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Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments

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The effects of pyrogenic carbon on the microbial diversity of forest soils were examined by comparing two soil types, fire-impacted and non-impacted, that were incubated with laboratory-generated biochars. Molecular and culture-dependent analyses of the biochar-treated forest soils revealed shifts in the relative abundance and diversity of key taxa upon the addition of biochars, which were dependent on biochar and soil type. Specifically, there was an overall loss of microbial diversity in all soils treated with oak and grass-derived biochar as detected by automated ribosomal intergenic spacer analysis. Although the overall diversity decreased upon biochar amendments, there were increases in specific taxa during biochar-amended incubation. DNA sequencing of these taxa revealed an increase in the relative abundance of bacteria within the phyla Actinobacteria and Gemmatimonadetes in biochar-treated soils. Together, these results reveal a pronounced impact of pyrogenic carbon on soil microbial community composition and an enrichment of key taxa within the parent soil microbial community.

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1. Introduction

Long-term carbon sequestration strategies are urgently being sought to counter the effects of rising levels of atmospheric carbon dioxide (CO_2). The production and application of pyrogenic, biomass-derived black carbon, or biochar, to soils have emerged as a viable tool for the stable, long-term storage of carbon in terrestrial ecosystems (Glaser et al., 2001; Lehmann et al., 2009; Marris, 2006). Whereas 80–90% of the carbon in uncharred biomass remineralizes within 5–10 years, the carbon in biochar undergoes slower degradation with carbon half-lives in the order of 10^3–10^7 years (Zimmerman, 2010). In addition, biochar amendments can enhance soil fertility while reducing water and fertilizer needs (Glaser et al., 2001; Lehmann et al., 2009; Chan et al., 2007; Glaser, 2007). The cause of this fertility enhancement is likely related to the ability of the biochar to absorb and exchange nutrients and natural organic matter. Prior research has suggested that changes in soil microbial community composition may also play an important role. First, ancient pyrogenic carbon-enriched soils, such as Amazonian Dark Earths (Terra Preta), have been shown to contain greater microbial biomass and, in some cases, greater diversity than surrounding non-enriched soils (Kim et al., 2007; Jesus et al., 2009; O'Neill et al., 2009). Second, filamentous microbes, including fungi, have been observed to infiltrate biochar particles over time, thus facilitating their degradation (Hockaday et al., 2007). Lastly, in soils amended with yeast and glucose derived pyrogenic biochar, increases in fungal and Gram-negative bacterial biomass have been observed using phospholipid fatty acid analyses (Steinbeiss et al., 2009).

In this current study, we expanded upon these previous biochemical studies by using molecular methods to examine the overall taxonomic changes in bacterial community structure due to experimental pyrogenic carbon amendments. We hypothesized that the soil type and source of the pyrogenic carbon would influence the microbial composition and target the growth of specific taxa within the soil community. To address this hypothesis we compared changes in biomass and microbial diversity within the soil communities and assessed whether these changes varied with pyrogenic carbon type and soil pre-conditioning to pyrogenic carbon sources.

2. Materials and methods

2.1. Soil sampling and biochar synthesis

Soils were collected in November 2008 from the surface layers (upper 10 cm) of two forests in north-central Florida. The first site

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was an upland hardwood forest located within a residential area near Gainesville, FL and had no historical record of burning (latitude: 29.6011; longitude: −82.3623). This unburned forest soil had an organic carbon content of 3.8 wt%, was primarily sandy and had no visible evidence of charcoal. The second sampling site was primarily a longleaf-slash pine-palmetto (i.e. pine flatwood) forest located in the Austin Cary Memorial Forest (ACMF), near Gainesville, FL (latitude: 29.7340; longitude −82.2171) that has been managed through controlled burns carried out periodically since the 1950s and annually since 1982. The ACMF burned forest soil was also sandy but had visible flecks of charcoal and an organic carbon content of 2.5 wt%. The unburned and burned forest soils were classified as Entisols and had sand contents of 76.1 and 98.4%, clay contents of 18.2 and 1.6%, and pH of 5.4 and 5.9, respectively.

The soils were augmented with two synthesized biochars derived from Laurel oak heartwood (*Quercus laurifolia* Michx.) and Eastern gama grass (*Tripsacum dactyloides* (L.) L.). The oak and grass biochars were generated by combusting 10–20 g of the material in a 0.04 m^3 standard oven under full atmosphere for 3 h at 250 °C (hereafter denoted Oak250) or by pyrolyzing 5–10 g of the biomass under N_2 in a custom-built pyrolyzer (5.5 cm diameter × 50 cm length pipe) for 3 h at 650 °C (hereafter denoted Oak650 or Grass650). The chars were lightly crushed with a mortar and pestle and sieved into a uniform size fraction of between 250 μm and 2 mm and sterilized in an autoclave. All biochars were then rinsed briefly with sterile water to remove fine ash.

The chemical characteristics of these biochars have been previously described (Zimmerman, 2010; Kasozi et al., 2010). Briefly, the Oak650 and Grass650 biochars were nanoporous (225 and 77 m2 g^{-1}; BET surface area, respectively) but all the biochars were microporous (331–528 m^2 g^{-1} CO_2 adsorption surface area). The Oak250, Oak650, and Grass650 biochars had organic C weight contents of 55, 79, and 64%, volatile contents of 66, 21, and 33%, respectively, and a pH of 3.5, 9.1 and 10 respectively. The pH of all soil and biochar mixtures ranged between 6.1 and 6.3.

2.2. Soil incubations

To assess the effects of biochar on the microbial soil community structure, each soil type was incubated in triplicate with and without biochar supplements. Soils were aliquoted (3 g) into individual glass vials with gas permeable covers. A subset of the soils was amended with 300 mg of oak (Oak250 and Oak650) and grass (Grass650) biochar. Sterilized distilled water (1.2 mL) was added to each vial at time zero to bring soils to their maximum holding capacity. The biochar-treated soils along with the unamended controls were incubated at 32 °C for 188 days in the dark. These conditions were chosen to optimize growth of heterotrophic microbes. Two additional replicate incubations were conducted in parallel for cell cultivation and CO_2 respiration analyses. These parallel incubations were carried out in sterilized 12 mL borosilicate vials with rubber septum and were scaled down to one-third size (1 g soil; 100 mg biochar; 0.4 mL water).

2.3. CO_2 evolution

To monitor microbial respiration within the soil treatments, CO_2 evolution was measured from the parallel incubations and compared on Day 62, 109 and 188 of the incubations. Headspace CO_2 was measured by purging, using CO_2-free air as a carrier gas, into an automated CO_2 coulometer (UIC Inc., Joliet, IL), leaving the vials refilled with CO_2-free air for continued incubation. The analytical detection limit for CO_2, determined using acidification of $CaCO_3$ standards, was found to be 0.1 μg C. 'Total' CO_2 evolution was the sum of three individual CO_2 measurements over the 188 days, whereas the 'mean' evolution rate was the mean of each of the three individual respiration rates calculated as evolved CO_2 divided by the number of days incubated since the last measurement.

2.4. Quantitative real time PCR

The microbes of the treated and untreated soils were assessed using quantitative real time PCR (qPCR). Genomic DNA was extracted in quadruplicate from approximately 100 mg of each soil treatment using the MoBio DNA Powersoil kit (Carlsbad, CA), quantified using the Quant-iT PicoGreen kit (Invitrogen, Carlsbad, CA) and normalized. Total recovery of genomic DNA ranged from 300 to 800 ng of DNA per 100 mg of sample material. Amplification and detection of the small subunit (SSU) ribosomal RNA (i.e. 16S rRNA) gene by qPCR were performed using the Roche Light Cycler-480 platform (Roche Diagnostics, Indianapolis, IN) in quadruplicate and the mean values were calculated. PCR reactions were run in 20 μL total volume containing final concentrations of 1× SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN), 0.4 μg mL^{-1} BSA, 0.5 μM each primer and 5 ng template DNA. The primers used in this study targeted the 16S rRNA genes of organisms from the domain Bacteria and have been previously employed for community composition analysis in soils (Fierer et al., 2005). The bacterial primer set included the forward primer EUB338 (5′-ACTCCTACGGGAGGCAGCAG; Lane, 1991) and the reverse primer EUB518 (5′-ATTACCGCGGCTGCTGG; Muyzer et al., 1993). The reaction conditions included incubation at 95 °C for 10 min followed by 30 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. A melting curve analysis was performed at 95 °C for 30 s; 65 °C, for 30 s with continuous monitoring at 95 °C to ensure that the derived fluorescent measurements were PCR products and not primer-dimers. DNA standards were generated from 16S rRNA amplicon library of unburned forest soil generating a 5-point curve. Copy numbers were based on the standard curve and an assumption that the average molecular mass of double stranded DNA is 660 g per mol (Fierer et al., 2005).

2.5. Cultivation of bacteria and Actinobacteria

Subsets of the soil treatments (0.1 g) were stirred with 10 mL of distilled water for 5 min. These suspensions were serially diluted and culturable cells were quantified using the pour plate method. Briefly, 1 mL of each dilution was added to 9 mL of agar-media maintained at 45 °C and then poured into sterile Petri dishes. To assess the non-fastidious culturable bacterial community a Nutrient agar containing (w/v) 0.3% beef extract, 0.5% peptone, 0.8% NaCl, and 1.5% agar adjusted to pH 6.5 was used. Soil treatments plated on the Nutrient agar were incubated at 37 °C for 2 d before quantification. Actinobacteria were cultivated using an arginine–glycerol–salt (AGS) media (El-Nakeeb and Lechavalier, 1963; Küster and Williams, 1964) that contained (w/v) 0.1% arginine, 1.25% glycerol, 0.1% K_2HPO_4, 0.1% NaCl, 0.05% $MgSO_4$, 0.001% $Fe_2(SO_4)_3$, 0.0001% $CuSO_4$, 0.0001% $ZnSO_4$; 0.0001% $MnSO_4$, and 1.5% agar at pH 6.9. The AGS plates were incubated at 37 °C for 7 d. The arginine–glycerol–salt (AGS) medium used in this study has been shown to be selective for the isolation of cultivable soil bacteria from the phylum Actinobacteria (i.e. actinomycetes) (El-Nakeeb and Lechavalier, 1963) as L-arginine as been shown to be a selective nitrogen source for actinomycetes. Although it is possible that non-target organisms may be capable of using the L-arginine as a nitrogen source the high carbon to nitrogen ratios in AGS media also helps reduce background bacteria levels (Labeda and Shearer, 1990), and Actinobacteria have a unique cell and colony morphology that facilitates their identification.

2.6. Automated ribosomal RNA intergenic spacer analysis (ARISA)

Microbial community fingerprinting of the biochar-treated soils was performed using ARISA, as previously described (Havemann

and Foster, 2008) to compare the relative diversities of the biochar-treated soil communities. The method exploits the length and sequence heterogeneity of the intergenic transcribed spacer (ITS) region located between the small (16S) and large (23S) subunit rRNA genes in the rRNA operon (Fisher and Triplett, 1999; Mora et al., 2003). This method generates bacterial ITS profiles of target communities, thereby providing a broad comparative overview of the microbial diversity (Fisher and Triplett, 1999; Mora et al., 2003; Havemann and Foster, 2008). Briefly, ARISA was performed on all samples using universal bacterial primers S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18, in which the 5′ and 3′ ends of the primers were complementary to positions 1452 and 1472 of the 16S rRNA gene and positions 133 and 115 of the 23S rRNA gene of *Escherichia coli*, respectively (Ranjard et al., 2001; Cardinale et al., 2004). Genomic DNA was extracted in triplicate as previously described and the PCR amplification conditions were conducted as previously described (Havemann and Foster, 2008).

Upon amplification 1 μL of each PCR reaction mixture was loaded into a DNA 1000 Lab-on-a-chip according to manufacturer's protocol (Agilent Technologies, Santa Clara, CA). The samples were then analyzed with an Agilent 2100 Bioanalyzer and 2100 Expert Software, which determined the area and size of each peak based on an internal standard (15 bp and 1500 bp) and external ladder. To exclude background fluorescence threshold levels were manually set at 20 fluorescent units for all samples. The peak sizes were compared among all treatments and, according to the kit instructions, were considered to be the same if within ±5% and then averaged to obtain the sizes reported in Table 2. Peaks of interest were then recovered, cloned and sequenced as previously described (Havemann and Foster, 2008). Recovered 16S–23S rRNA – ITS sequences were analyzed to identify the most similar accessioned DNA sequences using the basic local assignment and search tool (BLASTn) algorithm of the National Center for Biotechnology Information (Altschul et al., 1997). All sequences recovered in this study were deposited in the GenBank database under accession numbers GU195786 to GU195955.

2.7. Nested PCR–ARISA

To independently confirm the Actinobacteria ITS sequencing results, and to specifically track shifts in actinobacterial community composition associated with biochar amendment, a nested PCR–ARISA was used. The soil genomic DNA extracts previously analyzed by bacterial ARISA were PCR amplified in triplicate using an Actinobacteria-specific forward primer that targets the 235 position in the 16S rRNA gene (Stach et al., 2003; Inbar et al., 2005) and the ARISA L-D-Bact-132-a-A-18 primer (Ranjard et al., 2001; Cardinale et al., 2004). PCR reactions contained (final concentrations) 1× GoTaq Flexi reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 160 μM deoxynucleoside triphosphates, 100 μg per mL bovine serum albumin, 400 nM of each primer, 20 ng of genomic DNA, 2.5U of GoTaq Flexi DNA polymerase (Promega) and water. The reaction mixtures were incubated for 3 min at 94 °C followed by 25 cycles of amplification at 94 °C for 1 min, 53 °C for 30 s, and at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The products of this primary round of PCR were then used as the template material for a second round of "nested" PCR using domain Bacteria ARISA primers and conditions as previously described above. The products of the nested PCR–ARISA were then run on an Agilent lab-on-a-chip as described above and the resulting ITS profiles were directly compared to domain Bacteria ARISA profiles using the Agilent analysis software.

2.8. Statistical analysis of ITS data

Once peak sizes were determined to be significant then the peak areas were imported into Primer 6 software package (version 6.1.10; PRIMER-E, Ivybridge, UK) and normalized using the log ($X + 1$) pre-treatment function. Samples were analyzed by Bray–Curtis similarity tool in the Primer 6 software (Legendre and Legendre, 1998) that generates a resemblance matrix and clustered in the complete linkage mode with the default parameters (5% significance; mean number of permutations, 1000; number of simulations 999) to generate percent similarity.

3. Results

Biochars produced in the laboratory under varied conditions were added to two distinct soils, one with no historical record of burnings (unburned) and another that had been annually exposed to prescribed burns over the past century (burned). Community analyses were conducted through cultivation, quantitative PCR (qPCR), bacterial community fingerprinting using ARISA and DNA sequencing of the bacterial intergenic spacer (ITS) region located between the 16S and 23S rRNA genes.

3.1. Bacterial biomass and metabolic activity with pyrogenic carbon amendments

Both the qPCR and direct plating results indicated there was a decrease in the number of bacteria in unburned soils when treated with low temperature Oak250 biochar, though respiration rates increased (Table 1). Although unsurprisingly the cultivation approach recovered, on average, less than 1% of the total soil bacteria as inferred from qPCR data of bacterial 16S rRNA gene abundance. In contrast, both unburned and burned forest soils showed an increase in the 16S rRNA gene copy number and cultivated cells with addition of biochars produced at higher temperatures (Table 1).

Plate counts of culturable colony-forming units (CFUs) of bacteria and Actinobacteria were positively correlated ($r = 0.91$; $p = 0.5$) to each other in most treatments suggesting that these taxonomic groups responded similarly to biochar amendment (Table 1). The bacteria and the Actinobacteria were specifically targeted for direct plate counting to assess whether there were broad enumeration differences between the total bacteria and one of the dominant phyla in soils, Actinobacteria. Actinobacteria cultivated on selective media comprised approximately one-third of the CFU in all incubations except one. Substantial increases in CFUs of both Bacteria and Actinobacteria were also observed when oak or grass biochars prepared at 650 °C (Oak650 and Grass650, respectively) were added to unburned soils.

Measured CO_2 evolution rates were constant over time indicating that microbial populations remained active during the incubation period (Table 1). After accounting for the very low abiotic carbon oxidation of biochar, as indicated by the evolved CO_2 measured from sterilized char-only incubations, amendments of low temperature and grass biochar were found to significantly increase microbial C respiration while high temperature oak biochar additions resulted in no significant change in respiration regardless of soil type (Table 1).

3.2. Shifts in community composition

The application of ARISA enables a rapid assessment of changes in the microbial community by exploiting the size heterogeneity of the ITS region between bacterial lineages (Deka and Mishra, 1983; Fisher and Triplett, 1999; Ranjard et al., 2001; Havemann and Foster, 2008). Bands of interest were recovered, cloned and sequenced generating 170 sequences for this study. A dendrogram (Fig. 1) based on the presence and absence of peaks within ARISA profiles (Table 2) indicated that the parent soil type was a major

Table 1
Comparison of microbial respiration and cell quantification of soil treatments in the presence and absence of biochar. Gene copy numbers and CFUs are per gram of soil; SEM refers to standard error of the mean.

Treatment		CO_2 evolution rate		16S rRNA gene copy number	Colony-forming units	
Soil	Biochar	Total[a] (μg C/6 mo)	Mean rate (μg C/day)	Bacteria ($\times 10^{10} \pm$ SEM)	Bacteria ($\times 10^{5} \pm$ SEM)	Actinobacteria ($\times 10^{5} \pm$ SEM)
–	Oak250	200 ± 96	1.07 ± 0.5	0	0	0
–	Oak650	73 ± 13	0.39 ± 0.1	0	0	0
–	Grass650	39 ± 6	0.21 ± 0.0	0	0	0
Unburned	–	2275 ± 202	12.1 ± 4.3	3.4 ± 1.1	31.8 ± 1.4	10.8 ± 0.2
Unburned	Oak250	3559 ± 39	18.9 ± 0.2	1.2 ± 0.2	24.2 ± 1.0	2.8 ± 0.1
Unburned	Oak650	2968 ± 92	15.8 ± 0.5	3.4 ± 0.9	87.7 ± 4.4	23.3 ± 0.1
Unburned	Grass650	4634 ± 64	24.7 ± 0.3	3.2 ± 1.0	118.7 ± 12.0	55.0 ± 3.5
Burned	–	2474 ± 88	13.2 ± 0.5	2.6 ± 0.5	37.9 ± 0.5	59.9 ± 0.6
Burned	Oak650	2344 ± 61	12.5 ± 0.3	4.1 ± 0.6	103.5 ± 10.0	47.5 ± 3.2

Notes: 16S rRNA gene – gene that encodes for the small subunit of the ribosomal RNA molecule; Oak250 – Laurel oak combusted in atmosphere at 250 °C; Oak650 – Laurel oak pyrolyzed under N_2 at 650 °C; Grass650 – Eastern gama grass pyrolyzed under N_2 at 650 °C.
[a] Total and mean rate of evolved CO_2 for three measurements made during the six-month incubation (n = 188 days).

determinant in microbial community composition. The unburned and burned soil samples clustered into two distinct populations with a shared overlap of only 18%. The highest level of similarity was observed from the parent unburned forest soil and unburned soils treated with Grass650 and Oak650; approximately 30% similarity was observed between all the replicates. Within each treatment, however, the percent similarity of the six replicates was much higher ranging between 40% and 60%. Amplification of the ITS region generated 16 significant peaks from the unamended, unburned forest soil. Incubation of the unburned soils with each of the biochars showed an overall loss of peak diversity (Table 2). Only six significant peaks were detected in the unburned soils incubated with Oak250 biochar, 12 peaks were detected in soils incubated with Oak650, and 14 peaks in the Grass650.

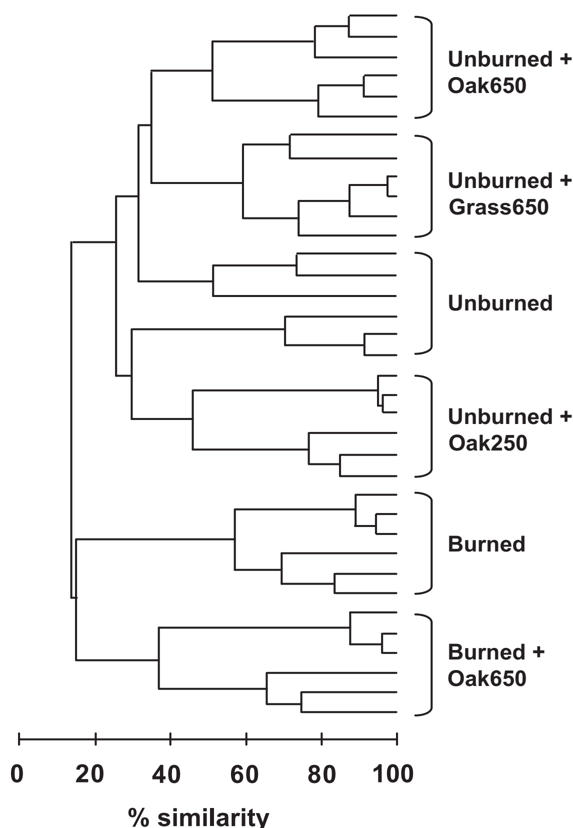

Fig. 1. Dendrogram based on the percent similarity of the ARISA profile of the parent and biochar-treated soils. Abbreviations: Unburned, unburned forest soil; Unburned Grass650, unburned forest soil incubated with grass biochar prepared at 650 °C; Unburned Oak650, unburned soil incubated with oak biochar prepared at 650 °C; Unburned Oak250, unburned forest soil incubated with oak biochar prepared at 250 °C; Burned, burned forest soil; Burned Oak650, burned forest soil incubated with oak biochar prepared at 650 °C.

Despite the overall decrease in peak frequency in biochar-amended soils, there were significant increases in peak areas at 620, 660, and 1028 bp (Table 2) indicating increases in the relative abundance of the species associated with these peaks. Fewer peaks in biochar-treated soils were also seen in those soils collected from the pine flatwood forest that experienced annual prescribed burns, with only six of its 15 recovered peaks also occurring when treated with Oak650 biochar. Therefore, augmentation of soils with biochars enriched only a subset of bacteria within the soil community; even in soils acclimated to the presence of pyrogenic carbon.

3.3. Taxa-specific enrichments in pyrogenic carbon amended soils

Six ARISA bands (374, 660, 700, 850, 900, and 1028 bp) were chosen for further sequencing analysis based on their change in peak area in response to biochar additions. Some of the bands that were not significant in the parent soil profiles were enriched in the biochar-treated soils (374, 660, 850, 1028), whereas the 900 bp band, detected in the parent unburned soil, was not detected in any of the biochar-treated soils. Atleast six clones from each treatment (range 6–19 clones) were sequenced and putative classification was performed by BLAST analysis of the ITS region sequence. A total of eight phyla (Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospira, Proteobacteria, and WS3) were represented by sequences recovered from the six representative ITS bands (Table 3). The relative abundance of the phylum Gemmatimonadetes in unburned soils increased when incubated with Oak650 and Grass650 biochar. The actinobacterial community in these soil samples was highly diverse, and sequences from eight different families were recovered (Table 3). Within the Actinobacteria, only one family Patulibacteraceae was identified in both the control and biochar-amended soils. Sequences and ARISA bands of the remaining actinobacterial families were only found in those soils exposed to natural or laboratory-produced pyrogenic carbon (Oak250, Oak650, Grass650), indicating that these organisms were heavily enriched during the amendments with biochar and were below detection limit in the initial soils. The majority of the recovered sequences shared similarity to those recovered from other forest soil communities such as the long-term soil productivity site at Skulow Lake, British Columbia and mineral-spiked neocaledonian soils (Hilscher et al., 2009). The increase in relative diversity of Actinobacteria was independently supported with nested PCR–ARISA using Actinobacteria-specific primers (Fig. 2).

Table 2
Comparison of ARISA peak area profiles of various soil treatments in the presence and absence of biochar amendments. Mean area is given only for those peaks with values above the 20 fluorescent unit threshold, which were considered significant; PF refers to the peak frequency or the number of the replicates that contained the peak; not significant refers to peaks that were present but were below the 20 fluorescent unit threshold level.

Peak size (bp)	Non-impacted forest soil				Fire-impacted forest soil	
	Unburned (PF)	Unburned + Oak250 (PF)	Unburned + Oak650 (PF)	Unburned + Grass650 (PF)	Burned (PF)	Burned + Oak650 (PF)
296	Not significant			38.35 (5)		
307	Not significant			41.59 (4)		
346					33.39 (3)	
374[a]	Not significant		29.5 (3)	25.59 (2)	25.29 (1)	
411	27.04 (1)				21.33 (1)	
424	30.8 (3)		52.49 (3)	50.97 (6)	91.67 (6)	
440	28.27 (2)			28.14 (5)		
454	27.66 (4)		30.25 (3)		52.29 (6)	
471	34.53 (3)	22.4 (3)			20.29 (1)	
481					33.04 (4)	
494	25.05 (2)			101.67 (6)	38.17 (5)	25.12 (1)
512	33.23 (3)		23.12 (1)	63.9 (3)		
535	36 (5)	34.96 (2)	35.97 (2)	39.62 (2)	104.24 (6)	51.6 (5)
567	30.95 (3)					
595	38.9 (5)		41.91 (3)		201 (6)	55.61 (6)
620	Not significant	117.44 (3)	43.39 (1)	79.95 (2)	25.67 (4)	39.8 (3)
660[a]	46.85 (5)	226.81 (3)	64.66 (6)	26.65 (2)	Not significant	93.04 (3)
700[a]	251.37 (5)	110.75 (6)	90.81 (6)	48.08 (6)	124.33 (3)	52.51 (3)
754	54.83 (3)		44.84 (1)	117.34 (6)	304.74 (6)	110.9 (4)
850[a]	52.47 (5)		95.47 (6)	82.22 (4)	Not significant	118.21 (6)
900[a]	66.62 (4)				42.1 (3)	
1028[a]	22.82 (1)	44.02 (6)	77.04 (3)	44.87 (3)	121.28 (6)	

[a] *Notes*: Peaks selected for DNA sequencing. Oak650 refers to Laurel oak biochars generated at 650 °C; Oak250 refers to Laurel Oak biochars prepared at 250 °C; and Grass650 refers to biochars prepared at 650 °C (see Methods section for details).

Untreated unburned soils had a lower number of Actinobacteria-specific peaks when compared to unburned soils amended with Oak650, as well as a pronounced shift in the community structure (Fig. 2). Burned forest soils, when supplemented with biochar, showed no significant increase in diversity of the Actinobacteria.

4. Discussion

The results of this study supported our working hypothesis that the soil type and source of the pyrogenic carbon influences the microbial composition and targets the growth of specific taxa within the soil community. Specifically, our results provided evidence that: (1) different biochars elicit variable metabolic responses in microbial populations; (2) biochar amendments induce taxon-specific shifts in the microbial biomass and diversity; (3) Gemmatimonadetes and Actinobacteria are enriched in soils that contain natural or added pyrogenic carbon; and (4) prior biochar exposure influences microbial community structure in response to additional pyrogenic carbon.

Pyrogenic carbon amendments had a pronounced impact on the structure and metabolic activity of the soil microbial communities. This impact was dependent on the type of soil and biochar. Unburned forest soils with no history of prescribed burning exhibited lower microbial numbers and diversity in response to low temperature biochars whereas both soils types (burned and unburned) were positively impacted by high temperature biochars and exhibited an increase in microbial number and diversity. These differences in the metabolic responses between biochar treatments likely reflect the accessibility of the C source. The more labile 250 °C biochar may have provided a readily utilizable C source for respiration, while the more refractory 650 °C biochars (Oak650 and Grass650) have a higher surface area and may have provided a suitable new habitat to foster microbial growth (Zimmerman, 2010). Additionally, increases in microbial numbers and growth in the fire-impacted soils suggest that these communities are predisposed to the utilization of pyrogenic carbon due their prior exposure as a result of periodic prescribed burns. These trends in metabolism can be further explained by previous studies that show that biochar made from grasses and at lower temperatures are generally more biologically labile, containing relatively greater proportions of aliphatic organic matter compared to hardwood and high temperature chars that are more aromatic (Baldock and Smernik, 2002; Hilscher et al., 2009). We have also found greater release of nutrients (N and P) from grass and lower temperature biochars (Zimmerman, unpublished), and this may also be a driver of the observed increases in microbial activity.

In addition to changes in metabolic responses, the analysis of the microbial community composition in experimental soils demonstrates that biochar amendments enrich for specific taxa within the whole soil community, and the enriched taxa vary with biochar and soil type. The observed increases in the relative abundance of sequences from bacteria of the phyla Gemmatimonadetes and Actinobacteria suggest an active role for these groups of organisms in soil pyrogenic C metabolism. Furthermore, the community composition analyses in this study are consistent with the hypothesis that biochar amendment to soil reduces microbial diversity, both at the total bacterial level and taxon-specific level (i.e. Actinobacteria). Although ARISA cannot specifically assess soil species richness, as multiple sequences can be present in each visualized band, our previous work has shown a correlation between ITS ARISA band diversity and species richness estimated with 16S rRNA gene sequence library analysis (Havemann and Foster, 2008). Our findings are contrasted by previous findings showing a 25% increase in species richness in Terra Preta compared to adjacent non-anthropogenic forest soils (Kim et al., 2007). The discrepancy between the lower diversity results of the ARISA profiling and those of Kim et al. (2007) may reflect the use of a single source of pyrogenic carbon, the lack of supplemental organic matter or nutrients that may have been used in the production of the Terra Preta, or because the incubations conducted in this study were closed systems, which limited microbial transport, and may not have been long enough for ecological adaptation and diversification to occur.

Table 3
Selected sequencing results from cloned ARISA intergenic spacer bands amplified from soil treatments. Band size is reported in base pairs. Of the selected bands, only those treatments with three or more than significant replicates were cloned and sequenced. Max ID refers to the percent similarity to the most closely related organism.

Band Size	Soil treatment	# of clones match	Phylum	Class	Family	Most closely related organism (BLAST)	Accession #	Max ID (%)
374	Unburned	7	*Proteobacteria*	*Gammaproteobacteria*	*Coxiellaceae*	*Coxiella burnetii* CbuK_Q154	CP001020	93
		2	*Actinobacteria*	*Actinobacteria*	*Patulibacteraceae*	Uncultured *Patulibacter* sp. LTSP_BACT_P2F24	FJ551115	82
		1	*Actinobacteria*	*Actinobacteria*	–	Uncultured actinomycete clone, strain CIU50-10	AJ417003	86
		1	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	Uncultured *Gemmatimonas* sp. LTSP_BACT_P6M15	FJ552614	88
		1	*Proteobacteria*	*Gammaproteobacteria*	*Methylococcaceae*	*Methylococcus capsulatus* str. Bath	AE017282	92
	Unburned + Oak650	5	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	Uncultured *Gemmatimonas* sp. LTSP_BACT_P1D10	FJ550711	81
		4	*Actinobacteria*	*Actinobacteria*	*Patulibacteraceae*	Uncultured *Patulibacter* sp. clone LTSP_BACT_P2F24	FJ551115	93
		1	*Actinobacteria*	*Actinobacteria*	*Frankiaceae*	Uncultured *Frankiaceae* LTSP_BACT_P5D22	FJ552064	89
	Unburned + Grass650	7	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	Uncultured *Gemmatimonas* sp. LTSP_BACT_P1D10	FJ550711	80
		1	*Actinobacteria*	*Actinobacteria*	*Frankiaceae*	Uncultured *Frankiaceae* LTSP_BACT_P1D06	FJ550707	93
		1	*Firmicutes*	*Bacilli*	*Bacillaceae*	Uncultured *Bacillus* sp. JAB EEA1 2006	DQ376548	95
		1	WS3	–	–	Uncultured candidate division WS3 LTSP_BACT_P6J07	FJ552538	86
660	Burned	4	*Acidobacteria*	*Acidobacteria*	*Solibacteraceae*	Uncultured *Solirubrobacter* sp. LTSP_BACT_P2D15	FJ551073	95
		4	*Proteobacteria*	*Alphaproteobacteria*	–	Uncultured bacterium clone 9992 – wastewater	AY484732	84
		1	*Actinobacteria*	*Actinobacteria*	*Patulibacteraceae*	Uncultured *Patulibacter* sp. LTSP_BACT_P4I23	FJ551826	95
		1	*Proteobacteria*	*Alphaproteobacteria*	–	Uncultured *Rhodospirillales* LTSP_BACT_P5J01	FJ552173	92
		1	*Proteobacteria*	*Gammaproteobacteria*	–	Uncultured *Chromatiales* I50-072ITS	AY934374	87
		1	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadaceae*	Uncultured *Xanthomonadaceae* LTSP_BACT_P3K24	FJ551525	100
	Burned + Oak650	3	*Actinobacteria*	*Actinobacteria*	*Mycobacteriaceae*	Uncultured *Mycobacterium* sp. LTSP_BACT_P6B24	FJ552371	100
		3	*Proteobacteria*	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Bradyrhizobium* sp. BTA1	AF324181	88
		2	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	Uncultured *Gemmatimonas* sp. LTSP_BACT_P5J03	FJ552175	91
		1	*Actinobacteria*	*Actinobacteria*	*Acidimicrobiaceae*	Uncultured *Acidimicrobium* sp. LTSP_BACT_P5O15	FJ552294	98
		1	*Actinobacteria*	*Actinobacteria*	*Micromonosporaceae*	*Micromonospora echinospora* ATCC 15835	AY524043	95
		1	*Actinobacteria*	*Actinobacteria*	*Conexibacteraceae*	Uncultured *Conexibacter* sp. LTSP_BACT_P5O20	FJ552299	88
		1	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiaceae*	Uncultured *Burkholderia* sp. LTSP_BACT_P6A05	FJ552330	100
700	Unburned	3	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclaceae*	Uncultured *Rhodocyclaceae* LTSP_BACT_P1G19	FJ550788	96
		2	*Proteobacteria*	*Betaproteobacteria*	–	Uncultured betaproteobacterium LTSP_BACT_P2I22	FJ551162	84
		1	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacteraceae*	*Caulobacter vibrioides* JAB NFA1 03	AY713459	92
		1	*Proteobacteria*	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Bradyrhizobium* sp. BTA1	AF324181	87
	Unburned + Oak650	7	*Nitrospira*	*Nitrospira*	*Nitrospiraceae*	*Candidatus Nitrospira defluvii* Contig5882	EU559167	93
		6	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclaceae*	Uncultured *Denitratisoma* sp. LTSP_BACT_P1A04	FJ550635	92
		1	*Proteobacteria*	*Deltaproteobacteria*	*Desulfomicrobiaceae*	*Desulfomicrobium baculatum* DSM 4028	CP001629	86
		1	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	Uncultured *Gemmatimonas* sp. LTSP_BACT_P5H11	FJ552138	96
	Burned	10	*Proteobacteria*	*Alphaproteobacteria*	*Rhodocyclaceae*	Uncultured *Denitratisoma* sp. LTSP_BACT_P1A04	FJ550635	92
		3	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiaceae*	*Burkholderia vietnamiensis* G4 chromosome 1	CP000614	96
		2	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcaceae*	*Anaeromyxobacter* sp. Fw109-5	CP000769	82
		1	*Acidobacteria*	*Acidobacteria*	*Acidobacteriaceae*	*Uncultured Acidobacteriaceae* LTSP_BACT_P2N06	FJ551236	96
		1	*Actinobacteria*	*Actinobacteria*	*Pseudonocardiacea*	*Microbispora bispor*	U83912	87
		1	*Proteobacteria*	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Bradyrhizobium* sp. BTA1	AF324181	88
		1	*Proteobacteria*	*Alphaproteobacteria*	*Bradyrhizobiaceae*	*Rhodopseudomonas* sp. TUT3626	AB498848	91
850	Burned + Oak650	3	*Chloroflexi*	*Caldilineae*	*Caldilineaceae*	Uncultured *Caldilineaceae* LTSP_BACT_P4G20	FJ551779	79
		2	*Actinobacteria*	*Actinobacteria*	–	Uncultured actinomycete 16S rRNA gene	AJ417011	93
		1	*Actinobacteria*	*Actinobacteria*	*Conexibacteraceae*	Uncultured *Conexibacter* sp. clone LTSP_BACT_P4M14	FJ551912	87
		2	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiaceae*	*Burkholderia phytofirmans* PsJN	CP001053	85
		1	*Acidobacteria*	*Acidobacteria*	*Acidobacteriaceae*	Uncultured *Acidobacteriaceae* LTSP_BACT_P1E18	FJ550741	89
		1	*Proteobacteria*	*Alphaproteobacteria*	*Methylobacteriaceae*	*Methylobacterium extorquens* DM4	FP103042	92
		1	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacteraceae*	*Caulobacter crescentus* CB15 complete genome	AE005673	95

Table 3 (*continued*)

Band Size	Soil treatment	# of clones match	Phylum	Class	Family	Most closely related organism (BLAST)	Accession #	Max ID (%)
900	Unburned	2	*Acidobacteria*	*Acidobacteria*	–	Candidatus *Koribacter versatilis* Ellin345	CP000360	93
		1	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonadaceae*	Uncultured *Sphingomonas* sp. LTSP_BACT_P6E0	FJ552425	99
		1	*Proteobacteria*	*Alphaproteobacteria*	*Xanthobacteraceae*	*Azorhizobium caulinodans* ORS 571	AP009384	80
		1	*Proteobacteria*	*Betaproteobacteria*	*Rhodocyclaceae*	Uncultured *Rhodocyclaceae* clone LTSP_BACT_P1G19	FJ550788	95
1028	Unburned + Oak250	3	*Proteobacteria*	*Alphaproteobacteria*	–	Uncultured alphaproteobacteria MB13F01	AY033325	90
		2	*Acidobacteria*	*Acidobacteria*	*Acidobacteriaceae*	*Acidobacterium capsulatum* ATCC 51196	CP001472	94
		1	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadaceae*	*Pseudomonas putida* strain ATCC 8209	DQ095205	93

Note: Oak650 refers to Laurel oak biochars generated at 650 °C; Oak250 refers to Laurel Oak biochars prepared at 250 °C; and Grass650 refers to biochars prepared at 650 °C (see Methods section for details).

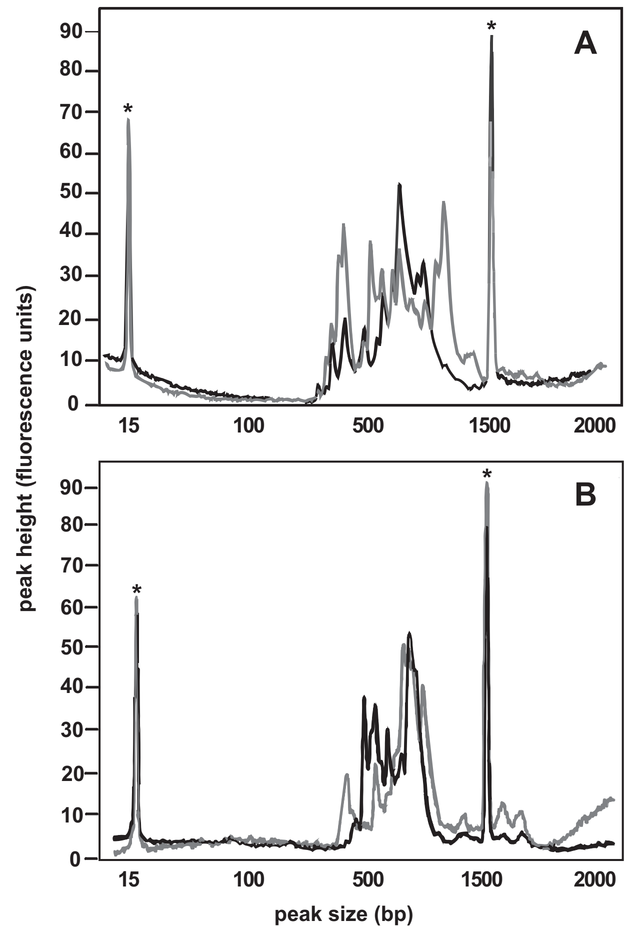

Fig. 2. ARISA profiles of Actinobacteria community that compares peak height to non-linear, peak sizes in base pairs (bp). Comparison between (A) unburned forest soil (black) and unburned soil treated with oak biochar prepared at 650 °C (gray); and (B) burned forest soil (black) and burned soil amended with oak biochar prepared at 650 °C (gray). Specific size markers (15 and 1500 bp) that were added to each ARISA replicate are denoted by asterisks.

Similar microbiological trends were also seen in our cultivation and qPCR results in the presence of pyrogenic carbon. For the fire-impacted soils, more CFUs were recovered from the Actinobacteria-specific media than from the general-purpose media. The greater Actinobacteria CFU recovery in the burned soils compared to the total CFUs may reflect the natural variability in the soils or may simply reflect the enhanced culturability of the Actinobacteria on taxa-specific media compared to general-purpose media. Despite certain well-known limitations of plate cultivation techniques (e.g. Amann et al., 1995), the CFU results do independently confirm the ARISA and qPCR molecular results indicating that the Actinobacteria are naturally elevated in the BFS soil. The increase in Actinobacteria relative abundance was not surprising, as members of this phylum have been shown to grow readily on carbon-rich refractory materials (O'Neill et al., 2009). In contrast, a significant decrease in the abundance of CFUs on both media types was observed when oak biochars, prepared at 250 °C (Oak250), were added to unburned forest soil. Although this suggests a reduction in microbial populations in soils amended with Oak250, respiration results indicate that the remaining community was metabolically active (Table 1). The decrease in CFU abundance, however, may also result from lowered recovery of cells from the incubations due to the absorbent nature of biochar (Kasozi et al., 2010). Overall, these results are consistent with the hypothesis that the Actinobacteria thrive in

soil with natural or added pyrogenic carbon. Enrichments of Actinobacteria have been previously detected under the charcoal layers of fire-treated soils (Bääth et al., 1995). However, the results of the present study suggest that, in addition to biomass, the relative diversity of Actinobacteria taxa also increases with pyrogenic carbon additions.

Although this survey of microbial taxa changes during incubations with pyrogenic carbon in soils is clearly not comprehensive, it does demonstrate that biochar amendment influences the diversity and dominant taxa of soil microbial communities, and that amendment enriches a subset of the parent soil microbial community. Even those fire-impacted soils that have experienced annual prescribed burns exhibited changes to the microbial diversity in response to biochar amendments, though it is not clear how much pyrogenic carbon was actually added to soils during prescribed controlled burns in these ecosystems (Robertson and Ostertag, 2006). Possible causes of the ecological shifts in the relative abundance of the taxa observed may include: enrichment of opportunistic taxa that can metabolize the refractory carbon or other nutrients supplemented by the biochar; toxic effects on a portion of the microbial community as a result of biochar addition; changes to the physiochemical state of the soil environment including pH, mineral content, pore and particle size, as well as water and nutrient availability, all of which have been shown to influence the bacterial community composition in soils (Carson et al., 2009). Further work will be required to determine the relative importance of these factors in altering microbial community composition due to pyrogenic carbon amendments of soils, the role that these changes may have on enhancing soil fertility, and the longer-term effects of biochar on the microbial community structure and activity.

Lastly, we demonstrate that molecular-based approaches, such as nested PCR–ARISA, can serve as effective tools to track these enrichment of key taxa within the soil community and responses to soil treatment. Elucidating the specific changes to the microbial community structure that occur during pyrogenic carbon additions with more in-depth and comprehensive molecular (e.g. SSU rRNA gene pyrosequencing) and biochemical approaches (for review see Kirk et al., 2004) will facilitate the development and application of biochars that may promote or inhibit targeted soil microbes to carry out specific environmental functions of value, such as increasing soil fertility and facilitating long-term carbon storage.

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