

DNA extraction methodology for biochar-amended sand and clay

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Abstract Studies on the molecular ecology of microbial communities in soils amended with biochar are potentially biased by changes in soil DNA extraction efficiency caused by DNA adsorption to biochar. To examine the extent to which this might occur, we evaluated a kit-free method for soil DNA extraction as well as MoBio's PowerSoil® commercial kit with and without phenol/chloroform/isoamyl (PCI) modification. DNA extraction efficiencies were quantified for soils amended with four biochar types that were added to either clay or sand soils at a rate of 3 % (w/w). Prior to extraction, all soil treatments were spiked with bacteria transformed with plasmids carrying a green fluorescent protein (*gfp*) gene, as a molecular marker. DNA purity and yield were assessed spectrophotometrically and fluorometrically. Quantitative PCR (qPCR) was utilized to enumerate *gfp* copy numbers extracted per gram soil. Results showed that qPCR-quality DNA could be efficiently extracted from biochar-amended soils using the PowerSoil® kit with or without PCI modification. However, the modified protocol resulted in an average of approximately 3 times greater yield than the provided protocol. Of the tested methods, none demonstrated reduced DNA extraction efficiency or purity when amended with the varying biochars.

Keywords DNA extraction · Soil · Biochar · Sand · Clay · PowerSoil · Kit-free

Introduction

The ability of biochar to enhance soil fertility will strongly correlate with its effect on soil biota and biological processes. Several studies have highlighted interactions between biochar and microbial biomass, activities, diversity, pathogenicity, and nutrient interactions (Kim et al. 2007; Warnock et al. 2010; Kolton et al. 2011; Harel et al. 2012; Harter et al. 2013; Farrell et al. 2014; Song et al. 2014; Sun et al. 2014). As the volume of research being conducted on these processes increases, it is vital to ensure that methodology addresses some key concerns in working with biochar. The extraction of DNA from terra preta soils and biochar-amended soils can be problematic due to reduced DNA extraction efficiencies and PCR amplification rates as compared to similar nonamended soils (O'Neill 2006; Jin 2010). Any inherent bias from biochar's presence could thereby lead to misleading results when using molecular methods to analyze microbial community structures and functions in biochar-amended soils. It can be expected that the presence of biochar will affect soil DNA extraction in a variety of ways. Biochar surface cation exchange properties vary for materials produced in different conditions and from different feedstocks and further change over time with aging. This could lead to DNA adsorption through cation-bridging-type interactions with the negatively charged phosphate backbone of DNA (Franchi et al. 2003). Biochar can also alter soil pH, which has been shown to affect DNA sorption to soil surfaces (Ogram et al. 1988; Chan et al. 2008). Furthermore, components of soil organic matter can contaminate extracted DNA with inhibitors of PCR-based, downstream applications (Watson and Blackwell 2000). If biochar adsorbs or releases

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components that are similar to these humic compounds, these substances could interfere with extraction reagents or copurify with DNA. Also, biochar offers additional protective pore spaces and improves aggregate stability, and this may protect cells from complete lysis during DNA extraction (Kimetu and Lehmann 2010).

In previous work, researchers have had generally good success in using MoBio's PowerSoil® kit to extract high yields of pure DNA from biochar-amended soils (Jin 2010; Harter et al. 2013; Leite et al. 2014). In a prior study, Leite et al. (2014) used commercial kits for comparison of DNA extraction methodologies for biochar-amended sand and found the PowerSoil® protocol to be the most effective. Here, we expand on this work to offer improved modifications to the PowerSoil® kit and compare this methodology to a more cost-effective, kit-free protocol (Griffiths et al. 2000). DNA recovery from soils with high-clay content has been problematic (Frostegård et al. 1999). Thus, here, we use soils with a range in clay content to reflect potential clay-biochar-DNA interactions. The current study reports extraction efficiencies for both clay and sand soils amended with a variety of biochars, based on recovery of plasmid DNA from a bacterium added to the sample. Our results provide the basis for recommending methods with broad applicability for nonbiased DNA extraction from biochar-amended soils.

Materials and methods

Soils and biochar

Four types of biochar were amended to soils at a rate of 3 % (*w/w*). The 3 % (*w/w*) rate represents a small increase over common field and pot application rates of 1–2 % (*w/w*) (Beesley et al. 2011). These biochar materials were chosen based on their variation in pH and Brunauer–Emmett–Teller (BET) specific surface area (SSA). The four biochars were prepared from palm fronds at 600 °C (palm 600), pinewood at 300 °C (pine 300), coconut shells at 300 °C (nut 300), and pistachio nut shells at 600 °C (shell 600). Pyrolysis conditions and biochar characterization were described previously (Hale et al. 2015). The biochar pH's and SSA's are provided in Table 1. Prior to addition to soils, biochars were crushed and sieved through a 2-mm mesh, to insure uniformity. Soils used for this study included a Willows silty clay, collected from Yolo County, CA and a Dello loamy sand collected from Merced County, CA. Soil characteristics (Table 1) were acquired from National Resources Conservation Services Soil Survey soil databases (Soil Survey Staff 2014). All soils and biochars were air-dried prior to treatment with inoculum or negative control (0.85 % NaCl).

Preparation of internal standard and inoculation

Strain *Pseudomonas putida* UW4 is a representative of a ubiquitous genus of bacteria found in many soil environments and has a fully sequenced genome (Duan et al. 2013). Electrocompetent UW4 cells were prepared according to a procedure described previously (Choi et al. 2006). Cells were transformed with plasmid pSMC21, a derivative of pSMC2 developed by Bloemberg et al. (1997) using standard techniques (Hale et al. 2014). Integration of pSMC21 was verified by sequencing of PCR products amplified from the *gfp* gene.

Liquid inoculum consisted of cultures grown overnight to late log phase, washed, and suspended in sterile 0.85 % NaCl. Forty grams of amended and nonamended soils were spiked with 6 ml of UW4-pSMC21 liquid inoculum, which had a concentration of 2×10^9 CFU ml⁻¹. Soil extractions were performed 24–48-h postinoculation. To determine the copy number of *gfp* per cell, the *acdS* gene, which is present in UW4 as a single copy, was used for comparison. DNA was extracted from UW4-pSMC21 cells using DNAzol® Reagent (Invitrogen, Carlsbad, CA, USA) and used as template for qPCR reactions. The qPCR conditions and primer sets used for enumeration of *gfp* from pSMC21 and *acdS* from *P. putida* UW4 were described previously (Park and Crowley 2005; Hale et al. 2015). The *gfp* gene existed in UW4-pSMC21 cells at approximately 19 copies per cell. Based on the CFU ml⁻¹ of the spiked culture, the *gfp* gene was added to soils at an approximate rate of log₁₀ 9.86 copies *gfp* g⁻¹ soil.

DNA extraction protocols

A kit-free protocol that allows rapid extraction of both RNA and DNA from soils (Griffiths et al. 2000) was compared with a commercial kit. All buffers and materials for the kit-free protocol were prepared as described by Griffiths et al. (2000) with the following exceptions: 0.5 g each of 0.5-mm glass beads (Sigma-Aldrich) and 0.1 mm zirconia/silica beads (BioSpec Products, Inc) were added to 2-ml screw-top microcentrifuge tubes. Then, 0.5-g inoculated soil was added to each tube along with the suggested hexadecyltrimethylammonium bromide (CTAB) buffer. Centrifugation steps were carried out at 14,000×*g* for 6 or 12 min, rather than the suggested 5 and 10 min at 16,000×*g*. Final pellets were allowed to air-dry, 10–15 min, and resuspended in 60-μl ultra-pure DI water. This protocol copurifies RNA, which can inhibit PCR and reduce qPCR efficiency. Thus, for this work, coextracted RNA was degraded in all samples by treatment with 1-μl 10 μg ml⁻¹ RNase for 10 min at room temperature.

The MoBio PowerSoil® provided protocol was followed according to the manufacturer's instructions. However, the DNA was eluted with only 60 μl to allow for direct comparisons with other tested protocols. In another treatment, the

Table 1 Characteristics of biochars and soils

Material	pH	SSA ^a (m ² g ⁻¹)	Sand-silt-clay (%)	Organic matter (%)
Biochar				
Shell600	9.06±0.44	261.6±20.9	NA	NA
Palm600	10.13±0.01	196.4±16.3	NA	NA
Nut300	6.88±0.14	0.04±0.02	NA	NA
Pine300	4.66±0.05	9.6±0.8	NA	NA
Soil				
Willows silty clay	7.4–8.4	ND	5–45–50	0.5–1.0
Dello loamy sand	7.4–9.0	ND	79–17–4	0.1–0.3

^a BET specific surface area, *ND* not done, *NA* not applicable

MoBio PowerSoil[®] protocol was modified to improve yields based on recommendations by MoBio Laboratories. The modified protocol (PS-PCI) involved replacement of 200 µl of solution 1 with 25:24:1 phenol/chloroform/isoamyl alcohol, pH 8 (AMRESCO, Solon, OH, USA) prior to initial cell lysis. Cell lysis was performed using a FastPrep[®] FP120 cell disrupter, speed setting 5 m s⁻¹, for 45 s (Qbiogene, Carlsbad, CA, USA). Upon addition of solution C4, a mixture of equal parts lysate, solution C4, and 100 % ethanol was vortexed and washed through the spin column 650 µl at a time. Then, a mixture of 300 µl C4 and 370-µl 100 % ethanol was used to wash each column, followed by a wash step with 500-µl 100 % ethanol, and a final wash step with 500-µl solution C5. The 60-µl DNA elutions were treated with 1 µl of 10 µg ml⁻¹ RNase to remove RNA that is concurrently extracted using this protocol.

There were three replicate DNA extractions performed per soil-amendment combination for each protocol used. Calculations for DNA and gene copy number quantities were based on the air-dried dry weight of the amended and nonamended soils.

Assessment of DNA concentration and purity

Representative samples of all DNA extractions were visualized using gel electrophoresis and imaged on a gel documentation system (Gel Doc 2000TM) using Quantity One 4.2 software (BioRad Laboratories, Hercules, CA). All extractions were tested in duplicate for purity (260/230 and 260/280 ratios) and concentration using a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The DNA concentrations were also verified in duplicate with a fluorometric method using the Qubit[®] dsDNA HS assay kit and a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Lastly, qPCR was performed to verify the copy number of *gfp* present in all samples. All qPCR protocols and data analysis were performed within the standards outlined by the MIQE guidelines (Bustin et al. 2009). Reactions using the SsoAdvanced universal SYBR[®] Green Supermix were run on

a MyiQ[®] Thermal Cycler (BioRad Laboratories, Hercules, CA). Primers that targeted the *gfp* gene sequence, solutions for qPCR standard curves, and cycle and melt curve analysis conditions were described previously (Hale et al. 2015). All qPCR reactions were prepared in duplicate and were performed on each individual DNA extraction. To ensure that qPCR data reflected the DNA extraction efficiency, rather than presence of inhibitors, the presence of qPCR inhibitors was evaluated for each protocol. One microliter DNA extracted from noninoculated soils or DI water (control) was added to reactions in which PSMC21 served as the template. We did not determine reduced *gfp* gene amplification in the presence of the extracted DNA (data not shown).

Statistical analyses

SigmaPlot 11.0 was used to generate the qPCR data plot and to perform all ANOVA and Tukey's honestly significant difference tests, which were applied to compare varying methods (Systat Software, San Jose, CA, USA). Analyses compared DNA concentration and yield variables within each soil type independently. Dunnett's tests were used to compare biochar-amended soils to nonamended soils and were performed using JMP 11 (SAS Institute, Cary, NC, USA). All analyses were run using means of four technical replicates, totaling three replicates per sample.

Results and discussion

It was hypothesized that the presence of biochar would decrease DNA extraction efficiency. However, all of the protocols evaluated here showed no reduction in DNA extraction efficiency or purity for DNA extracted from soils amended with biochar. There were no significant differences between any of the biochar amendments and control, nonamended soils, with respect to results for Qubit concentration, 260/280 and 260/230 ratios, and NanoDrop concentrations (Dunnett's test, $P < 0.05$, Table 2). The results of the *gfp*

Table 2 DNA purity and yield results from NanoDrop (ND) and Qubit assays

Soil	Kit-free			PowerSoil provided			PowerSoil PCI modified			
	280/280	260/230	ND ng g ⁻¹ soil	280/280	260/230	ND ng g ⁻¹ soil	280/280	260/230	ND ng g ⁻¹ soil	
	Qubit ng g ⁻¹ soil	Qubit ng g ⁻¹ soil	QuBit ng g ⁻¹ soil	Qubit ng g ⁻¹ soil	QuBit ng g ⁻¹ soil	QuBit ng g ⁻¹ soil	Qubit ng g ⁻¹ soil	QuBit ng g ⁻¹ soil	QuBit ng g ⁻¹ soil	
Sand										
No biochar	2.09±0.02a	2.21±0.01a	10978.0±1187.8a	1.70±0.07b	1.48±0.14b	1821.6±131.7b	2.09±0.06c	1.99±0.08c	4374.6±228.7c	3117.0±888.1b
Shell600	2.10±0.02a	2.13±0.04a	12001.4±481.5a	1.58±0.09b	1.48±0.10b	1731.9±323.6b	1.95±0.03c	1.92±0.04c	3238.4±270.8c	2296.3±383.7b
Palm 600	2.14±0.05a	2.22±0.05a	12643.1±2013.9a	1.77±0.06b	1.55±0.23b	1895.2±370.9b	1.87±0.07c	1.76±0.15c	3045.2±948.8c	2165.7±318.3b
Nut300	1.99±0.06a	2.29±0.05a	6543.5±3492.3a	1.76±0.07b	1.77±0.10b	1702±155.6b	1.89±0.05c	1.73±0.09c	3275.2±841.6c	2250.3±213.0b
Pine300	2.06±0.06a	2.38±0.21a	6187.0±3066.9a	1.74±0.04b	1.59±0.13b	1713.5±213.1b	1.96±0.02c	1.87±0.06c	3560.4±774.6c	2438.9±201.3b
Clay										
No biochar	1.25±0.09a	1.75±0.11a	533.6±56.7a	1.88±0.08b	2.17±0.16b	2419.6±454.5b	1.88±0.03b	1.63±0.01c	3010.7±67.2c	3378.2±167.1c
Shell600	1.15±0.12a	1.15±0.10a	494.5±72.4a	1.88±0.04b	2.06±0.37b	2074.6±149.9b	1.87±0.07b	1.69±0.14c	2709.4±763.5c	3037.8±1119.8c
Palm 600	1.20±0.19a	1.22±0.11a	690±76.8a	1.91±0.05b	1.80±0.14b	2194.2±176.1b	1.91±0.04b	1.82±0.15c	3939.9±233.7c	3914.6±449.4c
Nut300	1.15±0.02a	1.02±0.11a	770.5±91.0a	1.85±0.01b	1.91±0.11b	2026.3±110.6b	1.84±0.06b	1.59±0.02c	3788.1±381.1c	3933.9±653.2c
Pine300	1.28±0.06a	0.99±0.11a	897±49.8a	1.74±0.02b	1.79±0.06b	1918.2±53.9b	1.86±0.04b	1.52±0.02c	3829.5±285.0c	4473.0±184.1c

Values represent means and standard errors of three extractions. Assays were performed in duplicate

Different letters represent significant differences of assays between different protocols, conducted separately for clay and sand

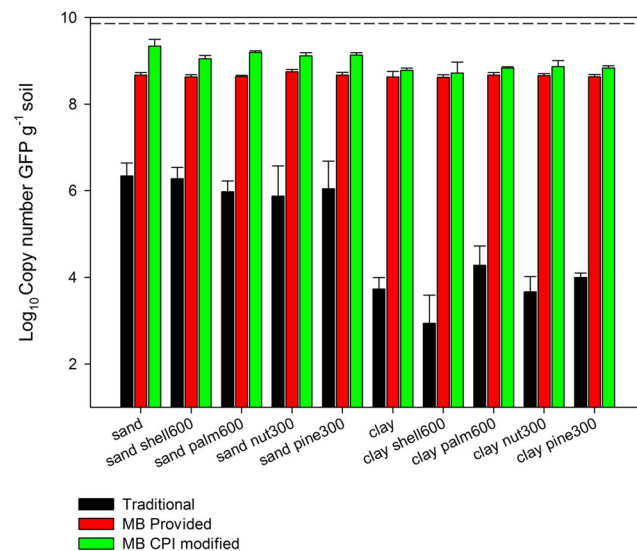


Fig. 1 Quantitative PCR-based enumeration of internal standard, *gfp*. The dashed line corresponds to the copy number of *gfp* added per gram soil. Values represent means and standard errors from triplicate DNA extractions. Different letters indicate significant differences in *gfp* copy numbers for separate analyses of clay and sand soils (Tukey's, $P < 0.05$)

enumeration qPCR assay are presented in Fig. 1, in which the dashed line represents the *gfp* copy number spiked per gram soil. For all protocols, there were no significant differences in \log_{10} *gfp* copy numbers determined for extractions from any of the biochar-amended soils, as compared to the nonamended controls (Dunnett's test, $P < 0.05$). This was true for both soil types. The varying pH's of the studied biochars should reflect a range in cation exchange capacities (CEC's) of these materials (Silber et al. 2010). Furthermore, the varying SSA's correspond to variable porosities and adsorption capacities of the biochars (Chen et al. 2008; Yu et al. 2010). Hence, these materials represent a wide array of potential difficulties that occur while working with biochar-amended soils. However, the effects of biochar aging were not reflected using the newly prepared biochar (stored less than 1 year). During the process of aging in soils, biochar surfaces are typically oxidized, develop more surface charges, and adsorb metals (Cheng et al. 2008; Joseph et al. 2010; Spokas 2013). This will change the adsorption capacities of the biochars and could also affect DNA recovery (Major et al. 2010; Hale et al. 2011).

The direct DNA imaged using gel electrophoresis is provided in Supplementary Fig. 1. The NanoDrop and Qubit assays contrast regarding the yield of the DNA extractions (Table 2). The Qubit assay may depict the most accurate results as this method only detects double-stranded DNA (dsDNA), as opposed to all DNA, RNA, and free nucleotides, and similar results have been reported previously (Ahn et al. 1996; Simbolo et al. 2013). The results of the fluorometric assay better correlate with the DNA band intensities imaged after gel electrophoresis and the qPCR results, providing further evidence of the higher accuracy associated with the

dsDNA-specific assay. Furthermore, NanoDrop results are sensitive to contamination from ethanol, and residual ethanol may have resulted in falsely high 260/280 ratios and concentrations determined for the kit-free extractions.

The NanoDrop assay showed significant differences between protocols for all variables attained (260/230, 260/280, and concentrations), with the exception that the 260/280 ratios between the 2 PowerSoil® protocols were not significantly different in the clay soils (Tukey's, $P < 0.05$) (Table 2). When comparing the 2 PowerSoil® protocols, it is important to note that the 260/230 ratios indicate that greater purity was attained using the PS-PCI protocol with the sand, but the purity was lower using this technique in clay (Table 2). This provides further justification that extraction methods should always be optimized prior to experiment commencement. Based on concentration values obtained from the Qubit assay, DNA yields from the different protocols were shown to vary between soil types. With the sand soil, there was a significant difference in the concentration of extractable DNA between the kit-free protocol and PowerSoil® protocols, but no significant difference between the 2 PowerSoil® protocols (Table 2; Tukey's, $P < 0.05$). With the clay soils, there were significant differences in extracted DNA among all of the extraction protocols, with the PS-PCI protocol providing the best outcomes (Tukey's, $P < 0.05$). Extraction efficiencies were also compared based on *gfp* copy numbers. In sandy soils, there were significant differences in *gfp* copy numbers using all 3 protocols, again with the PS-PCI protocol provided greatest yield (Fig. 1; Tukey's, $P < 0.05$). In contrast, when DNA was extracted from the clay soils, there were significant differences only between the kit-free and PowerSoil® protocols, but no significant difference between the 2 PowerSoil® protocols was determined (Fig. 1; Tukey's, $P < 0.05$). Overall, the modified PowerSoil® protocol averaged 3 ± 0.7 times more quantifiable *gfp* copy number per gram soil than the provided PowerSoil® protocol.

Jin (2010) found that PowerSoil® provided highly pure DNA with sufficient yields. Yet, these yields were reduced, and yield reduction was positively correlated with biochar amendment rate (Jin 2010). As manufacturers have optimized soil DNA extraction kits to work with notably problematic clays, it may be that these alterations resulted in improved efficacy with biochar-amended soils. Previously, the addition of 25:24:1 PCI to MoBio's PowerSoil® kit reduced DNA extraction efficiency and PCR amplification of 16 s rRNA gene sequences from Terra Preta soils (O'Neill 2006). TaqPolymerase was shown to be less efficient or inhibited in the presence of phenol (Katcher and Schwartz 1994). Hence, incorporation of PCI with the initial step and the additional wash steps involved in the PS-PCI protocol may be important to better ensure the complete removal of phenol prior to elution. Furthermore, in this study, DNA was extracted within 48 h of inoculation. Thus, extraction efficiencies could be

lower in cases of longer biochar-microbe interactions. However, in previous studies in which biochar materials were precolonized by inoculum, DNA extraction efficiencies were still high (Hale et al. 2014, 2015).

While the PowerSoil® protocols offered significant improvements, the kit-free protocol did yield DNA that amplified during qPCR, an assay typically sensitive to inhibitors. It also showed no bias from biochar amendments. A similar protocol, based on that of Griffiths et al. (2000), was utilized with success for assessment of biochar-amended soils (Anderson et al. 2014). Of the protocols tested, both PowerSoil® protocols provided DNA of high yield and high purity. However, the PS-PCI protocol provided improvements in most of the variables assayed. This protocol would likely be ideal if working with problematic soils, such as those with low microbial biomass or high levels of PCR inhibitor compounds. While this modified protocol worked well to improve yield and quality in many cases, in all experimentation, it is recommended that DNA extraction methodology be chosen carefully and with consideration of the soil type and desired analyses to be performed with the DNA product (Bakken and Frostegård 2006).

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