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DNA extraction efficiency from soil as affected by pyrolysis temperature and extractable organic carbon of high-ash biochar

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ABSTRACT

Biochar additions to soil have, in some cases, been shown to reduce the DNA extraction efficiency, but the mechanisms remain unclear and commonly used high-ash biochars have not been investigated. We studied the effects of pyrolysis temperatures (300 or 700 °C), post-pyrolysis extractable organic carbon (separating acetone extractable C, AeC), and soil incubation on DNA extraction efficiency using high-ash swine manure biochar. We used quantitative PCR to measure the extraction efficiency of an internal DNA standard (*Aliivibrio fischeri*) added to samples before extraction. DNA extraction efficiency from biochars decreased by 39% as pyrolysis temperature increased from 300 to 700 °C ($p < 0.05$). AeC from biochar 300 °C increased DNA extraction efficiency for biochars made at both pyrolysis temperatures and when added to biochar 700 °C, the extraction efficiency increased by 52% ($p < 0.05$). Incubation in soil increased DNA extraction efficiency from isolated biochar particles by up to 28% (main effect $p < 0.05$). However, biochar-soil mixtures had up to 24% lower DNA extraction efficiency compared to what would be predicted based on a simple mixing model of incubated soil and separated biochars. Biochar pyrolysis temperature, extractable C, and incubation with soil were all associated with changes in DNA extraction efficiency. The differences in DNA extraction efficiency indicated that caution must be exercised when comparing microbial abundance and diversity with different biochar additions, even for high-ash biochars.

1. Introduction

Biochar additions to soil have been shown to significantly alter microbial abundance and diversity (Kim et al., 2007; Graber et al., 2010; Khodadad et al., 2011; Kolton et al., 2011; Harter et al., 2014; Whitman et al., 2016). Such changes play important roles in biogeochemical cycling, with possible implications for N_2O (Harter et al., 2014) and CO_2 emissions (Whitman et al., 2016), or pathogen resistance (Graber et al., 2010; Kolton et al., 2011). Assessments of microbial diversity increasingly relies on DNA extraction approaches (Nesme et al., 2016). The efficiency of DNA extraction can vary depending on the extraction procedure and sample properties (Feinstein et al., 2009; Guo and Zhang, 2013; Leite et al., 2014; Sagar et al., 2014). Even without biochar additions, soils are known to present many challenges due to their heterogeneity and the presence of compounds that interfere with both DNA extraction and subsequent DNA amplification (Fitzpatrick et al., 2010; Albers et al., 2013). Prior studies of the impact of biochar on DNA extraction have mainly focused on woody (Hale and Crowley, 2015) and crop residue (Jin, 2010) biochars, while less is known about high-ash biochars. Hale and Crowley (2015) evaluated DNA extraction efficiency in soil to which biochars with different pH and surface area were added using the recovery of plasmid DNA. Biochar additions to soil did not reduce the efficiency of DNA extracted 1–2 days after addition of the inoculum compared to unamended soils and neither pH nor surface area had an effect on extraction (Hale and Crowley, 2015). In contrast, Jin (2010) showed that the

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recovery of DNA added to corn-stover biochar without soil decreased extraction efficiency by an order of magnitude, but did not study the mechanisms. These contradictory results also indicate that biochar interaction with soil may play a role in modifying DNA extraction efficiencies, but changes over time have not been studied.

Several mechanisms may contribute to biochar's reduction of DNA extraction efficiency, including: (1) ligand exchange between the phosphate backbone of DNA and hydroxyl (-OH) groups (Pietramellara et al., 2009); (2) cation bridging between negatively charged functional groups and DNA molecules (Nguyen and Elimelech, 2007); (3) hydrophobic interactions (Saeki et al., 2011); and (4) entrapment in small pores within the biochar structure. Biochar polarity and surface area vary depending on pyrolysis temperature (Spokas, 2010; Tsai et al., 2012). Higher pyrolysis temperatures have been shown to increase the retention of DNA (Wang et al., 2014) and would be expected to decrease DNA extraction efficiency when added to soil. In addition, changes during exposure to soil, including oxidation (Cheng et al., 2006) and adsorption of organic matter (Nguyen et al., 2009), would be expected to diminish the effect of biochar in reducing DNA extraction efficiency if hydrophobic adsorption is the main effect (Cheng et al., 2014). Due to the low number of studies, contradictory findings, and lack of information about key biochar properties such as pyrolysis temperature or extractable organic matter, the underlying mechanisms require further investigation.

Our objectives were to: (1) quantify the magnitude of bias in DNA extraction efficiency from a high-ash biochar; (2) assess the effects of pyrolysis temperature and extractable organic matter on DNA extraction efficiency; and (3) investigate how biochar exposure to soil changes the DNA extraction efficiency from biochar and its mixture with soil. We hypothesized that (i) the studied high-ash biochar has a moderate effect on DNA extraction efficiency; (ii) higher pyrolysis temperature will significantly decrease DNA extraction efficiency due to higher surface area and hydrophobicity; (iii) removal of extractable organic matter from biochars will decrease DNA extraction efficiency due to changes in surface sites and hydrophobicity; and (iv) exposure to soil will increase DNA extraction efficiency.

2. Materials and methods

2.1. Biochar preparation and characteristics

Biochar materials were produced by pyrolysing autoclaved swine manure at two temperatures, 300 or 700 °C (B300 and B700, respectively), using a modified muffle furnace under an argon atmosphere. The heating rate was 2.5 °C min^{-1} and the residence time was 0.5 h. After pyrolysis, the biochar materials were ground and sieved to between 1 and 2 mm. Proximate analysis of B300 and B700 was conducted using a modified method from ASTM D1762-84 Chemical Analysis of Wood Charcoal (Enders et al., 2012). The moisture, fixed carbon, and volatile matter contents are given in Table S1. In order to separately test differences between the more readily degradable carbon at the surface of the biochar and the pore structure of the biochar, acetone was used to extract C from the biochar surface. Anhydrous acetone (100%) was used to extract C from B300 and B700 (biochar to acetone ratio of 1:10 g to ml). The biochar-acetone mixtures were shaken end-over-end for 24 h. The biochar particles and C extracted by acetone (herein called 'acetone extractable C', AeC) were separated by vacuum filtration. The resulting extracted B300 and extracted B700 (biochars without AeC) are hereafter referred to as exB300 and exB700, respectively. The AeC that was extracted from B300 and B700 by acetone are referred as AeC300 and AeC700, respectively. In addition, in order to separate the effects of degradable carbon from the biochar and biochar structure, the AeC from B300 (AeC300) was added to the exB700 and the AeC from B700 (AeC700) was added to the exB300, resulting in biochar samples hereafter referred to as: exB300 + AeC700 and exB700 + AeC300. While these types of biochar would not be used in application, these treatments allow for the measurement of the relative contributions of surface-bound carbon sources and the underlying biochar structure to DNA extraction efficiency. Excess acetone residues were removed thoroughly by drying at 25 °C in a fume hood and subsequently with vacuum at 25 °C. The AeC extraction rate was calculated using the following equation and resulting data presented in Table S1:

AeC extraction rate (%) = AeC300 or AeC700 extracted from biochar (mass)/biochar prior to acetone extraction (mass) × 100.

The six unincubated biochar materials were ball-milled to a homogenous fine powder for chemical analyses. Elemental composition (C, H, O and N) was analysed using a Thermo Delta V isotope ratio mass spectrometer (IRMS) interfaced to a NC2500 elemental analyzer (Sercon Ltd., Cheshire, UK). The pH was measured in deionized water at a biochar to water ratio of 1:10 (w/v). Ash content was obtained by heating the biochar at 750 °C for 6 h and calculated as: Ash (%) = (weight of ash)/(weight of biochar) × 100. The BET (Brunner–Emmet–Teller) surface area was measured by N_2 adsorption at −196 °C using a Micromeritics ASAP 2020 (Micromeritics, Norcross, GA, USA) after degassing at 200 °C for a minimum of 2 h. The biochar characteristics are given in Table 1. Functional groups were characterized by Fourier Transform Infrared (FTIR) spectroscopy using a Bruker Vertex 70 (Bruker, Ettlingen, Germany) connected with PikeGladiATR (Pike, Madison, WI, USA). In this study, the pH of all unincubated biochar materials was adjusted to 7.6, (equal to the initial pH of untreated B300) using HCl or NaOH to reduce any potential effects of pH on DNA extraction efficiency. The produced biochars without incubation were then stored at −80 °C prior to DNA extraction experiment.

2.2. Soil and biochar incubations

Mineral soil (a Mardin channery silt loam-a coarse-loamy, mixed, active, mesic Typic Fragiudept) was collected from 0 to 0.2 m depth from a forest hillside (42°27′46.4″N, 76°23′10.6″W) located in Freeville, NY, USA, and then air-dried, crushed, and sieved to <1 mm. The soil pH, total organic C, and total organic N were 5.39, 6.2 g kg^{-1} and 0.9 g kg^{-1}, respectively. The biochar materials were added to soil at 50 g kg^{-1} (5%, w/w) and mixed thoroughly prior to incubation. Three replicates of each treatment were incubated in darkness at 30 °C for 150 d. The moisture of each sample was adjusted and maintained at 50% water holding capacity. Soil without biochar constituted the control. After 150 days of incubation, the biochars were removed from the soil using two methods, either manually removed using tweezers or by floating on water. The details are provided in the Supporting Information. The incubated biochar-amended soils and incubated biochars extracted from soil samples were also stored at −80 °C prior to the DNA extraction experiment.

2.3. Internal standard and primers

Based on a method developed by Smets et al. (2016), a marine bacterium not found in soils, *Aliivibrio fischeri,* was selected to use as the internal standard. The *A. fischeri* strain ATCC 7744T was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was grown according to the procedures described by Tavares et al. (2010). Briefly, *A. fischeri* was grown on

Table 1
pH, elemental composition, ash content, molar H/C ratios and surface area of biochar samples.

Property (units)	Biochar type					
	B300	exB300	B700	exB700	exB300 + AeC700	exB700 + AeC300
pH (water)	7.60	7.73	11.89	11.86	7.61	11.84
N (mg g^{-1})	35.1	36.6	22.4	23.0	37.5	22.7
C (mg g^{-1})	482.5	435.9	408.1	419.3	443.6	452.4
O (mg g^{-1})	251.8	260.5	203.9	200.5	253.8	180.8
H (mg g^{-1})	53.0	42.8	07.2	08.4	45.1	20.7
Ash (% w/w)	31.5	32.4	56.9	56.8	32.0	55.7
H/C (mol mol^{-1})	1.32	1.18	0.21	0.24	1.22	0.55
BET surface area (m^2 g^{-1})	1.39	5.95	44.2	40.2	3.83	0.29

tryptic soy agar (BD Difco™, Franklin Lakes, NJ) amended with 3% NaCl at 25 °C. After culturing, the DNA from *A. fischeri* was extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Then, multiple *A. fischeri* DNA extracts were combined and the concentration determined using a Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Specific primers for *A. fischeri* targeting the 16S rRNA gene were designed using DECIPER (Wright et al., 2014) and their predicted target confirmed using Primer3 (Untergasser et al., 2012) and NCBI BLAST (Camacho et al., 2009). The forward and reverse primers selected were: Af-F: 5′-GCGGAAACGACTTAACTGAACC-3′ and Af-R: 5′-GAAGGTCCCCCTCTTTGGTC-3′, respectively. Primer specificity was verified by polymerase chain reaction (PCR) amplification of *A. fischeri* DNA and DNA extracted from biochar-amended soil with and without *A. fischeri* DNA added (Fig. S1). The PCR reaction and amplification conditions are given in the Supporting Information. Although other studies have used an internal standard (e.g., plasmids) together with qPCR to measure DNA extraction efficiency (Mumy and Findlay, 2004; Hale and Crowley, 2015), this approach uses the 16S rRNA gene which is often targeted for sequencing and thus would complement existing methods for quantitative sequencing (Smets et al., 2016).

2.4. DNA extraction and qPCR

DNA extractions were performed in triplicate for all incubated and unincubated samples, using the MO BIO PowerSoil® DNA Isolation Kit. Before extraction, 1 μl of *A. fischeri* DNA (4 ng μl^{-1}) was added to each PowerSoil Bead tube containing 200 mg unincubated biochar. Preliminary DNA extractions were performed for each type of incubated sample to determine the amount of internal standard to add. A range of 0.5–1.5 μl of *A. fischeri* DNA, calculated to target ~0.5% of the total DNA extracted, was then added to each tube containing either 200 mg incubated biochars or 500 mg biochar-amended soils. Immediately afterwards, DNA was extracted following the manufacturer's instructions. The *A. fischeri* 16S rRNA gene copy numbers were determined using quantitative PCR (qPCR) with a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). The qPCR reagents (20 μl volumes) contained 2.0 μl DNA template and 1.0 μl of each primer (10 μM) (IDT, San Jose, CA, USA), 10 μl Fast Plus EvaGreen qPCR Master Mix (Biotium, Hayward, CA, USA) and 6 μl DNase/RNase free water. The thermal cycling program used for qPCR was: initial denaturation (98 °C for 2 min), 40 cycles of fluorescence quantification; including, denaturation (98 °C for 30 s), annealing (60 °C for 30 s), extension (72 °C for 30 s), followed by final extension (72 °C for 60 s) and melt curve analysis. Each qPCR run included triplicate wells for each sample, standard and negative control. *Aliivibrio fischeri* DNA extracts were PCR-amplified using the primers developed, run on a 2% agarose gel, and the targeted amplicon extracted using the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). The concentration of *A. fischeri* DNA was quantified using fluorospectrometry (QuantiFluordsDNA System, Promega, Madison, WI, USA) and serially diluted to generate the qPCR standard curve.

The copy number of total 16S rRNA genes in the treatment sample DNA extracts and the DNA of *A. fischeri* used as the internal standard were measured by qPCR. DNA extraction efficiency was then calculated by the equation:

$$Ef(\%) = (C_m \times V_e) \Big/ \left(C_i \times V_f\right) \times 100$$

where Ef (%) is the DNA extraction efficiency, C_i (copy number μL^{-1}) is the initial copy number of 16S rRNA genes, V_f (μL) is the volume of *A. fischeri* DNA spiked, C_m (copy number μL^{-1}) is the measured copy number of 16S rRNA and V_e (μL) is the volume of DNA extracted. To assess the potential effects of inhibitors, *A. fischeri* DNA was also spiked into a tube of DNA extracted from a B700 sample prior to amplification and quantification using qPCR (Fig. S2).

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was performed to test the significance of differences ($p < 0.05$) in the DNA extraction efficiency between unincubated biochars and biochars isolated from soil after incubation. Three-way ANOVA was conducted to examine the effects of pyrolysis temperature, acetone extraction and soil incubation on the efficiency of DNA extraction from added biochars. A *t*-test was used to test the significance of differences ($p < 0.05$) between DNA extraction efficiency from unincubated and soil incubated biochars and between predicted and measured extraction efficiency from soil-biochar mixtures.

3. Results

3.1. Internal standard primer specificity

The primers for the internal standard, *A. fischeri*, successfully amplified the *A. fischeri* DNA sample and did not amplify DNA extracted from soil or biochar samples without *A. fischeri* DNA added (Fig. S1). Primers targeting the V4 region of the 16S rRNA gene amplified the *A. fischeri* DNA sample and both the soil and biochar DNA samples without *A. fischeri* DNA added (Fig. S1). Amplification of a single product using these primers was confirmed by the presence of a single peak during qPCR. Thus, the *A. fischeri* primers designed were specific to *A. fischeri* and suitable for use as an internal standard to test the efficiency of DNA extraction from amended soils.

3.2. Biochar characteristics

The molar H/C ratios in the biochars produced at 700 °C (B700,

exB700 and exB700 + AeC300) were lower than those in the biochars produced at 300 °C (B300, exB300 and exB300 + AeC700) (Table 1). These results were in accordance with the FTIR analyses that showed three sharp peaks (aromatic C=C) at 500-585 cm^{-1} occurring in biochars produced at 700 °C (Fig. 1). The lower molar H/C indicated a higher degree of fused aromatic C. The B300 and exB700 + AeC300 had higher molar H/C ratios than exB300 and exB700 (Table 1), respectively. This was also consistent with FTIR results that showed that the B300 and exB700 + AeC300 had more apparent aliphatic-CH (peaks at 2920 cm^{-1} and 2850 cm^{-1}) than exB300 and exB700 (Fig. 1), respectively, indicating that the presence of AeC300 reduced the overall biochar aromaticity. The B300 and exB700 + AeC300 had lower surface areas, followed by the exB300 and exB300 + AeC700, while the surface areas of the B700 and exB700 were the highest (Table 1). There was no detectable C extracted from the B700 (Table S1), however AeC700 was still used as a treatment to account for C that was present at lower than detectable levels, other compounds that would have dissolved in the acetone, and any effects of the treatment process.

3.3. DNA extraction efficiency from unincubated and incubated biochars

With unincubated biochar, the DNA extraction efficiencies from biochar pyrolyzed at 700 °C (B700) was much lower than that measured for biochar pyrolyzed at 300 °C (B300) (Fig. 2). This trend remained the same for the biochar with the AeC extracted (exB700 and exB300). The presence of AeC700 did not change extraction efficiencies as can be seen by the lack of difference in extraction efficiency between B700 and exB700 or between exB300 and exB300 + AeC700 (Fig. 2). In contrast, acetone extractable carbon from biochar pyrolyzed at 300 °C (AeC300) had a significant effect on DNA extraction efficiency. B300 had a significantly higher extraction efficiency than the sample without AeC300, exB300. Additionally, when AeC300 was added to exB700 (exB700 + AeC300), the extraction efficiency increased from 0.005% to 51.7% (Fig. 2).

Overall, increasing surface areas of the unincubated samples was associated with lower DNA extraction efficiency (r^2 = 0.75, $p < 0.01$ by simple exponential decay fitting) (Fig. 3).

Incubation increased ($p < 0.05$) DNA extraction efficiency for biochars pyrolyzed at 300 °C and without extractable C (exB300 and exB300 + AeC700) (Fig. 4). Three-way analysis of variance showed that pyrolysis temperature (300 or 700 °C), extractable organic carbon (no AeC, AeC700 and AeC300) and incubation all significantly ($p < 0.05$) affected DNA extraction efficiency (Table S2). Similar to the unincubated biochars, the DNA extraction efficiency in incubated B700 and exB700 was an order of magnitude lower (less than 4% in comparison to about 40%, $p < 0.05$) than other incubated biochars (Fig. S3). There were no significant differences ($p > 0.05$) in efficiency of DNA extraction between B300, exB700 + AeC300, exB300 and exB300 + AeC700 in the incubated treatments (Fig. S3). The DNA extraction efficiency in isolated biochars where AeC300 was present (B300 and exB700 + AeC300) was significantly ($p < 0.05$) higher than in those with AeC700 (B700 and exB300 + AeC700) or without AeC present (exB300 and exB700) (Table S3). There was no significant difference ($p > 0.05$) in DNA extraction efficiency as determined for biochars isolated from soil by the two different methods-floating on water or picking out with tweezers (Fig. S3).

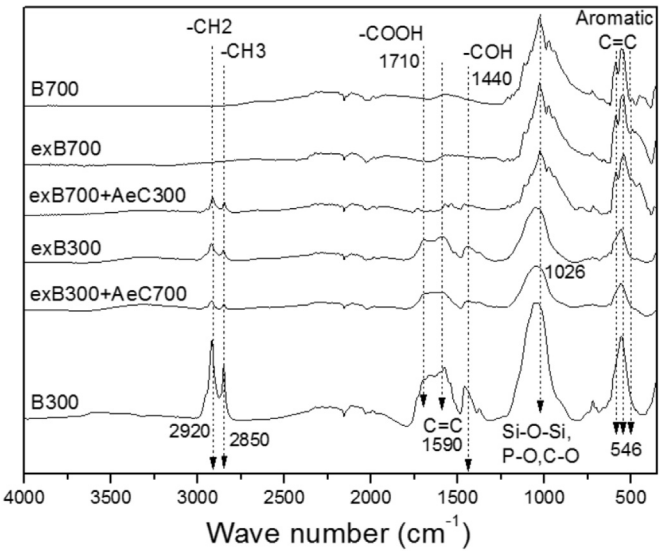

Fig. 1. FTIR spectra of unincubated biochars.

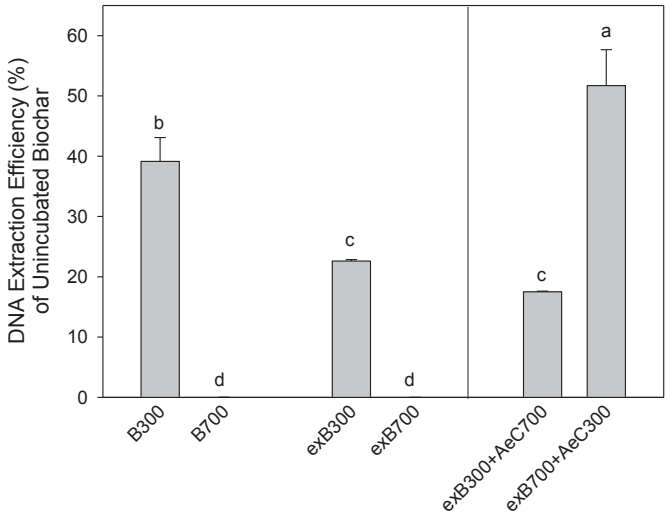

Fig. 2. Extraction efficiency of *Aliivibrio fischeri* DNA from unincubated biochars. Error bars represent the standard deviation among triplicate samples. One-way ANOVA ($p < 0.05$) was conducted to test for significance, different lower-case letters represent significant differences at the 0.05 probability level.

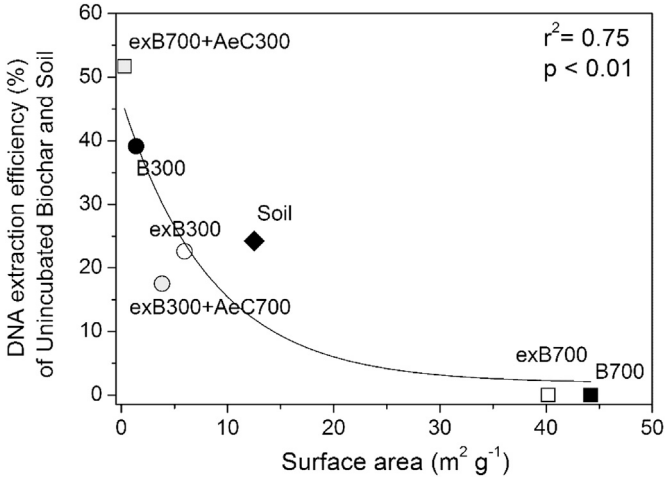

Fig. 3. Relationship between BET surface area and DNA extraction efficiency of *Aliivibrio fischeri* DNA from unincubated biochar and soil samples.

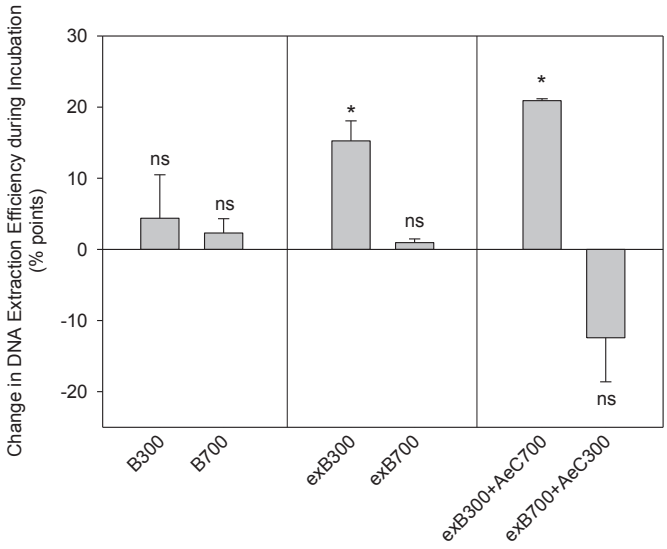

Fig. 4. Changes in DNA extraction efficiency (%) from biochars during incubation in soil, calculated by subtracting the extraction efficiency of unincubated biochars from the extraction efficiency of the incubated biochars. Incubated biochars were isolated from soil following 150 days of incubation. A *t*-test was used to determine significant differences between each pair of unincubated vs incubated treatment, "*" represents a significant difference ($p < 0.05$).

3.4. DNA extraction efficiency in biochar-amended soils

Incorporating biochar into soil decreased the soil DNA extraction efficiency of the soil-biochar mixture for B700, exB700 and exB300 + AeC700 ($p < 0.05$), yet not for B300, exB300 and exB700 + AeC300 ($p > 0.05$) (Fig. S4). The DNA extraction efficiency decreased with B700 and exB700 to a greater extent ($p < 0.05$) than any of the other biochar treatments. In addition, biochar added to soil had a larger negative (decreasing) effect on the extraction efficiency compared to what would be predicted based on a simple mixing model, based on mass, of incubated soils and biochar (Fig. 5). For instance, B700 added to soil actually decreased soil DNA extraction efficiency of the incubated biochar-soil mixture by 15.6%, whereas, based on the mixing model using incubated and isolated biochar, together with incubated soil alone, B700 should only decrease DNA extraction efficiency by 2.4%. The decreased DNA extraction efficiency, compared to what would be predicted based on the mass of the biochar, was significant for all types of biochar pyrolyzed at 700 °C (B700, exB700, and exB700 + AeC300) (Fig. 5).

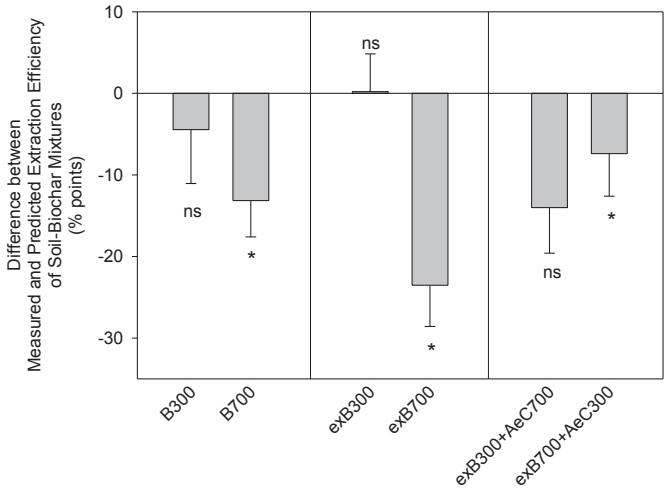

Fig. 5. Difference between measured and predicted DNA extraction efficiency (%) of *A. fischeri* DNA of soil-biochar mixtures (5% biochar by weight). Predicted DNA extraction efficiency was calculated using a simple mixing model of the measured extraction efficiency from incubated and isolated biochars and incubated soil (Predicted DNA extraction efficiency = DNA extraction efficiency (incubated soil) * 0.95 + DNA extraction efficiency (incubated and isolated biochar)* 0.05). The measured DNA extraction efficiency was measured directly from subsamples of soil-biochar mixtures after 150 days of incubation. A *t*-test was used to determine significant differences between the measured and predicted DNA extraction efficiency from soil-biochar mixtures, "*" indicates a significant difference ($p < 0.05$).

4. Discussion

4.1. Interferences during DNA extraction with biochar

The presence of PCR inhibitors, e.g. dissolved organic matter (Albers et al., 2013), in DNA extracts could interfere with measuring DNA extraction efficiency using qPCR. To test for this potential effect, we conducted a spike-recovery test by adding a known amount of DNA standard into the DNA extracted from B700 (the treatment with the lowest DNA extraction efficiency) prior to qPCR. Results showed that the copy number measured by qPCR did not differ significantly from the input copy number ($p > 0.05$) (Fig. S2), suggesting that the low DNA extraction efficiency observed was unlikely to be due to the presence of PCR inhibitors in the samples, but rather the interaction of DNA with biochar before and during DNA extraction.

Sample pH may also affect the interaction of DNA with different biochars. Therefore, we adjusted the pH of all the biochar materials to the same value (pH 7.6) to reduce the effects that pH may have had on DNA recovery. In general, DNA is more likely to sorb to sample constituents at low pH than high pH (Saeki et al., 2012). However, the reagents contained in the MOBIO PowerSoil® DNA Isolation Kit used in this study were all alkaline (pH ≈ 9.0), which would result in both the biochar and DNA being negatively charged. Thus, changes in pH were not expected to explain the low DNA recoveries observed here.

4.2. Differences in DNA extraction efficiency between biochar types

There are four potential mechanisms by which DNA can adsorb to biochar materials in soil and other matrices, and subsequently affect DNA extraction efficiency: (1) ligand exchange between the phosphate backbone of the DNA molecule and hydroxyl (-OH) groups on the surface of biochar as shown for -OH groups on soil minerals (Pietramellara et al., 2007), which could occur with biochar materials as they also have abundant -OH groups; (2) electrostatic adsorption between negatively charged DNA molecules and negatively charged surfaces of biochars by inorganic cation bridging as shown by Nguyen and Chen (2007) for soil particles; (3) hydrophobic interactions between non-polar DNA molecules or portions of these molecules and non-polar biochar surfaces (Pietramellara et al., 2009; Saeki et al., 2012); (4) entrapment of shorter DNA fragments in small pores of biochar.

The FTIR spectra showed that −OH and carboxyl (−COOH) groups were more abundant in biochars produced at the lower pyrolysis temperature (300 °C) (Fig. 1), suggesting that cation bridging or ligand exchange with −OH groups should be greater for B300 than B700. However, lower DNA extraction efficiencies were not observed in biochars produced at the lower pyrolysis temperature. Rather, higher surface area, as a result of, higher pyrolysis temperatures and concomitant increased porosity were likely mechanisms controlling DNA extraction efficiency. Such decreasing DNA extraction efficiency at higher pyrolysis temperatures was in agreement with previous studies showing that higher pyrolysis

temperatures were associated with higher DNA adsorption capacity (Wang et al., 2014).

Further evidence for adsorption to hydrophobic surface was provided by the higher DNA extraction efficiency when acetone-extracted C (+AeC300) was added to biochars. This may indicate that the presence of acetone extractable C (mainly aliphatic C) may have interfered with several of the potential adsorption mechanisms listed above. In addition, we observed that the aromaticity and the degree of fused aromatic C (indicated by molar H/C) and surface area (BET) increased at the higher pyrolysis temperature, while AeC decreased (Table 1), implicating these properties in lowering DNA extraction efficiency. Based on these observations, we suggest that non-polar adsorption of hydrophobic functional groups of DNA, promoted by greater surface area, was the most likely mechanism for observed lower extraction efficiencies due to biochar.

4.3. Changes in DNA extraction efficiency by biochar in soil over time

Differences between unincubated and incubated biochars indicate that changes occurring during the 150 day incubation affected the DNA extraction efficiency from isolated biochars (Figs. 2 and 4, Table S4). These changes could include both biotic and abiotic effects, including interaction between biochar and native soil organic matter or minerals (Zimmerman et al., 2011), differences in microbial growth (Luo et al., 2013) and production of extracellular polymeric substances (Laspidou and Rittmann, 2002). While specific measurements of these interactions were not made, many of these changes would be expected to decrease the available surface area of soil minerals and biochar by filling the pores and covering the surfaces and resulting in increased DNA extraction efficiency. The potential impact of these effects are supported by the observation that the DNA extraction efficiency significantly increased in the incubated soil and the incubated exB300 and exB300 + AeC700 compared to unincubated soil and biochars (Fig. 4 and Table S4). The DNA extraction efficiencies in the isolated biochars with AeC300 (B300 and exB700 + AeC300) did not change after incubation (Fig. 4 and Table S4). This may reflect potentially competing outcomes of microbial colonization, which may include a higher DNA extraction efficiency due to increased cell numbers and EPS adsorbed to biochar surfaces in comparison to a reduced DNA extraction efficiency due to further microbial degradation of aliphatic carbon and EPS decomposition products.

For soil amended with biochars pyrolyzed at 700 °C, the actual DNA extraction efficiency was lower than the efficiency calculated based on incubated soil and biochars separately (Fig. 5). This indicates that biochar additions to soil reduces DNA extraction efficiency through microbial processes beyond adsorption to biochar surfaces and that higher pyrolysis temperature can increase these effects. Given the observation that microbial processes may be responsible for greater extraction efficiency of the biochar particles over time, similar effects may be responsible for biochar effects on extraction efficiency from soil surrounding the biochar particles. Abiotic effects of biochar on surrounding soil that occur over time during the incubation could also promote changes in extraction efficiency directly or indirectly through effects on soil microorganisms.

4.4. Implications for soil-biochar studies

Even without biochar additions, total DNA content may not correlate with microbial biomass as determined in organic forest soils by chloroform fumigation-extraction or total extractable phospholipid fatty acids (PLFA) (Leckie et al., 2004), even though previous studies had reported that total DNA content can be used as an indicator of microbial biomass in mineral soils (Marstorp et al., 2000; Taylor et al., 2002). Thus, varying organic C properties in different soils may cause biases in DNA extraction efficiency, with concomitant adverse effects on downstream analyses.

Several recent studies have used DNA extraction and qPCR to evaluate whether biochar addition stimulated or inhibited soil microbial biomass and functional gene abundance (Yoo and Kang, 2012; Pereira et al., 2015; Ippolito et al., 2016; Whitman et al., 2016). However, estimated changes in microbial biomass based on DNA yield and qPCR may not always be attributed to the changes in soil physiochemical properties and microbial living environments caused by biochar incorporation. Instead, the differences in total DNA content and gene abundance may equally well be a result of differential DNA extraction efficiency, as shown in our study.

Studies evaluating DNA sorption to biochar alone have shown an order of magnitude reductions in DNA extraction efficiency (Jin, 2010) and increasing sorption with increasing pyrolysis temperature (Wang et al., 2014). In the only other study of DNA extraction efficiency from biochar-soil mixtures, yet without an assessment over time, Hale and Crowley (2015) report differences less than an order of magnitude between unamended soils and soils with 3% (w/w) biochar. Similarly in our study, using a high-ash biochar at 5% (w/w) the decreases in extraction efficiency compared to unamended soil were less than an order of magnitude. However, decreases in extraction efficiency over time, especially for high temperature biochar, were greater than what would be predicted based on a simple mixing model (Fig. 5), indicating that biochar may exert an effect on DNA extraction efficiency in soils that is larger than predicted based on the extraction efficiency of biochar and soil individually.

These findings suggest that caution should be taken when using techniques that rely on efficient DNA extraction (e.g., qPCR) to quantify microbial biomass in environmental samples, if they contain biochar, without appropriate correction. This may also extend to naturally occurring pyrogenic C that can be found in most soils world-wide (Lehmann et al., 2008; Bird et al., 2015), which share many characteristics with biochar materials.

Due to these potential methodological biases, internal standards should be used with quantitative measures of DNA yield and gene abundance. Biases in other microbial biomass analyses should also be considered and quantified using internal standards (Thies, 2015). PLFA extraction efficiency is also reduced in the presence of biochar (Gomez et al., 2014), even though overall PLFA community profiles appear to be less impacted by biochar (Plaza et al., 2015). Similarly, correction factors need to be considered for fumigation-extraction when working with biochar-rich soils (Liang et al., 2010). Alternative extraction techniques should also be evaluated to address some of these biases and can include multiple bead beating steps (Feinstein et al., 2009) and addition of sorption site competitors (Paulin et al., 2013).

5. Conclusions

In summary, we found that DNA extraction efficiency from soil decreased even in the presence of high-ash biochar with unexpected changes over time. Increased aromaticity and increased surface area of the studied biochars were associated with lower DNA extraction efficiency likely due to hydrophobic adsorption of DNA. These results highlight that DNA extraction efficiency can be significantly affected not only by different organic C amounts but also by organic C of different structure and composition, which may strongly influence quantitative comparisons of microbial abundance from soils containing biochar or pyrogenic C deposited during vegetation fires. Further work should apply internal

standards to quantify biases in characterizing microbial community structures from soils with biochar additions and a wide range of organic C forms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.08.016.

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