)	2009	2010
-----Peroxidase activity ($\mu\text{mol product g}^{-1} \text{h}^{-1}$)-- ----- (LSD = 1.03)			
0	0	0.72 a	0.90 a
	6	0.76 a	1.12 a
	12	0.59 a	0.87 a
	18	0.62 a	0.85 a
12.5	0	3.84 a	0.89 a
	6	0.60 b	1.02 a
	12	0.55 b	1.37 a
	18	0.36 b	1.11 a
25	0	3.90 a	0.89 a
	6	0.41 b	0.87 a
	12	0.44 b	1.09 a
	18	0.69 b	0.94 a
50	0	5.40 a	0.86 a
	6	0.60 b	0.88 a
	12	0.89 b	0.92 a
	18	0.96 b	0.93 a
75	0	2.39 a	0.70 a
	6	0.88 b	0.87 a
	12	1.01 b	1.18 a
		0.88 b	1.02 a

In summary, biochar trenching did not appear to negatively impact the activities of enzymes involved in cellulose decomposition. In contrast, peroxidase enzyme activity was elevated in biochar material compared to soil, indicating that the aromatic and recalcitrant nature of biochar stimulated the microbial production of peroxidases. However, peroxidase activity declined over time so that there was no difference between trenched biochar and soil in 2010. This may be due to exhaustion of degradable C substrates in biochar and thus loss of enzyme activity, or due to the mixing of biochar with soil and thus the dilution of high enzyme activity from biochar with low enzyme activity from soil.

Microbial community FAMES

The total mass of microbial FAMES extracted from soil was affected by sampling distance in 2009 but not in 2010 (significant distance \times year interaction) (Table 8). In 2009, FAME biomass was lowest within the trench, and highest in soils 12 and 18 inches from the trench, suggesting that biochar initially had a lower microbial biomass than soil on a per g basis. For control plots, microbial biomass may have been lower in trenched soil than soil nearby the trench due to the physical disturbance that occurred during trenching. By July 2010, these effects had diminished, and FAME biomass was equivalent across all sampling distances. Except for inside the trench, FAME biomass in soil was lower in 2010 than in 2009, but the reasons for this year effect are not known. This result is not unusual, though, and reflects the dynamic properties of microbial communities as they respond to changes in their environment, even on an annual scale.

The FAME result contrasts with the MBC pattern described earlier, which found greater MBC in the trench than in the nearby soil (Table 6). Because FAMES are an actual component of microbial biomass (e.g., fatty acids within cell membranes and storage compounds), the FAME results may reflect actual biomass trends whereas the MBC results reflect trends in respiration activity. However, it is possible that FAME extraction efficiency was lower in biochar than in soil samples, due to the ability of biochar material to absorb extractants and organic chemicals (perhaps including fatty acids). More research is needed to identify the best method for determining microbial biomass in biochar.

Table 8. Total biomass, Shannon's diversity index, and number of microbial fatty acid methyl esters (FAMES) from soils sampled at increasing distance away from trenched soil (control) or biochar. Values are means of three replicate plots and five biochar rates, sampled in either July 2009 or 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Year	Sampling distance from trench (inches)			
	0	6	12	18
-----FAME biomass (nmole g ⁻¹)-----				
----- (LSD = 68.2)				
2009	171 c	365 b	467 a	449 a
2010	213 a	265 a	199 a	199 a
-----FAME diversity-----				
----- (LSD = 0.10)				
2009	2.34 b	3.05 a	3.04 a	3.02 a
2010	2.60 b	2.90 a	2.88 a	2.89 a
-----Number of FAMES-----				
----- (LSD = 2)				
2009	16 b	33 a	32 a	31 a
2010	21 b	28 a	27 a	27 a

Diversity is a measure that encompasses both the number of species (in this case, the number of FAMES), and the distribution of these species/FAMES within the community. Communities with more species, and with species evenly distributed, are more diverse than communities with fewer species and/or having a few species dominating over rare species. Microbial FAMES can be used as an indicator for microbial species diversity, based on the assumption that diverse microbial communities will contain more types of FAMES and greater FAME diversity.

In this study, the interaction between sampling distance and year on FAME diversity and number was significant. In both years, the diversity and number of FAMES were significantly lower inside the trench than in soils outside the trench, regardless of the biochar trench rate (Table 8). The difference was dramatic in 2009, when the number of FAMES in the trench was one-half the number detected in soils 6, 12, and 18 inches away. While the trend continued in 2010, the differences in FAME diversity and number between inside and outside the trench were not as great, either due to soil from outside the trench mixing with soil/biochar inside the trench, and/or recovery and growth of microbial communities residing in trenched soil or biochar.

Microbial FAME diversity and number were also affected by the interaction between biochar trench rate and sampling distance from the trench (Table 9). Regardless of sampling year, FAME diversity and number in control plots (0 ton biochar/acre) were not affected by sampling distance, whereas for all other biochar rates, FAME diversity and number were significantly lower in the biochar inside the trench than in soil outside the trench.

Table 9. Shannon's diversity index and number of microbial fatty acid methyl esters (FAMES) from soils sampled at increasing distance away from trenched soil (control) or biochar. Values are means of three replicate plots sampled twice, in July 2009 and 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Biochar rate tons acre ⁻¹	Sampling distance from trench (inches)			
	0	6	12	18
-----FAME diversity----- -----				
(LSD = 0.15)				
0	3.00 a	3.00 a	2.97 a	3.00 a
12.5	2.57 b	2.99 a	2.98 a	2.95 a
25	2.58 b	2.96 a	2.95 a	2.88 a
50	2.34 b	2.97 a	2.92 a	2.94 a
75	1.96 b	2.94 a	2.98 a	2.99 a
-----Number of FAMES----- -----				
(LSD = 3)				
0	30 a	31 a	30 a	31 a
12.5	19 b	31 a	30 a	29 a
25	19 b	30 a	29 a	27 a
50	15 b	31 a	30 a	30 a
75	10 b	30 a	30 a	31 a

The relative abundance of FAME 16:1 ω 5c, a biomarker for arbuscular mycorrhizal fungi (AMF) was affected by the biochar rate \times sampling year interaction, as well as sampling distance from the trench. Initially, biochar trench rate had no effect on the AMF biomarker, whose relative abundance was similar among the five rates, regardless of sampling distance (Table 10). In 2010, however, the relative abundance of this biomarker increased significantly from the 0 ton/acre plots to the 50 tons/acre plots, and then declined again in the 75 tons/acre plots. These data suggest that biochar amendment to soil can enhance the proportion of the microbial community composed of AMF, but only to a certain point (50 ton/acre).

Table 10. Relative abundance of FAME biomarker for arbuscular mycorrhizal fungi (AMF) from field plots receiving increasing rates of biochar amendment. Values are means of three replicate plots and four sampling distances from trenches, sampled in either July 2009 or 2010. Within a row, means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Year	Biochar rate (tons acre ⁻¹)				
	0	12.5	25	50	75
-----AMF (mole %)------					

(LSD = 2.11)					
2009	2.71 a	3.21 a	3.33 a	3.21 a	3.62 a
2010	5.19 c	7.74 b	7.81 b	11.1 a	6.33 bc

When averaged across biochar rates and sampling year, the relative abundance of AMF biomarker was also affected by sampling position (Fig. 1). The percent AMF biomarker was lower inside the trench compared to soil sampled outside the trench. In control plots, trenching of soil resulted in a physical disturbance that likely disrupted AMF hyphal networks and reduced their population size compared to soil outside the trench. In plots receiving trenched biochar, biochar stimulated AMF populations in soil up to 18 inches away from the trench.

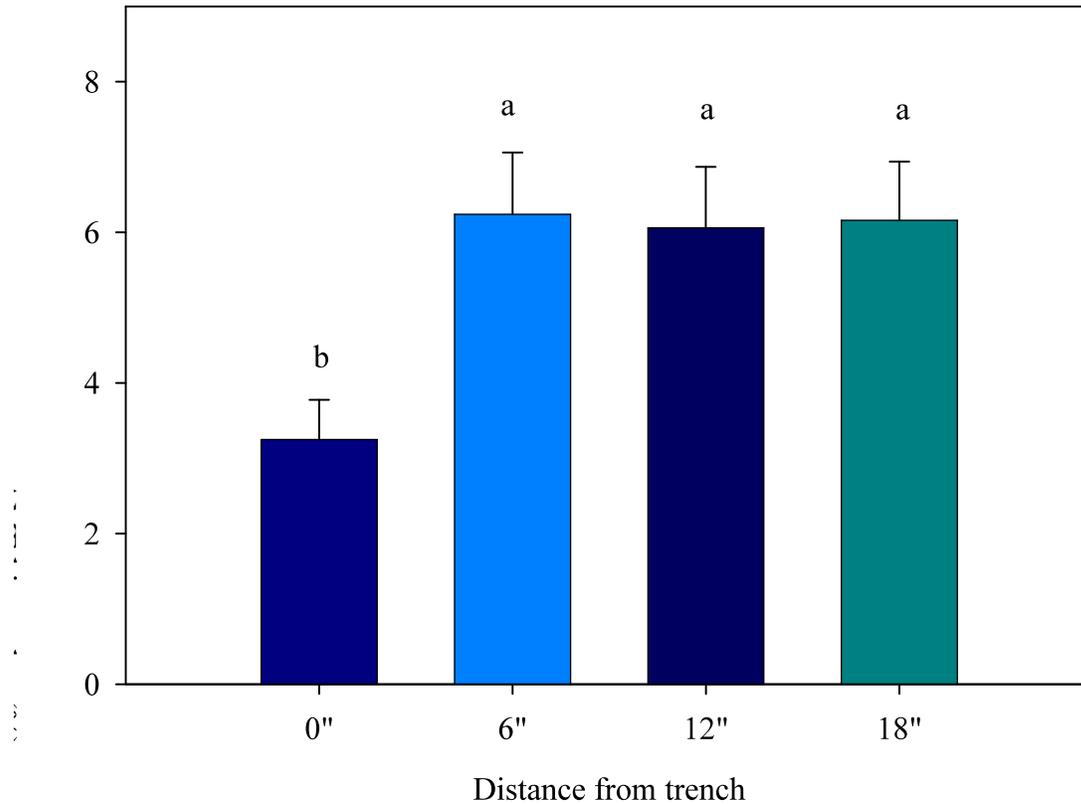


Fig. 1. Relative abundance (%) of FAME biomarker for arbuscular mycorrhizal fungi (AMF) as affected by sampling distance away from trenched biochar or soil. Data are means of five biochar rates, two sampling years, and three replicate field plots. means followed by different lower case letters are significantly different ($p < 0.05$, LSD mean separation test).

Microbial community structure was significantly affected by biochar rate, as indicated by multivariate analyses of community FAME data. In both years, microbial communities inside the trench (0") were significantly different from soil microbial communities according to the PCA and MRPP analyses (data not shown). This was because of the low diversity and number of FAMES in biochar compared to soils. When biochar samples were removed from the analysis, it was clearer to distinguish whether microbial community differences existed among the soil samples at 6, 12, or 18 inches from the trenches.

In 2009, microbial communities differed significantly from each other based on distance from the trench. Fig. 2 shows the 2009 community FAME patterns in a two-dimensional space, where each axis or PC represents a linear combination of all the FAMES, similar to a multiple linear regression. Communities that are similar to each other cluster together, such as the tight clustering of microbial communities from the 50 and 75 tons/acre biochar treatments (red and yellow symbols, respectively, in Fig. 2). Communities from these two treatments were structurally similar according to MRPP analysis. However, soil microbial community composition from the 50 tons/acre treatment was significantly different from the microbial community composition of the 0, 12.5, and 25 tons/acre treatments, and soil microbial community composition of the 75 tons/acre treatment was also significantly different from the microbial community composition of the 25 tons/acre treatment (green symbols).

Some of these patterns continued into July 2010. Despite some overlap in community structures as evidenced by the PCA in Fig. 3, the MRPP analysis revealed that overall, soil microbial communities from the 50 ton/acre biochar treatment remained significantly different from soil microbial communities of the 0 and 12.5 tons/acre treatments. In addition, soil microbial community composition from the 50 tons/acre treatment was now significantly different than community composition of the 75 tons/acre treatment. In both years, the differences among communities was mainly driven by the large proportion of AMF in soils from the 50 ton/acre treatment, with this FAME biomarker showing a strong negative correlation to PC 1 axis ($r = -0.86$) in 2010. This explanation is also supported by the data in Table 10.

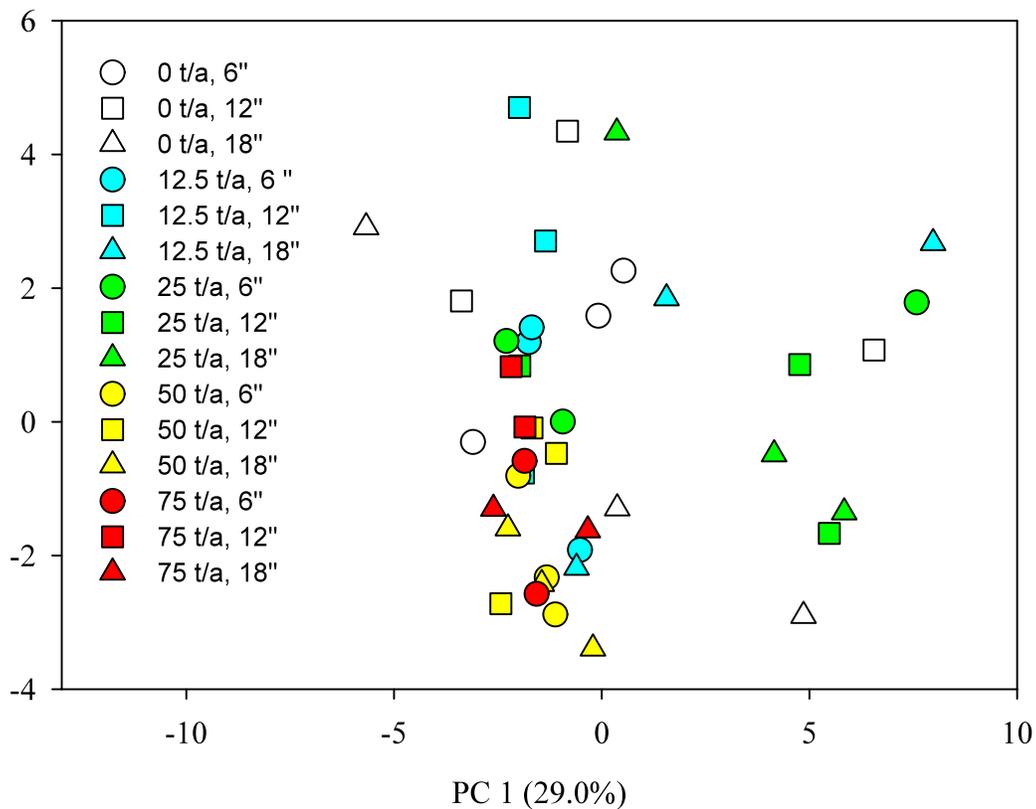


Fig. 2. Principal components analysis of microbial community fatty acid methyl esters (FAMES) extracted from field plot soils in July 2009. Biochar was trenched at rates ranging from 0-to-75 tons acre⁻¹, and soil samples were collected at increasing distances away from the trench (6, 12 or 18 inches) in each of three replicate plots. The percent variance explained by each principal component (PC) is shown in parentheses.

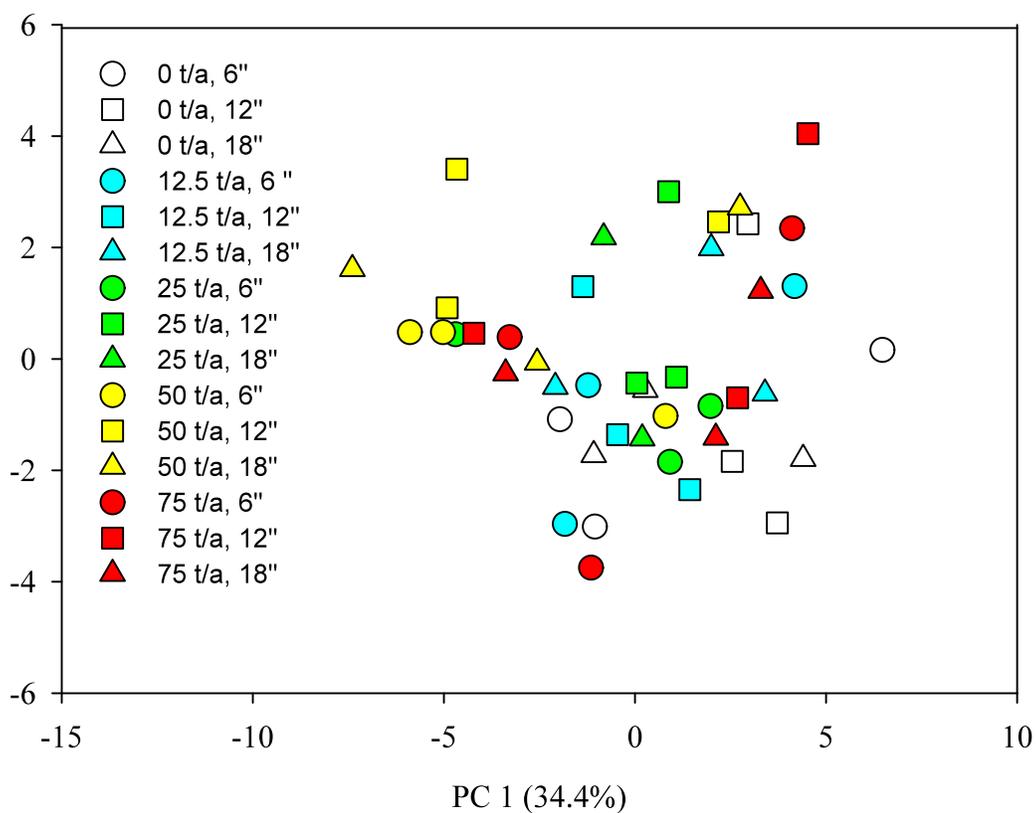


Fig. 2. Principal components analysis of microbial community fatty acid methyl esters (FAMES) extracted from field plot soils in July 2010. Biochar was trenched at rates ranging from 0-to-75 tons acre⁻¹, and soil samples were collected at increasing distances away from the trench (6, 12 or 18 inches) in each of three replicate plots. The percent variance explained by each principal component (PC) is shown in parentheses.

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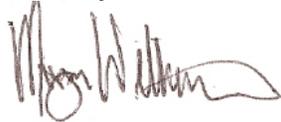
- h) **Biochar porosity:** Varies significantly by biochar type and age.
- i) **Forage yield:** We did not find that CQuest Biochar applied in trenches significantly impacted plant growth positively at rates up to 50T per acre, but did find insignificant negative responses at rates of 75 T per acre.

Additional years of data collection are needed to further explore the significance of these initial findings. In all fairness, our research protocol to monitor forage performance may have been slightly flawed given the fact that forage data was only collected from 18 inches on either side of the treatment trenches. Larger plots, or more replicates, are likely needed to better vet forage yield given the application methods tested.

- j) **Repeated soil testing and forage yield:** Completed
- k) **Data analysis:** Completed spring 2011
- l) **Final report:** Completed spring 2011. A final movie summing up what we have learned will be made available to the Colorado Department of Agriculture for use at it sees fit.

Thank you for the opportunity to conduct this important research. We will continue to keep you informed as our understanding of biochar evolves. If additional clarification is needed, please do not hesitate to contact me directly.

Sincerely,



Morgan Williams
Executive Director
Flux Farm Foundation

Table 2. Moisture content and microbial properties of trenched soil or biochar, and soil sampled 6, 12, or 18 inches from the trench. Samples were collected in July 2010, one year after trenching. Values are the mean of three replicate field plots

Biochar	Distance (inches)	Soil moisture (%)	MBC ($\mu\text{g g}^{-1}$)	SIR ($\text{mg CO}_2 \text{ g}^{-1} \text{ d}^{-1}$)	Peroxidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	Cellobioside ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	β -glucosidase ($\mu\text{mol product g}^{-1} \text{ h}^{-1}$)	FAME mass (nmoles g^{-1})	FAME richness (#)	FAME diversity (Shannon's index, H)	FAME AMF (relative mole %)
0	0	9.9	406	1.42	0.90	0.84	5.62	358	30	2.98	4.59
	6	10.1	402	1.28	1.12	1.37	7.78	256	29	2.93	6.25
	12	13.2	396	1.04	0.87	1.61	5.26	216	27	2.92	3.28
	18	9.8	402	1.28	0.85	1.46	7.15	260	29	2.97	6.64
12.5	0	11.8	415	1.78	0.89	1.25	7.64	163	23	2.77	7.12
	6	9.6	411	1.63	1.02	0.97	6.39	252	28	2.92	7.94
	12	8.4	394	0.95	1.37	1.84	9.29	239	28	2.91	8.90
	18	9.2	398	1.10	1.11	0.84	5.17	162	28	2.91	6.98
25	0	13.3	416	1.80	0.89	1.16	4.66	272	22	2.70	6.76
	6	9.9	400	1.19	0.87	1.46	6.52	244	29	2.92	8.76
	12	13.3	401	1.22	1.09	1.69	6.93	177	28	2.88	7.44
	18	13.9	396	1.04	0.94	1.44	8.34	160	24	2.79	8.31
50	0	22.6	403	1.30	0.86	2.29	14.8	150	18	2.46	2.46
	6	10.8	408	1.48	0.88	1.29	7.18	254	30	2.89	13.9
	12	9.7	407	1.48	0.92	1.18	6.69	198	27	2.80	14.1
	18	10.6	405	1.36	0.93	1.57	11.8	232	28	2.84	13.9
75	0	31.4	405	1.39	0.70	4.48	27.9	123	11	2.11	2.60
	6	11.0	403	1.31	0.87	1.57	5.49	317	27	2.83	7.37
	12	11.1	408	1.48	1.18	1.18	7.60	165	26	2.89	7.97
	18	10.4	403	1.31	1.02	1.45	7.03	179	28	2.94	7.39

MBC = microbial biomass C, SIR = substrate induced respiration, FAME = fatty acid methyl ester, AMF = arbuscular mycorrhizal FAME biomarker (16:1 ω 5c).

Where applicable, units are in per g (g^{-1}) dry soil or biochar.

Nd = no data.