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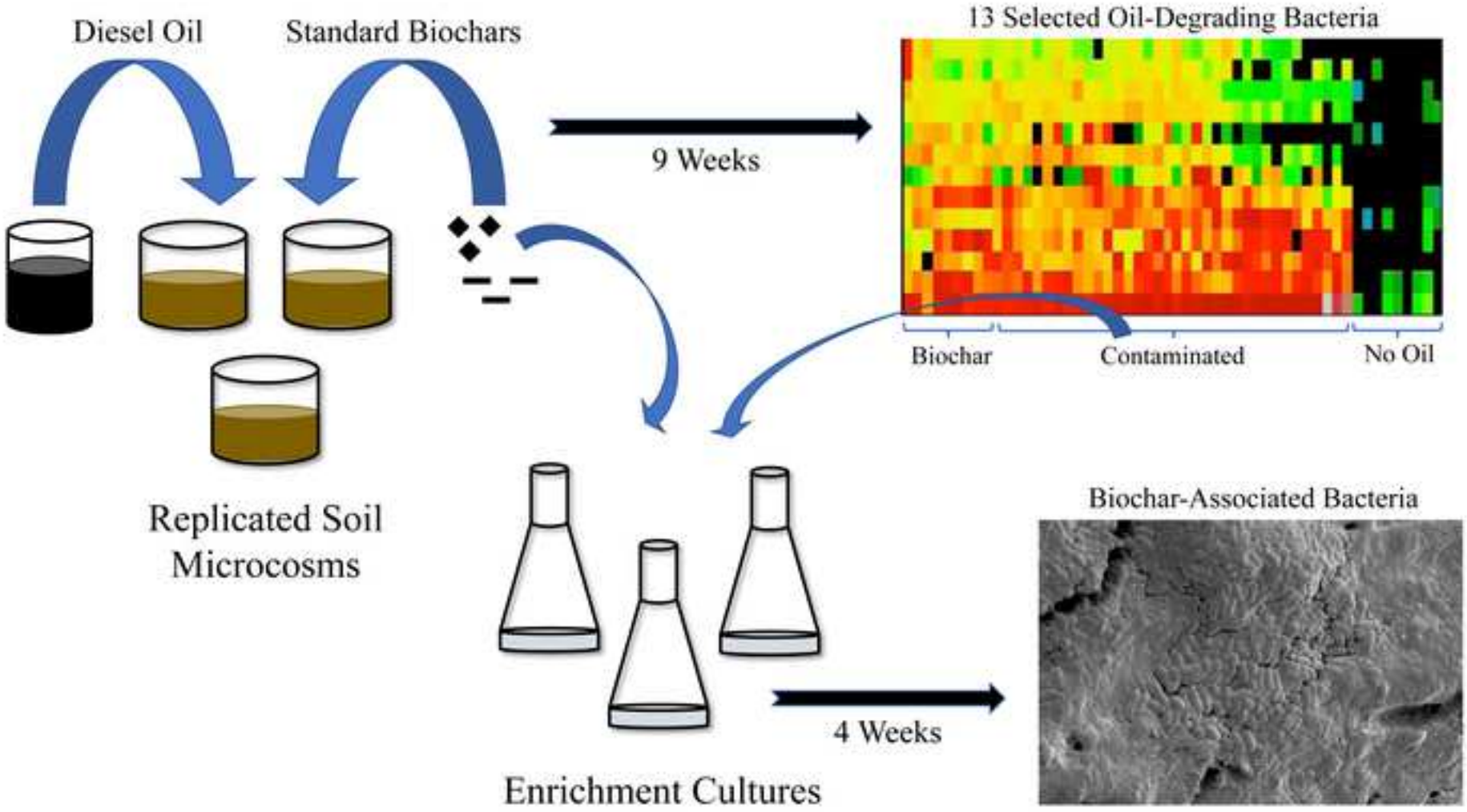
Graphical Abstract



HIGHLIGHTS

- Biochar was evaluated for the biostimulation of hydrocarbon contaminated soils.
- Amplicon sequence variants (ASVs) reveal specific soil microbial responses to oil.
- Oil amplifies 13 ASVs - putative hydrocarbon degraders - to high relative abundance.
- Standard biochars reduce soil diversity loss and are enriched in oil-selected ASVs.
- Biochar enriched with endogenous microbes is a promising approach to bioremediation.

ABSTRACT

Biochar has been proposed as a suitable biostimulant for the remediation of hydrocarbon contamination, and also has the potential to act as a carrier for hydrocarbonoclastic microorganisms which could bioaugment endogenous microbial communities. However, the evidence regarding the biostimulatory effects of biochars on hydrocarbon bioremediation is somewhat equivocal, possibly due to variability of the physicochemical properties of biochar and soil across studies. Here, we use standard biochars with defined properties produced from softwood pellets (SWP) and rice husk (RH) at pyrolysis temperatures of 550 ˚C or 700 ˚C to test the effects of biochar amendment on microbial community composition and hydrocarbon degradation in soil microcosms contaminated with diesel oil. Combining this approach for the first time with specific analysis of microbial community composition using amplicon sequence variants (ASVs), we find that oil contamination causes extreme short-term loss of soil microbial diversity, and highly-specific selection of a limited set of genera defined by 13 ASVs. Biochar ameliorates the short-term loss of diversity, and in the longer term (9 weeks), changes community composition in a type-specific manner. The majority of the 13 selected ASVs are further enriched on biochar particles, although SWP biochars perform better than RH biochar in enrichment of putative hydrocarbonoclastic *Aquabacterium* spp. However, complete degradation of normal (*n*) alkanes from the aliphatic hydrocarbon fraction is prevented in the presence of biochar amendment, possibly due to their adsorption onto the char surface. Furthermore, we show that putative hydrocarbon degraders released from diesel-amended soil can subsequently be enriched to high levels on SWP biochar particles in growth medium supplemented with diesel oil as the sole carbon source; these include selected ASVs representing the genera *Rhodococcus*, *Aquabacterium*, and *Cavicella*. This work suggests that

use of biochar pre-enriched with endogenous, conditionally-rare hydrocarbon degrading bacteria is a promising strategy for bioaugmentation of diesel-contaminated soils.

Keywords: Soil biostimulation; hydrocarbon contamination; biochar; bioaugmentation; conditionally-rare taxa; amplicon sequence variant

Abbreviations footnote: SWP550: softwood pellet biochar (550 ˚C); SWP700: softwood pellet biochar (700 ˚C); RH700: rice husk biochar (700 ˚C); ASV: amplicon sequence variant.

1. Introduction

Hydrocarbons are often recalcitrant, globally-distributed, high-priority pollutants found in soil or aquatic environments (Abbasian et al., 2015). They are produced naturally by biosynthetic activity and through geological processes; exploitation of the fossil fuels produced by the latter in the oil industry leads to anthropogenic contamination of the environment during exploration, extraction, processing and disposal (Hu et al., 2013). Biodegradation of these compounds by hydrocarbon-degrading (hydrocarbonoclastic) microorganisms under aerobic or anaerobic conditions is an important process in their removal from contaminated environments (Abbasian et al., 2015). However, it can be limited by the environmental conditions and/or the endogenous microbial species present at any given location, especially in soil environments (Fuentes et al., 2016). For instance, salinity can have a major effect on biodegradation, requiring the presence of halotolerant species to be overcome (Cao et al., 2020). To counteract these issues, approaches such as biostimulation and bioaugmentation have been proposed to enhance microbial activity (Wu et al., 2016). Biostimulation involves the stimulation of the endogenous microflora via the addition of nutrients such as nitrogen, phosphorous or organic carbon, or alteration of physicochemical parameters, whereas bioaugmentation is the approach of inoculating with cultures of allochthonous or autochthonous hydrocarbonoclastic microorganisms to enhance degradative capability (Galitskaya et al., 2016). In many studies, while biostimulation can be very effective in increasing the rate of hydrocarbon degradation, bioaugmentation can have a limited long-term effect (Fuentes et al., 2016; Galitskaya et al., 2016; Wozniak-Karczewska et al., 2019). The latter is a general phenomenon also associated with bioremediation of other toxic compounds such as organohalides (Beaudet et al., 1998), and may be because it is difficult for an exogenous species to become stably established in a competitive community environment (Free et al.,

2018). In addition, delivery of microbial inocula to contaminated environments, such as soil, *in situ* is potentially problematic. Some possible approaches to this issue, such as the stepwise use of pre-inoculated soil for inoculation of a larger volume (Innemanová et al., 2018), have been proposed.

Endogenous microbial community responses to hydrocarbon contamination can be both highly specific and taxonomically diverse. Marine studies, such as those following the Deepwater Horizon oil well blowout in the Gulf of Mexico suggested that a wide range of rare taxa expanded rapidly due to the event, out-competing major hydrocarbon degraders adapted to slow oil seepage (Kleindienst et al., 2016). These represent conditionally-rare taxa normally present within the rare biosphere fraction of the microbial community (Pedros-Alio, 2012; Shade et al., 2014). In soils, while it has been suggested that community responses are more diverse and less pronounced, highly specific blooms of genera such as *Alkanidiges* have also been reported (Fuentes et al., 2016). The abundances of primary hydrocarbon-degrading genes such as alkane 1-monooxygenase (*alkB*), naphthalene dioxygenase (*nah*) and phenol monooxygenase (*phe*) also increase as a result of these community changes (Liu et al., 2017). Again, the taxonomic identity of the enriched hydrocarbonoclastic bacteria can be diverse and contaminant- and site-specific, although genera such as *Rhodococcus* are commonly elevated in response to contamination (Hamamura et al., 2013).

One approach which has been suggested to achieve remediation, biostimulation and bioaugmentation of hydrocarbon-contaminated soils is the addition of biochar. Biochar is a porous, carbonaceous material produced by the pyrolysis of organic feedstocks under limiting oxygen, and has great potential in the field of environmental remediation (Oliveira et al., 2017). Its primary remediation effect, which covers a wide range of organic and inorganic pollutants,

84 is attributed to its physical and chemical adsorptive abilities, resulting in removal of toxic
85 compounds from the hydrophobic or aqueous phase (Ahmad et al., 2014). Optimal adsorption
86 for specific compounds is affected by both feedstock and pyrolysis temperature, and includes
87 the removal of hydrocarbons (Kong et al., 2018; Silvani et al., 2017; Tang et al., 2018). In
88 general, higher-temperature biochars show greater adsorption due to higher specific surface
89 area and stronger π–π bonding (Ni et al., 2020). However, while greatest for aromatic
90 hydrocarbons, sorption to biochar does not seem to compromise biodegradation of short-chain
91 normal (*n*) alkanes in short-term experiments in a sandy soil (Bushnaf et al., 2011). Moreover,
92 the porous structure of biochar forms an excellent habitat for microbial biofilms, where
93 colonizing organisms gain access to water, air and nutrients, potentially including adsorbed
94 compounds (Schnee et al., 2016; Zhu et al., 2017). The taxonomic composition of biochar
95 surface biofilms is affected by the feedstock used for pyrolysis, and can include enrichment of
96 hydrocarbonoclastic bacteria when their substrates are present (Kong et al., 2018). However,
97 immobilisation of some nutrients, including contaminants, on biochar surfaces may limit their
98 bioavailability (Jośko et al., 2013), and high biochar pH (>9 in some cases; (Mašek et al.,
99 2018)) may also limit hydrocarbon biodegradation.
100
101 Biochar also has the potential to be used as a microbial carrier (biochar augmented with
102 microorganisms), protecting the microbes within its matrix from both physicochemical stresses
103 and biological competition (Partovinia and Rasekh, 2018). However, somewhat equivocal
104 results have been obtained from attempts to bioaugment hydrocarbon remediation systems with
105 inoculated biochars: although faster removal of petroleum hydrocarbons from liquid medium
106 has been shown using biochar-attached *Acinetobacter* or *Corynebacterium* species (Chen et
107 al., 2016; Zhang et al., 2016), and biochar-associated *Mycobacterium gilvum* has been shown
108 specifically to enhance the removal of PAHs from soil (Xiong et al., 2017), no additional effect

of biochar-associated hydrocarbon degraders on petroleum hydrocarbon removal from contaminated soil above that of biochar alone was seen in an 84-day experiment (Galitskaya et al., 2016). It may therefore be difficult to separate the effects of biochar alone from those of biochar augmented with microorganisms, and comparison between published studies may be confounded by different pyrolysis conditions and feedstocks used for biochar production, and different soil properties. Many studies have also shown that bioaugmentation with allochthonous microorganisms does not significantly improve on the performance of the autochthonous microbial community (reviewed in (Maletić et al., 2019)).

Recent developments in microbial community metataxonomic analysis have enabled the highly-specific identification of exact Amplicon Sequence Variants (ASVs) via the application of denoising algorithms (Callahan et al., 2017; Callahan et al., 2016). These enable the more accurate detection of conditionally-rare taxa which are enriched from the rare biosphere in response to environmental changes or specific culture conditions. Similarly, standard biochars produced under defined conditions from a range of feedstocks have been developed to facilitate research into their differential properties in a variety of applications (Mašek et al., 2018). Here, we combine these two approaches for the first time to study the selection of specific microbial species by standard biochar types encompassing different feedstocks and pyrolysis temperatures in the presence of diesel oil contamination. We rationalised that we might improve the reliability of bioaugmentation approaches by selecting endogenous hydrocarbon degraders from soil communities contaminated with petroleum hydrocarbons by enriching on biochar particles. Hence, we studied soil microbial responses to hydrocarbon contamination in the presence of different types of standard biochar, to characterise the microbial signatures, including those of conditionally-rare

133 hydrocarbonoclastic bacteria, that define different biochar-amended soils and biochar-
134 associated biofilms.

2. Materials and methods

2.1. Soil microcosms

Soil microcosms were set up in February 2019 and incubated in the dark at room temperature for 9 weeks in closed 250 ml glass pots, giving sufficient time for microbial community responses, oil degradation and colonisation of biochar. Standard soil type 2.3 (0.66% organic carbon), purchased from LUFA (Speyer, Germany), was dried at 37 °C for 1 week prior to set-up, and for sterile controls, dried soil was autoclaved twice (2 cycles of 121°C for 15 minutes). Softwood pellet (SWP) and rice husk (RH) standard biochars (Mašek et al., 2018) were provided by UK Biochar Research Centre, The University of Edinburgh (https://www.biochar.ac.uk/standard_materials.php). Softwood pellet chars were broken into smaller particles (~0.25-0.5 cm length), and all chars were added to appropriate amounts of dried soil at 1% (w/w) to avoid complete hydrocarbon adsorption, prior to dispensing 200 g of soil per pot. Commercial diesel oil, weathered under an airflow of 0.4 m/s in a fume hood for 20 hours to volatilise short-chain hydrocarbons, was mixed into an emulsion with 30 ml (15% v/w) moisture, consisting of 2 ml fertilizer solution (15 g/l NH_4NO_3, 10.85 g/l K_2HPO_4) and 28 ml sterile distilled water, prior to addition to each individual microcosm and homogenization with a sterilized glass rod. The final concentration of diesel oil added was 7500 mg kg^{-1}. Six different treatments were applied in triplicate to soil microcosms: diesel-contaminated sterile soil (STD), uncontaminated soil (UC), diesel-contaminated soil (DO), contaminated soil amended with softwood pellet 550 °C biochar (DOSWP550), contaminated soil with softwood pellets 700 °C biochar (DOSWP700), and contaminated soil with rice husk 700 °C biochar (DORH700).

Soil (0.25 mg) from each microcosm was sampled with a sterile spatula at days 1, 14, 37 and 63 and frozen at -20° C prior to DNA extraction. Biochar was isolated from microcosms at day 63 and soil particles removed by washing 3 times with phosphate-buffered saline (PBS). Samples (20 g) for GC-FID hydrocarbon analysis were taken from starting uncontaminated and diesel-contaminated soil mixes and from each microcosm at days 37 and 63, and stored at -20° C prior to analysis.

2.2. Enrichment cultures

Enrichment cultures were inoculated with microorganisms isolated from microcosms at day 63. Microorganisms were released from 10 g soil particles by gently shaking at 180 rpm and room temperature in 90 ml 0.1% (w/v) sodium pyrophosphate containing sterile 3 mm glass beads (40 g/l) for 2 hours. Following settlement for 3 days at room temperature, 250 µl samples of the supernatant were taken for DNA extraction and stored at -20 °C. Microorganisms were enriched in sterilised Bushnell-Haas broth (0.2 g/l $MgSO_4$, 0.02 g/l $CaCl_2$, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, 1 g/l NH_4NO_3, 0.05 g/l $FeCl_3$, pH 7.0), supplemented with 0.1% (v/v) Wolfe's minerals and vitamins, in sterile glass flasks. Each flask contained 20 ml of supplemented Bushnell-Haas medium and 1% (w/v) of weathered diesel oil as a carbon source. Biochar at a concentration of 10% (w/v) and soil microorganism inoculum (200 µl) were added as appropriate. After 4 weeks of incubation in a shaker (120 rpm; 25 °C), unstained slide preparations of inoculated samples were observed in an optical microscope (Nikon Eclipse E200, bright-field illumination), and 500 µl samples from the flasks were stored at -20° C for further analysis.

2.3. DNA extraction and PCR amplification of 16S rRNA gene V4 regions

185

186 DNA from separated soil and biochar samples (~250 mg) and enrichment culture
187 samples (250 µl) was extracted using a QIAGEN DNeasy PowerSoil Kit and a QIAcube
188 Connect robot according to the manufacturer's instructions. Following manual addition of the
189 sample to the bead tube and supplementation with solution C1, cells and soil aggregates were
190 disrupted in a PowerLyzer 24 Homogenizer (QIAGEN) at 2000 rpm for 5 min and centrifuged
191 for 1 min at 13000 x g. Supernatants were transferred to the QIAcube Connect for the remaining
192 extraction steps, and purified DNA was eluted in 100 µl solution C6 and stored at -20 °C.
193

194 Variable V4 regions of bacterial and archaeal 16S rRNA genes were amplified using a
195 modified, barcoded 515F-Y/806R primer set (Caporaso et al., 2012), where the forward primer
196 contained the modification described by Parada *et al*. to remove biases against certain archaeal
197 taxa (Parada et al., 2016). PCR-grade water (Sigma), Taq polymerase buffer and $MgCl_2$
198 (Roche) were pre-treated with UV in a PCR cabinet [15 W UV light (λ = 254 nm); 15 mins]
199 prior to setting up reactions containing 1 x buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 250 µM
200 each primer and 0.05 U/µl Taq DNA polymerase (Roche). DNA (typically 1-10 ng) or PCR-
201 grade water (negative controls) were added for a final reaction volume of 25 µl. Cycling
202 conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C
203 for 90 s, with a final extension at 72 °C for 10 min. Entire PCR reactions were separated by
204 1% (w/v) TAE-agarose gel electrophoresis, visualized with ethidium bromide staining and
205 amplicons excised under UV light. DNA was purified from the gel fragments using a Wizard®
206 SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.
207 DNA concentrations were determined using a Quant-iT PicoGreen dsDNA Assay Kit
208 (Invitrogen), and amplicons were pooled in equimolar concentrations for Illumina MiSeq
209 sequencing.

2.4. Barcoded-amplicon MiSeq sequencing, bioinformatic and statistical analysis

Pooled, barcoded amplicons were sequenced in a single run of an Illumina MiSeq v2 sequencer by Edinburgh Genomics, yielding 250 bp paired-end reads. The resulting data were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2), version 2019.4 (Bolyen et al., 2019). Quality control did not show a need to trim the reads. Demultiplexed reads were denoised and paired using DADA2 (Callahan et al., 2016) with default parameters to generate a table of exact sequence features. A Naïve Bayes taxonomic classifier trained on 99% OTU sequences extracted from the 16S rRNA gene subset of the SILVA 132 database (Quast et al., 2013) by *in silico* PCR with the 515F-Y/806R primer set was used to assign taxonomy to the features via the q2-feature-classifier plugin (Bokulich et al., 2018). Best matches to ASVs of interest were identified by BLAST against the prokaryotic rRNA and non-redundant databases at NCBI.

Alpha diversity measures were calculated on rarefied feature tables using the q2-diversity plugin in QIIME2, while beta diversity was assessed via Bray-Curtis dissimilarity on relative abundance tables in Primer-E Version 6.1.12 (Primer-E, Ivybridge, UK), followed by visualization via Non-metric multidimensional scaling (NMDS) and Principal Components Analysis (PCoA) in the same software. PERMANOVA and PERMDISP tests on Bray-Curtis similarity matrices were performed using the PERMANOVA+ add-on to Primer-E (Anderson et al., 2008), with PERMDISP used routinely to confirm that positive PERMANOVA results were not the consequence of a dispersion effect. Relationships between similarity matrices based on the full set of taxa and an oil-selected subset of taxa were tested using a non-parametric version of the Mantel test (RELATE) in Primer-E,19 based on the Spearman

correlation coefficient. All permutation tests were performed using 999 permutations. Calculation of centred-log ratios and ANCOM analysis (Mandal et al., 2015) of differentially-abundant taxa between sample groups was performed in QIIME2 on un-normalized feature tables.

2.5. qPCR of the alkB gene

DNA extracts from contaminated soil (DO microcosms) at days 14 and 63 were used for cloning of the *alkB* gene. PCR reaction components were as for 16S rRNA gene amplifications, except that the *alkB* forward (5'-AACTACMTCGARCAYTACGG-3') and reverse (5'-TGAMGATGTGGTYRCTGTTCC-3') primers (Liu et al., 2017) were at a final concentration of 0.4 mM in a total reaction volume of 50 µl. PCR amplification was carried out at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, with a final extension at 72 °C for 10 min. Correct *alkB* amplicons of ~100 bp were purified from 1.5% (w/v) TAE-agarose gels, cloned into pCR2.1 using a TA cloning Kit (Invitrogen) according to the manufacturer's instructions and submitted for Sanger sequencing (University of Dundee, UK). A dilution series (10^1 to 10^{10}) of a verified *alkB* clone was used in 20 µl qPCR reactions containing 1x SYBR Green Master Mix (Roche), 2 mM $MgCl_2$ and 0.3 mM *alkB* forward and reverse primers in a LightCycler 96 (Roche), with cycling conditions adapted from Wang *et al*. (Wang et al., 2019): 900 s of pre-incubation at 94 °C, 40 cycles of 94 °C for 20 s, 50 °C for 30 s, 72 °C for 45 s, followed by melting at 95 °C for 10 s, 55 °C for 6 s and 97 °C for 1 s. Concentrations of *alkB* gene copies in test samples were determined using the standard curve in LightCycler 96 software.

2.6. Hydrocarbon analysis

Samples (10-15 g) of soil or soil plus biochar from microcosms at incubation days 37 and 63 were transferred to pierced plastic bags and freeze dried with an Alpha 1-4 LDplus freeze dryer (Martin Christ). The dried soil was pulverised and the total hydrocarbons extracted with a FOSS Soxtec^{TM} 8000 extraction unit. The pulverized samples were transferred into pre-extracted cellulose thimbles (size 33x80 mm) and extracted using 120 ml of an azeotropic mixture of dichloromethane/methanol (93:7; vol/vol) (Justwan et al., 2006). The thimble and sample were immersed in the boiling solvent for 130 minutes, then rinsed for another 130 mins at a temperature of 120 °C, and the solvent then allowed to drain from the sample for 3 mins. The solvent-extracted organic matter was transferred to 8 ml vials and evaporated under a gentle blow of nitrogen gas using a FlexiVap Heated Evaporator (Glas-Col) at 31 °C. The aliphatic hydrocarbon fraction of the extracts was separated by column chromatography using a 4 cm (column length) activated silica gel column as the stationary phase, and petroleum ether as the eluting solvent.

A 1 ml sample of the aliphatic fraction was transferred to a pre-ashed 2 ml vial, and gas chromatography (GC) analysis performed using a Thermo Scientific™ TRACE 1310 GC coupled to a flame ionization detector (GC-FID) with injection at 280 °C and FID temperature at 310 °C. The sample (1 µl) was injected using an autosampler in split/splitless mode. The oven temperature was ramped from 50 °C (held for 2 mins) to 310 °C at 5 °C/min and held at the final temperature for 21 mins. Helium was used as the carrier gas, with a constant flow rate of 1 ml/min, an inlet pressure of 50 kPa and the split set at 50 ml/min. The separation was performed on a 30 m x 0.25 mm fused silica capillary column coated with 0.25 µm 5% phenylmethylpolysiloxane (Aglient HP-5MS). The results were stored and analysed using Thermo Scientific™ Chromeleon software.

285

286 *2.7. Data availability*

287

288 The raw DNA sequences were deposited in the European Nucleotide Archive ENA

289 (www.ebi.ac.uk/ena) under BioProject accession number PRJEB39166.

3. Results

3.1. Biochar addition ameliorates diesel oil effects on microbial community diversity

Biochar particles were mixed into fertilised soil at a level of 1% (w/w) where applicable, and weathered diesel oil was added to triplicate soil microcosms set up using these soil mixtures at a level of 7500 mg kg^{-1} dry soil in all except untreated controls. During incubation at room temperature for 63 days, sterile sampling was performed at 2- to 4-week intervals, and DNA extracted from these samples was analysed by Illumina metataxonomic sequencing to determine changes in microbial community composition and alpha-diversity. Starting soil Shannon diversity was high, as expected, and remained in the range 9-10 in untreated microcosms throughout the experiment (Fig. 1). In contrast, diesel oil treatment caused a rapid decline in Shannon diversity to a value of 3.55 ± 0.91 (mean ± SD) within 14 days, followed by a gradual increase to 6.19 ± 0.25 by the end of the experiment, indicating oil-induced species loss and subsequent partial restoration of diversity and community evenness. In all biochar-supplemented microcosms, the initial drop in diversity was reduced (Fig. 1), reaching a minimum of 4.8-5.1 for the different biochars, followed by eventual recovery to levels similar to the unsupplemented, oil-treated microcosms. The reduced effect of oil on diversity in biochar-amended soil at day 14 is statistically significant (Kruskal-Wallis test: $H = 6.23$; $p = 0.013$). This suggests that the presence of biochar particles in soil protects the endogenous community from the effects of oil treatment, either by providing a protective habitat for microorganisms, or by adsorbing some of the added oil. Unsurprisingly, soil sterilised by autoclaving showed a drastic reduction in Shannon diversity to 1.59 ± 0.71 by day 14, although an apparent slight recovery to values >2 in these microcosms by later weeks was detectable (Fig. 1).

315

316 *3.2. Effect of diesel oil on soil community composition*

317

318 The entire metataxonomic dataset contained 19,947 Amplicon Sequence Variants
319 (ASVs), illustrating the high microbial diversity in this typical soil. To determine the
320 differential effects of oil treatment and biochar amendment on different taxa, taxonomy was
321 assigned to these ASVs; taxonomic data grouped at the class level are summarised in Fig. S1.
322 A total of 140 different classes were identified in the dataset, all but 11 of which contributed
323 to less than 1% of the total relative abundance (grouped as "Other" in Fig. S1). These rarer
324 classes accounted for >10% of the total community in starting soil and in uncontaminated and
325 day 1 microcosms, but for <5% of the community in diesel-treated and sterilised microcosms
326 from day 14 onwards, indicating that they were particularly impacted by these treatments.

327

328 In untreated and day 1 soils, the classes *Alphaproteobacteria* (relative abundance 22.5
329 ± 3.3%) and *Gammaproteobacteria* (16.2 ± 1.9%) dominated the community, with no other
330 individual class accounting for >9%. *Actinobacteria*, *Bacteroidia*, *Blastocatellia* (group 4) and
331 *Deltaproteobacteria* all made up 6-8.5% of these communities, and there were few changes in
332 the community composition in uncontaminated microcosms over time, although at the genus
333 level, *Sphingomonas* sp. declined from ~10% relative abundance to ~2% between day 1 and
334 day 63 (data not shown). Compared to these untreated communities, diesel oil treatment caused
335 a mean 3.4-fold increase in *Gammaproteobacteria* and a 1.9-fold increase in *Actinobacteria*
336 from days 14 to 63, while all other abundant classes declined by between 30%
337 (*Alphaproteobacteria*) and 87% (*Gemmatimonadetes*). Even more drastic changes were seen
338 in the community composition of autoclaved microcosms, with the notable observation of a
339 large (3.9-fold) increase in the relative abundance of sequences assigned to the class *Bacilli*.

340 Not unexpectedly, this may reflect the ability of some microorganisms to survive even the
341 harshest treatments (Otte et al., 2018), as discussed in more detail later (section 4.3).
342
343 Because metataxonomic datasets of this type exhibiting widespread changes of
344 community relative abundance are highly sensitive to the compositionality of the data (Gloor
345 et al., 2017), we employed the compositionality-insensitive ANCOM method (Mandal et al.,
346 2015) to detect genera which showed significantly altered abundance in diesel-treated
347 microcosms without biochar amendment compared to untreated controls between days 14 to
348 63. Of the >1,200 genera within the soil community dataset, ANCOM detected 8 genera which
349 were significantly elevated in diesel-treated microcosms (Fig. 2). The plot of ANCOM W score
350 against Centred Log Ratio (CLR) revealed that these genera were highly enriched in the
351 presence of diesel oil, while many other genera were moderately enriched in uncontaminated
352 soil and, to a lesser extent in diesel-contaminated soil.
353
354 The significantly enriched genera (Fig. 2) included abundant *Aquabacterium*,
355 *Nocardia*, *Cavicella* and *Rhodococcus*, and lower-abundance *Caulobacter* and uncultured
356 *Rhodospirillales* sequences. Collectively, these genera accounted for >55% of the microbial
357 community abundance in this set of microcosms, while all of them had a mean abundance of
358 <1% in untreated microcosms. Their enrichment accounts for the increased abundance of the
359 classes *Gammaproteobacteria* and *Actinobacteria* in oil-treated microcosms seen in Fig. S1.
360 In contrast, no taxa elevated in uncontaminated microcosms were detected as significant by
361 ANCOM, although three low-abundance genera, unclassified *Sphingomonadaceae*,
362 *Parafilimonas* and *Nitrosospira*, depleted by oil treatment fell just below the significance
363 threshold (Fig. 2). These data indicate that diesel oil causes a highly-specific enrichment of a

364 limited set of genera to high relative abundance, while at the same time depleting or eliminating
365 a large number of other genera within the soil community.
366
367 *3.3. Microbial communities of biochar-amended soil and biochar-associated taxa*
368
369 To test the effect of biochar amendment of soil microbial communities in the presence
370 of diesel oil, community (Bray-Curtis) similarity was compared between amended and
371 unamended soils from days 14 to 63. An NMDS plot revealed that, while all communities had
372 similar composition at day 14, they diverged over time (Fig. S2A). In addition to the
373 statistically-strong effect of time (PERMANOVA: Pseudo-F = 7.85, $p < 0.001$), biochar
374 amendment also had a significant effect on composition (PERMANOVA: Pseudo-F = 2.12, p
375 = 0.034). Although the NMDS plot suggested some clustering of samples from soils amended
376 with individual biochar types, this was not significant except for the comparison of rice husk
377 700˚C biochar-amended soil to unamended soil (pairwise PERMANOVA: t = 0.80, p = 0.044).
378 To visualise the effects of biochar amendment more clearly, day 63 samples, taken after biochar
379 had been present in the soil for the maximum length of time, were considered in isolation.
380 NMDS ordination (Fig. 3A) showed a clear clustering of unamended soils separately from
381 those supplemented with biochar (PERMANOVA: Pseudo-F = 2.63, p = 0.002), as well as an
382 effect of biochar type on community composition (PERMANOVA: Pseudo-F = 1.68, p =
383 0.005).
384
385 We then examined the microbial communities associated specifically with biochar
386 particles isolated from the microcosms, as compared to those in bulk soil. There was a clear
387 separation between soil microcosm communities at day 63 and those associated with biochar
388 particles isolated at the same time (Fig. 3B), with strong statistical support (PERMANOVA:

389 Pseudo-F = 3.22, $p < 0.001$). There was some evidence of clustering of biochar-associated
390 communities by biochar type, which was clearest when these communities were considered in
391 isolation (Fig. S2B). Again, this was supported by a PERMANOVA test (Pseudo-F = 3.14, p
392 = 0.002). As might be expected, alpha (Shannon) diversity at day 63 (Fig. S2C) was also
393 significantly lower in biochar-associated communities than in the corresponding soil
394 communities (Kruskal-Wallis test: H = 9.28, p = 0.002). While there appeared to be slightly
395 different levels of Shannon diversity between different biochar-associated communities, this
396 had no overall or pairwise significance, although RH700-associated communities had
397 significantly higher Pielou's evenness (Kruskal-Wallis H = 5.96, p = 0.05).
398
399 To exploit the resolution of ASVs, we performed analysis of the ASVs which were
400 enriched in diesel-treated microcosms over time and which were selected in the presence of
401 different types of biochar. Extraction of the top 15 most abundant ASVs for each sample at
402 days 14 to 63 (including uncontaminated microcosms) yielded a subset of 119 ASVs which
403 represented 76.9 ± 8.9% of the total sequences in contaminated samples, but only 20.9 ± 2.6%
404 of the sequences in uncontaminated samples. This disparity illustrates again the highly-specific
405 enrichment of a limited set of taxa in the presence of diesel oil. To simplify the data further for
406 detailed comparison, we restricted our analysis to the top 5 most abundant ASVs in each
407 sample, a set of 33 features representing 10.2 ± 1.4% of total sequences in uncontaminated
408 samples, but 60.6 ± 12.4% of the sequences in contaminated samples. Thirteen of these ASVs,
409 assigned to the genera *Aquabacterium*, *Rhodococcus*, *Cavicella*, *Nocardia*, *Sphingomonas*,
410 *Sphingopyxis*, *Caulobacter* and *Noviherbaspirillum* were highly abundant in most or all diesel-
411 treated samples, but rare or undetectable in uncontaminated samples, as illustrated by a
412 heatmap (Fig. 4; features 1-13). The remarkable overlap of these taxonomic assignments with
413 the genera found to be significantly enriched in contaminated samples by ANCOM (Fig. 2)

414 suggests that in most cases, individual species are specifically selected in the presence of oil.
415 Moreover, beta-diversity analysis based solely on the distribution of these 13 ASVs reproduced
416 closely the patterns seen in Fig. 3 based on day after treatment, biochar amendment, sample
417 type (soil vs. biochar) and biochar type (ρ = 0.843-0.947; $p < 0.001$ in all cases). Hence these
418 13 species are fundamental to the soil community response to diesel contamination and biochar
419 amendment.
420
421 To analyse the ability of these highly-selected species to colonise the surfaces of
422 different types of biochar particles, we calculated the centred-log ratios of the ASV abundances
423 on biochar samples isolated from contaminated microcosms at day 63, compared to bulk soil
424 isolated from the same microcosms (Fig. 4). In general, the CLR values were close to zero,
425 consistent with the observation that the abundant diesel-selected taxa were found in both types
426 of sample. However, the three most abundant *Aquabacterium* ASVs (i.e. ASVs 1, 3 and 4)
427 were noticeably less abundant on RH700 biochar particles, compared to slight enrichment on
428 SWP biochar particles, whereas ASV-7 (*Aquabacterium citratiphilum*) was strongly enriched
429 on both types of SWP biochar, but slightly depleted on RH700 biochar. ASV 9 (*Cavicella*) was
430 specifically enriched on SWP700 biochar compared to the other types. Across all of these 13
431 ASVs, the mean CLR was positive for SWP550 and SWP700 biochars (0.68 and 0.87
432 respectively), but negative for RH700 biochar (-0.39). Considering all ASVs in the dataset,
433 enrichment on SWP550 and SWP700 biochars was significantly correlated ($r = 0.78$, $p =$
434 0.009), but enrichment patterns between RH700 biochar and either of the SWP biochars were
435 negatively correlated, albeit below the significance threshold (Fig. S3). These data suggest that
436 distinct properties of the RH biochar inhibit its colonisation by representative soil
437 microorganisms compared to SWP-derived chars, and this applies to some of the highly-
438 selected diesel-responsive taxa such as *Aquabacterium* sp. Other potentially-relevant abundant

taxa showing differential colonisation included no. 27 (*Cavicella*), while a *Pseudonocardia* species (no. 31) was enriched only on 700 °C biochars (Fig. S3). Consistent with the patterns of colonisation being char-specific, only a single low-abundance *Pseudomonas* ASV was identified as a biomarker of biochar communities across all types, with 3 other low-abundance ASVs diagnostic of soil samples (Fig. S4).

3.4. Biochar amendment prevents further diesel degradation after day 37

As biochar particles added to soil in the presence of diesel oil seem to be colonised by a specific subset of soil microorganisms, including putative hydrocarbon degraders, we sought to characterise the effect of biochar amendment on oil degradation in the soil environment. We therefore analysed the hydrocarbon profile of contaminated soil sampled at the start of the experiment and at days 37 and 63 in sterile, unsterilised and biochar-amended microcosms by GC-FID. Comparison of the GC-FID chromatograms showed that *n*-alkanes (n-$C_{>14}$) showed significant degradation in unsterile soil over 63 days, whereas the isoprenoids (e.g. pristane and phytane) were recalcitrant (Fig. S5A-C), as observed previously in aerobic environments (Dawson et al., 2013; Miller et al., 2019). Fig. S5D shows the ratio of pristane and phytane to the n-C_{17} and n-C_{18} *n*-alkanes in these samples. In unsterilised microcosms, the ratio increases significantly at each time point, indicating faster degradation of the *n*-alkanes compared to the more recalcitrant isoprenoids. Sterile microcosms also show a similar trend, but with a lower biodegradation rate. This is possibly connected to the survival of some potential hydrocarbon degraders as noted above, although this effect lacks statistical significance. Interestingly, microcosms amended with biochars showed similar degradation at day 37 to unamended microcosms, but no statistically-significant change in degradation thereafter. A likely explanation for this is that a fraction of the *n*-alkanes, including n-C_{17} and n-C_{18}, was

464 preferentially adsorbed to the biochar surface and became resistant to further degradation on
465 this timescale.
466
467 To correlate these biochar effects on hydrocarbons with those on hydrocarbonoclastic
468 bacteria, we performed qPCR for the alkane 1-monooxygenase (*alkB*) gene in biochar-
469 amended soils throughout the experiment, and on biochar particles isolated on day 63.
470 Compared to unamended, diesel-treated soil, soils amended with biochars showed reduced
471 copies of *alkB* at day 14, though the difference was only significant for SWP700 biochar
472 amendment (Fig. 5A). In contrast, biochar-amended soils showed significantly higher levels of
473 *alkB* at day 37, especially for low-temperature biochar (SWP550). By day 63, copies of the
474 gene had declined in all soil samples, whereas uncontaminated soil contained $<7 \times 10^3$
475 copies/ng DNA throughout the experiment. These results are consistent with a delayed
476 response by *alkB*-containing soil bacteria to diesel oil in the presence of biochar. Final SWP
477 biochar particles themselves contained similar levels of *alkB* to bulk soil at day 63, although
478 RH biochar retained significantly more copies of the gene (Fig. 5A).
479
480 *3.5. Enrichment of hydrocarbon degraders extracted from contaminated microcosms in the*
481 *presence of biochar*
482
483 The microcosm data suggest that biochar can act as a sorbent for hydrocarbon
484 components of diesel oil, and may also be a habitat for hydrocarbon-degrading soil
485 microorganisms, which are enriched upon diesel contamination. Therefore, we sought to test
486 whether these selected species could be further enriched by culture in minimal medium
487 supplemented with diesel oil, with or without biochar amendment. Microorganisms were
488 released from soil particles isolated from pooled day 63 diesel-treated microcosms, with or

without biochar amendment, by shaking with glass beads followed by settlement (see Materials and Methods). Released microbial communities contained a mix of species related to, but distinct from, the soils and biochar samples in the mature microcosms (Fig. 6); the sample released from SWP700 microcosms had a composition noticeably distinct from the other samples. These isolated communities had significantly lower richness than the starting soils ($p = 0.005$) or biochar samples ($p = 0.009$), indicating that only a subset of the starting species were released efficiently. Genus-level taxonomic analysis of these inoculum samples showed high proportions of *Pseudomonas*, *Aquabacterium*, *Cavicella* and unclassified *Burkholderiaceae* and *Micrococcaceae*, with the latter elevated in the SWP700 inoculum (Fig. S6). There was also a high proportion (20-40%) of rare genera present.

These microbial communities were used to inoculate flasks containing sterilised Bushnell-Haas medium with 1% (w/v) weathered diesel oil as the sole carbon source. Biochars to 10% (w/v) and inocula (1:1000) were added as shown in Fig. S6. Flasks were incubated at room temperature for 28 days, after which time significant microbial growth in the medium and on biochar surfaces was visible both by direct visual and microscopic observation. Uninoculated enrichments also showed smaller amounts of growth, which may represent outgrowth of organisms present either on biochar particles or in the oil itself; however, mean DNA concentrations isolated from these flasks were ~10-fold lower than those from inoculated flasks. The uninoculated enrichments also contained distinct microbial communities (Fig. 6), which were dominated by *Dietzia* or *Mycobacterium* species that were rare or absent in both the inocula and inoculated enrichments (Fig. S6).

Inoculum communities were much more diverse than enrichment communities (mean Shannon diversities of 6.0 and 3.5 respectively; Kruskal-Wallis test $H = 7.385$; $p = 0.007$),

514 indicating that enrichment in the presence of diesel oil was highly selective for a subset of taxa.
515 Interestingly, enrichments in the presence of biochar contained different microbial
516 compositions from enrichment in medium plus oil alone (Fig. 6), with higher levels of
517 *Rhodococcus* species which were only moderately enriched in liquid medium (Fig. S6). This
518 suggests that members of this genus preferentially associate with biochar surfaces. There was
519 also a noticeably distinct composition of species associated with SWP-derived biochars (richer
520 in *Aquabacterium*) compared to the RH700 biochar (enriched in *Acinetobacter*). Although the
521 SWP700 inoculum exhibited a divergent taxonomic composition, inoculating a Bushnell-Hass
522 medium-SWP700 biochar enrichment with the inoculum derived from unamended microcosms
523 instead resulted in a similar final enrichment composition, although this enrichment contained
524 almost 50% *Rhodococcus* (Fig. S6). Supporting the selectivity of enrichment in the presence
525 of biochar, char-amended enrichments tended to have lower Shannon diversity than enrichment
526 in liquid medium. These enrichments also contained significantly higher levels of the *alkB* gene
527 as determined by qPCR (Fig. 5B); lower levels of *alkB* were also detectable in SWP biochar-
528 amended uninoculated enrichments.
529
530 Finally, we constructed an ASV-level heatmap of the top 5 most abundant features for
531 each sample in the enrichment experiment (not including uninoculated cultures). These 22
532 features comprised 49 ± 12% of all sequences in inoculum samples, but 74 ± 10% of all
533 sequences in enrichment samples, highlighting again the selectivity of enrichment. Of the 10
534 most abundant ASVs in the enrichment experiment dataset (Fig. 6), 7 were also present in the
535 set of 33 most abundant ASVs from the microcosm experiment (Fig. 4; indicated by numbering
536 in Fig. S7), with ASV nos. 2 (a; *Rhodococcus*), 7 (b; *Aquabacterium citratiphilum*) and 1 (e;
537 *Aquabacterium*) highly abundant in some or all enrichments. The first two of these ASVs,
538 along with *Acinetobacter* and *Parvibaculum* sequences (c and g) rare in the microcosms, were

539 elevated to higher levels in enrichments than in the original inocula (CLR values >3; Fig. 6),
540 while some ASVs belonging to the genera *Massilia*, *Brevibacillus* and *Pseudomonas* were
541 absent or less abundant in enrichments amended with biochar (Fig. S7; ASVs k-n). Notably,
542 the *A. citratiphilum* sequence (ASV b) seemed to be specific to enrichments amended with
543 softwood pellet-derived biochar.
544
545

4. Discussion

There are several important conclusions from our soil microcosm experiments. Firstly, our methods reveal that the selection of taxa from microbial communities of pristine soil exposed to diesel oil for the first time is remarkably specific. At the level of ASVs (100% sequence identity of the 16S rRNA gene V4 region), >60% of all the sequences in all diesel-contaminated samples map to just 33 individual sequence features, and the 13 most abundant of these features are all rare or undetectable in uncontaminated soil. These represent clear examples of conditionally-rare taxa which respond rapidly (within 14 days) to diesel contamination. Such rapid, short-timescale shifts in community abundance are rare in the literature on oil-contaminated soils: while they have been documented at the level of 97% OTU clusters mapping to the genus *Alkanindiges* (Fuentes et al., 2016), many other studies describe less dramatic enrichments over longer timescales (Bell et al., 2013; Yang et al., 2014). Our data imply a rapid, function-driven reassortment of the microbial community, initially associated with a major loss of alpha-diversity, but culminating in an oil-adapted community specific to this soil type. This pattern represents a version of the ecological phenomenon of community succession (Tipton et al., 2019), in which early colonisers (survivors) of the contaminated state are supplemented by additional adapted species as the soil recovers (Fig. 4); the resulting "mature" community constitutes a rich source of organisms for bioaugmentation.

Secondly, we show that biochars added to these contaminated soils also become populated by many of the taxa elevated due to oil contamination, and this may be connected with the adsorption of a proportion of the added hydrocarbons by the biochar particles. There is evidence of some specificity of biochar type in selection of certain taxa from this highly-

571 enriched population. Finally, we have shown that it is possible to enrich a subset of these
572 species from contaminated soil on biochar particles in liquid medium containing oil, which
573 suggests a possible strategy for bioaugmentation of contaminated soils using endogenous
574 microorganisms.
575
576 *4.1. Taxa specifically selected by diesel contamination*
577
578 Key taxa selected in the contaminated microcosms and enrichment cultures are detailed
579 in Table 1. The most abundant ASV in contaminated microcosms (1 in Fig. 4) is assigned to
580 the genus *Aquabacterium* and reaches a mean abundance of 20% in all contaminated samples.
581 However, it makes up part of the rare community (mean 0.1%) in uncontaminated microcosms,
582 and is undetectable in the starting soil sample. While not quite as striking as the >60% relative
583 abundance of *Alkanindiges* sp. observed in diesel-contaminated microcosms by Fuentes *et al*.
584 (Fuentes et al., 2016), this is still a remarkable and consistent level of selection by oil exposure.
585 A second *Aquabacterium* ASV (ASV-3; Fig. 4) is almost universal (mean 5.3%) in
586 contaminated microcosms and undetectable in both bulk soil and uncontaminated microcosms.
587 The closest match to both of these sequences in the NCBI 16S rRNA sequence database is
588 *Aquabacterium commune* strain B8 (Table 1). There are two other diesel-selected ASVs
589 assigned to the genus *Aquabacterium* in the dataset (ASV-4 and ASV-7 in Fig. 4) which are
590 almost universal in contaminated samples (means 5.2% and 2.5% respectively), and these are
591 very sporadically detected or undetectable in uncontaminated microcosms and bulk soil. The
592 first of these sequences is 98.8% identical to *Aquabacterium commune* B8, while the second is
593 100% identical to *Aquabacterium citratiphilum* strain B4 (Table 1). Finally, an *Aquabacterium*
594 sequence elevated in some contaminated microcosms, but absent from both uncontaminated

595 samples and biochar particles in contaminated microcosms (ASV-24 in Fig. 4), matches to both
596 *Aquabacterium commune* B8 and *Aquabacterium parvum* strain B6 (Table 1).
597
598 The three *Aquabacterium* strains named above were first isolated from drinking water
599 systems, (Kalmbach et al., 1999), but the genus has subsequently been shown to be dominant
600 in some oil-contaminated soils (Jechalke et al., 2013; Pham et al., 2015), and the genome
601 sequence of an *n*-alkane-degrading strain, NJ1, is available (Masuda et al., 2014). Exact
602 matches to the ASV sequences identified in our study have been found in many environmental
603 soil and water samples based on entries in the NCBI non-redundant database, including oil-
604 polluted soil (the *A. citratiphilum* sequence ASV-7). This latter sequence, unlike the other
605 *Aquabacterium*-related ASVs, is also abundant in diesel enrichment cultures (Fig. S7). Hence,
606 our data suggest that several closely-related members of the genus *Aquabacterium* involved in
607 *n*-alkane degradation are selected by exposure to diesel oil.
608
609 Members of the genera *Rhodococcus* and *Nocardia* (family *Nocardiaceae*) are well-
610 known hydrocarbon degraders (Finnerty, 1992; Luo et al., 2014), and ASVs mapping to these
611 genera were strongly enriched in contaminated microcosm samples (Fig. 4; ASV-2 and ASV-
612 6 respectively). These ASVs are universally present in our contaminated samples at 4-6.5%
613 mean abundance, but rare or sporadic in the uncontaminated microcosms and undetectable in
614 bulk soil. The *Rhodococcus* sequence, which is also highly abundant in enrichment cultures
615 (Fig. S7; ASV-a) is identical to that of *Rhodococcus erythropolis* ATCC 4277 (Table 1), a
616 known hydrocarbon degrader which can utilize alkanes ranging from hexane to heptadecane
617 (Ludwig et al., 1995). The *Nocardia* sequence is identical to those of several *Nocardia* species
618 including *Nocardia ignorata*, which although a potential pathogen has also been isolated from
619 oil-contaminated soil in Kuwait (Rodríguez-Nava et al., 2005).

An ASV assigned to the genus *Cavicella* (Fig. 4; ASV-5), which is universally present (mean 4.9% abundance) in contaminated samples, but only sporadically detected in uncontaminated soil, is most closely related to *Cavicella subterranea* strain W2.09-231 in the NCBI 16S rRNA sequence database (Table 1). *Cavicella* is a novel genus isolated from a deep aquifer in 2015 (França et al., 2015) and a second *Cavicella* ASV (Fig. 4; ASV-9), which becomes common in contaminated samples from day 37 onwards, shows 100% identity to the same *Cavicella* strain. This genus has recently been reported to be elevated in soils in response to crude oil contamination (Xue et al., 2020), suggesting that it contains novel hydrocarbonoclastic bacteria. Both ASVs are also abundant in inocula derived from contaminated soil (Fig. S7; ASV-j and ASV-h), but less abundant in enrichment cultures. Again, the data suggest that at least two closely-related members of this genus are involved in hydrocarbon degradation in our study.

A single *Sphingomonas* ASV (Fig. 4; ASV-8) increased in abundance from day 37 in contaminated samples, yet was undetectable in uncontaminated samples. Its closest match is *Sphingomonas insulae* DS-28 (Table 1) isolated from soils (Yoon et al., 2008). *Sphingomonas* sp. were also enriched in Deepwater Horizon-impacted sediments, (Looper et al., 2013) and are known degraders of PAH compounds (Asaf et al., 2020). Similarly, other ASVs or genera identified as enriched in contaminated samples (Figs. 2 and 4) are either known hydrocarbon degraders or enriched in oil-contaminated sites, including *Caulobacter* (Yergeau et al., 2012), *Sphingopyxis* (Verma et al., 2020) and *Noviherbaspirillum* (Lin et al., 2013). It is likely that the most strongly-selected ASVs in diesel-contaminated microcosms (assigned to *Aquabacterium*, *Rhodococcus*, and *Cavicella*) are involved in the alkane degradation observed by GC-FID during the microcosm experiment, given the close correspondence between their

645 relative abundance over time and the abundance of the alkane 1-monooxygenase (*alkB*) gene
646 as determined by qPCR (Fig. 5A).
647
648 *4.2. Taxa specifically selected by enrichment on diesel oil as a carbon source*
649
650 Consistent with their enrichment in diesel-contaminated soil, the *Rhodococcus* and
651 *Aquabacterium* ASVs -1(e), -2(a) and -7(b) were also abundant in enrichments containing
652 diesel oil as the sole carbon source (Figs. 7 and S7). This illustrates the potential for using
653 enriched extracts from a given soil type to inoculate biochar particles in the laboratory, prior
654 to application of this inoculated biochar for bioremediation *in situ*. However, other abundant
655 ASVs from the oil-selected set of 13 observed in soil microcosms were not abundant in the
656 enrichments, and while in some cases this was probably due to their poor survival in the soil
657 extracts, the *Sphingomonas* and *Cavicella* species ASV-5(j), ASV-8(l) and ASV-9(h) had low
658 abundance in enrichment cultures despite being abundant in the extracts. This may reflect poor
659 culturability under the enrichment conditions, although ASV-9 did grow in one enrichment
660 culture. One other species with lower abundance in the original microcosms,
661 *Pseudarthrobacter* ASV-19(d), was highly abundant in soil extracts but reduced in the
662 enrichment cultures, although it remained universally present (Fig. S7). This sequence is 100%
663 identical to *Pseudarthrobacter phenanthrenivorans* sp. nov. (Table 1), a phenanthrene-
664 degrader isolated from creosote-contaminated soil (Kallimanis et al., 2009).
665
666 As might be expected, there are some other taxa which are rare or undetectable in the
667 inoculum samples, but abundant in the enrichment cultures (Fig. S7). One of these,
668 *Acinetobacter* ASV-c, was abundant in most enrichments (mean 13.5%), but occurred
669 sporadically in inocula (mean 0.05%). Its sequence showed a 100% match to 16 different

Acinetobacter sequences, including the diesel-degrading strain *A. oleivorans* NR1 (Table 1; (Jung et al., 2010)). Similarly, a *Parvibaculum* sequence (ASV-g) was abundant in most enrichments (mean 4.5%), but rare or absent in inocula (mean 0.13%). Its closest match (94.9%) was to *Parvibaculum indicum* strain P31 (Table 1), a member of a deep sea water PAH-degrading consortium (Lai et al., 2011); *Parvibaculum* sp. were also identified as key alkane-degrading species in Deepwater Horizon-impacted sediments (Looper et al., 2013). Other known hydrocarbonoclastic species which were enriched more sporadically in the cultures included several *Pseudomonas* sp. (ASVs -f, -m, -n, -q and -v), two *Sphingobium* sp. (ASVs -p and -t) and an *Acidovorax* sequence (ASV-o) seen only in the presence of 700˚C biochars. *Acidovorax* strains isolated from contaminated soils have previously been shown to be active in PAH degradation (Singleton et al., 2018).

4.3. Effect of biochar on colonisation by specific taxa

By using different biochars with known standard properties, we are able to draw some conclusions about the role of biochar physicochemical properties in colonisation by hydrocarbonoclastic bacteria. Being derived from a different feedstock, the RH700 has the most distinct properties compared to the SWP biochars, and this is consistent with our taxonomic data that clearly separate it from the SWP chars. Final soil community composition in RH700-amended microcosms is slightly distinct from that in SWP-containing soil, although the difference between the biochar-associated communities is clearer (Fig. 3). In general, the abundant diesel-selected ASVs were less enriched on RH700 biochar than on the SWP biochars, and this was especially true of the *Aquabacterium* spp. which make up 4 of the 10 most abundant ASVs in contaminated microcosms (Figs. 4 and S3). Two of these species which were selected in enrichment culture also showed reduced abundance in RH700-amended

695 cultures compared to those containing SWP biochar (Fig. S7). RH700 char has a 50-fold higher
696 ash content than the SWP chars (Mašek et al., 2018), has a high silica content and is slightly
697 hydrophilic, which may affect oil adsorption. Likewise, RH700 has ~2-fold lower carbon
698 content (47 wt%, compared to 85-90 wt% for SWP chars). However, there is no indication that
699 it affects alkane degradation differently from the SWP biochars (Fig. 5). The pH of the RH
700 biochar is also strongly alkaline (9.8, compared to 7.9-8.4 for the SWP biochars), and it
701 contains 3-fold more potassium than the SWP chars (Cabeza et al., 2018; Mašek et al., 2018),
702 which may impose selection against specific bacterial species. Biochar can also influence
703 microbial community composition by interfering with bacterial cell-cell communication (Gao
704 et al., 2016). However, in enrichment cultures, all three types of biochar show greater copy
705 numbers of the *alkB* gene involved in hydrocarbon degradation, compared to unamended
706 culture (Fig. 5).
707
708 Overall, the two SWP biochars showed similar enrichment of ASVs from diesel-
709 contaminated soil (Figs. 3, 4 and S3), suggesting that pyrolysis temperature does not make a
710 significant difference to colonisation by these taxa for this feedstock. SWP700 has the highest
711 BET surface area of the three chars (162 m^2/g, compared to 26.4 m^2/g for SWP550 and 42 m^2/g
712 for RH700), which may enhance oil adsorption, although this is due to the presence of
713 micropores of ~10 Å size (Wong et al., 2019), which will be too small for bacteria to colonise.
714 However, differences in oil adsorption may affect microbial diversity indirectly. SWP700 char
715 particles isolated from soil microcosms at day 63 exhibited the lowest Shannon diversity of the
716 three types, while RH700 biochar showed the highest diversity and significantly greater
717 evenness (Fig. S2), despite its lower C content. Our overall conclusion is that SWP biochars
718 show better colonisation by the main potential hydrocarbon degraders in this soil type, while
719 RH700 biochar shows more general microbial association and may select against

720 *Aquabacterium* spp. Hence SWP biochars would be preferred as a matrix for bioaugmentation
721 in this context.
722
723 *4.4. Species dynamics and hydrocarbon degradation in sterilised microcosms*
724
725 Analysis of hydrocarbon degradation in sterilised soil microcosms suggested a slow
726 rate of *n*-alkane degradation (Fig. 5), although the changes over time were below the
727 significance threshold and much smaller than those in unsterilised soil. Likewise, taxonomic
728 changes occurred in the sterilised microcosms over time compared to sterilised bulk soil,
729 suggestive of residual microbial activity (Fig. S1). Although repeated autoclaving is an
730 effective sterilisation strategy, hydrocarbon degradation and other microbial activity have been
731 observed in autoclaved soils and sediments over similar time periods (Lu et al., 2019; Otte et
732 al., 2018), suggesting that abiotic controls in such studies are not completely free of microbial
733 activity. Prolonged autoclaving has also been shown to allow limited microbial survival and
734 PAH degradation in soils, in comparison to treatment with toxic $HgCl_2$ (Wang et al., 2011a).
735
736 Major taxa (>5% mean abundance) in the sterilised microcosms were assigned to the
737 genera *Luteibacter*, *Massilia* and *Pseudomonas* and to the family *Micrococcaceae*, and while
738 undetectable in the starting sterilised soil, were also detected in unsterilised microcosms,
739 especially those contaminated with diesel oil: this suggests survival and outgrowth of these
740 organisms in the presence of hydrocarbons. In support of this hypothesis, the genera
741 *Luteibacter, Massilia* and *Pseudomonas* include members with reported hydrocarbon-
742 degrading abilities (Gutierrez et al., 2015; Lu et al., 2019; Mahjoubi et al., 2013). In contrast,
743 uninoculated enrichment cultures were rich in *Dietzia* spp. and *Mycobacterium* spp. which
744 were undetectable in soil and microcosms; this suggests that these organisms may have been

745 present in the diesel oil itself, or possibly on added biochar particles. *Dietzia* and
746 *Mycobacterium* species have both been shown to have hydrocarbon degradation ability (Kim
747 et al., 2015; Wang et al., 2011b), and have been observed at high abundance in hydrocarbon-
748 contaminated soils amended with biochar (Gielnik et al., 2019).
749
750 **5. Conclusions**
751
752 In conclusion, by using exact amplicon sequence variant analysis methods, our study
753 shows the extreme species selectivity of soil community responses to hydrocarbon
754 contamination. Just 13 highly-selected ASVs, mapping to 8 known hydrocarbon-degrading
755 genera, characterise the oil-, time- and biochar-dependent changes in the structure of the highly
756 diverse soil community. These conditionally-rare taxa are likely to be soil type-specific,
757 making them prime candidates for bioaugmentation into similar types of soil to bioremediate
758 hydrocarbon contamination. Similar specificity is seen in the interactions of these enriched
759 species with biochar particles, with softwood pellet-derived biochars enriching the majority of
760 these species further, while rice husk-derived biochar is particularly counter-selective for
761 *Aquabacterium* spp. Enrichment experiments with diesel oil as the sole carbon source showed
762 that softwood pellet biochar could be used to further enrich *Aquabacterium* and *Rhodococcus*
763 species isolated from the contaminated soil, and was more effective than liquid enrichment
764 alone in increasing copy numbers of the *alkB* gene.
765 Our work suggests a bioremediation strategy in which autochthonous conditionally-
766 rare taxa could be isolated from a given soil type, enriched on biochar particles in a liquid-
767 based culture system, and then used to carry out bioaugmentation for *in situ* bioremediation of
768 the soil. This would allow application of hydrocarbon degraders likely to thrive within the
769 target environment, especially in cases of acute contamination where the endogenous

community may not be able to respond sufficiently. Such an approach takes account of both the composition of the endogenous community, to ensure enrichment with compatible taxa likely to persist in the environment, and the appropriate type of standard biochar to achieve their enrichment and stable delivery. It would therefore be expected to have advantages over the application of exogenous species, which may not become stabilised in the environment, or of biochar alone, which would facilitate only biostimulation and adsorption unless colonised *in situ*. Key challenges for such an approach are an ability to culture the necessary species in an enrichment, preparation of sufficient amounts of inoculant for a field site, and delivering the inoculated biochar particles at scale. Important next steps for this research are to test the effectiveness of pre-inoculated biochar particles - compared to uninoculated biochar, biochar inoculated with exogenous hydrocarbon degraders and liquid inoculum without biochar - in contaminated soils at scale.

CRediT authorship contribution statement

Zhansaya Assil: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original Draft, Visualization; **Onoriode Esegbue:** Methodology, Investigation, Resources, Writing - Review & Editing, Supervision; **Ondřej Mašek:** Conceptualization, Methodology, Writing - Review & Editing; **Tony Gutierrez:** Conceptualization, Methodology, Writing - Review & Editing; **Andrew Free:** Conceptualization, Methodology, Formal analysis, Writing - Original Draft, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

References

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924 Herbaspirillum canariense and Herbaspirillum psychrotolerans as Noviherbaspirillum
925 soli comb. nov., Noviherbaspirillum aurantiacum comb. nov., Noviherbaspirillum
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Table 1

ASVs specifically selected by diesel contamination in microcosms and in enrichment cultures.

ASV	Best Match	Accession no.	Identity (%)
1/e	*Aquabacterium commune* B8	NR_024875	99.2
2/a	*Rhodococcus erythropolis* ATCC 4277	NR_119125	100
3	*Aquabacterium commune* B8	NR_024875	99.6
4	*Aquabacterium commune* B8	NR_024875	98.8
5/j	*Cavicella subterranea* W2.09-231	NR_145637	97.2
6	*Nocardia ignorata* DSM 44496	NR_117326	100
7/b	*Aquabacterium citratiphilum* B4	NR_024871	100
8/l	*Sphingomonas insulae* DS-28	NR_044187	99.2
9/h	*Cavicella subterranea* W2.09-231	NR_145637	100
19/d	*Pseudarthrobacter phenanthrenivorans* sp. nov.	NR_042469	100
24	*Aquabacterium parvum* B6	NR_024874	99.2
c	*Acinetobacter oleivorans* NR1	NR_102814	100
g	*Parvibaculum indicum* P31	NR_116565	94.9

Notes: ASV numbers and letters refer to those in Figs. 4 and S6 respectively. The identity of the closest matching sequence in the NCBI 16S rRNA sequence database is shown, together with the corresponding accession no. and % identity.

Figure legends

Fig. 1. Changes in Shannon diversity from days 1-63 in uncontaminated soil microcosms, and in microcosms treated with diesel and amended with rice husk 700 ˚C (RH700), softwood pellets 550 ˚C (SWP550) or softwood pellets 700 ˚C (SWP700) biochar. Sterilised, diesel-treated microcosms are also shown. Diversity was determined at a rarefaction depth of 9,500 sequences, and values are mean ± SD (n = 3).

Fig. 2. (A) ANCOM analysis of differential abundance of genera between uncontaminated and diesel-treated microcosm soil across days 14-63 (n = 9 for each group). The centred-log ratio (CLR) is negative for genera elevated in the uncontaminated microcosms, and positive for those elevated in the diesel-treated microcosms. W is the ANCOM significance score, and genera which violate the null hypothesis are shown in black. The genus *Cavicella*, which has a high positive CLR but no significance, is annotated. (B) Mean % abundances are calculated for soil samples from all microcosms for these days (n = 9). *W score not significant.

Fig. 3. NMDS ordination plots of Bray-Curtis similarity between microbial communities in diesel-treated microcosms. (A) Soil samples from triplicate unamended and biochar-amended microcosms at day 63. (B) Soil and biochar samples from triplicate biochar-amended microcosms at day 63.

Fig. 4. (A) Heatmap of the top 5 most abundant ASVs for each sample from days 1-63 in uncontaminated soil microcosms (UC), and in microcosms treated with diesel (DO) and amended with rice husk 700 ˚C (RH700), softwood pellets 550 ˚C (SWP550) or softwood pellets 700 ˚C (SWP700) biochar. The most accurate taxonomy assigned to each ASV is shown,

along with an arbitrary ID corresponding to its rank abundance in this set of samples in parentheses. (B) Differential abundances of the top 13 diesel-selected ASVs on SWP550, SWP700 and RH700 biochar particles compared to the corresponding amended soil at day 63. Negative CLR values indicate reduced abundance on biochar particles compared to in the corresponding soil.

Fig. 5. Graphs of *alkB* gene copy number per ng DNA determined by qPCR for samples from (A) diesel-amended microcosms on days 14, 37 and 63, and biochar particles isolated on day 63; (B) enrichment cultures on day 28. Values are means (n = 3) and error bars represent standard deviations. Statistical significance (*: $p < 0.05$; **: $p < 0.01$) of comparisons within day groups in panel A (compared to DO for soil samples and RH700 for biochar samples) and between DO and biochar enrichments in panel B is indicated. Uncontaminated or uninoculated samples are not compared to others.

Fig. 6. (A) NMDS ordination plot of Bray-Curtis similarity between microbial communities in day 63 microcosms, inocula and enrichments. Microcosm samples (triangles) were extracted to yield inocula (grey circles) for enrichments. Inoculated enrichments at day 28 are annotated as biochar-amended or liquid only (DO). Communities from uninoculated enrichments (open circles) are also shown. (B) Mean % abundances are calculated for the top 10 ASVs in enrichment (n = 5) and inoculum (n = 4) samples. Negative CLR values imply reduced abundance in enrichment samples. IDs correspond to those shown in Fig S7, with numbers corresponding to the IDs of the same ASVs in Fig. 4 in parentheses.

Figure 1

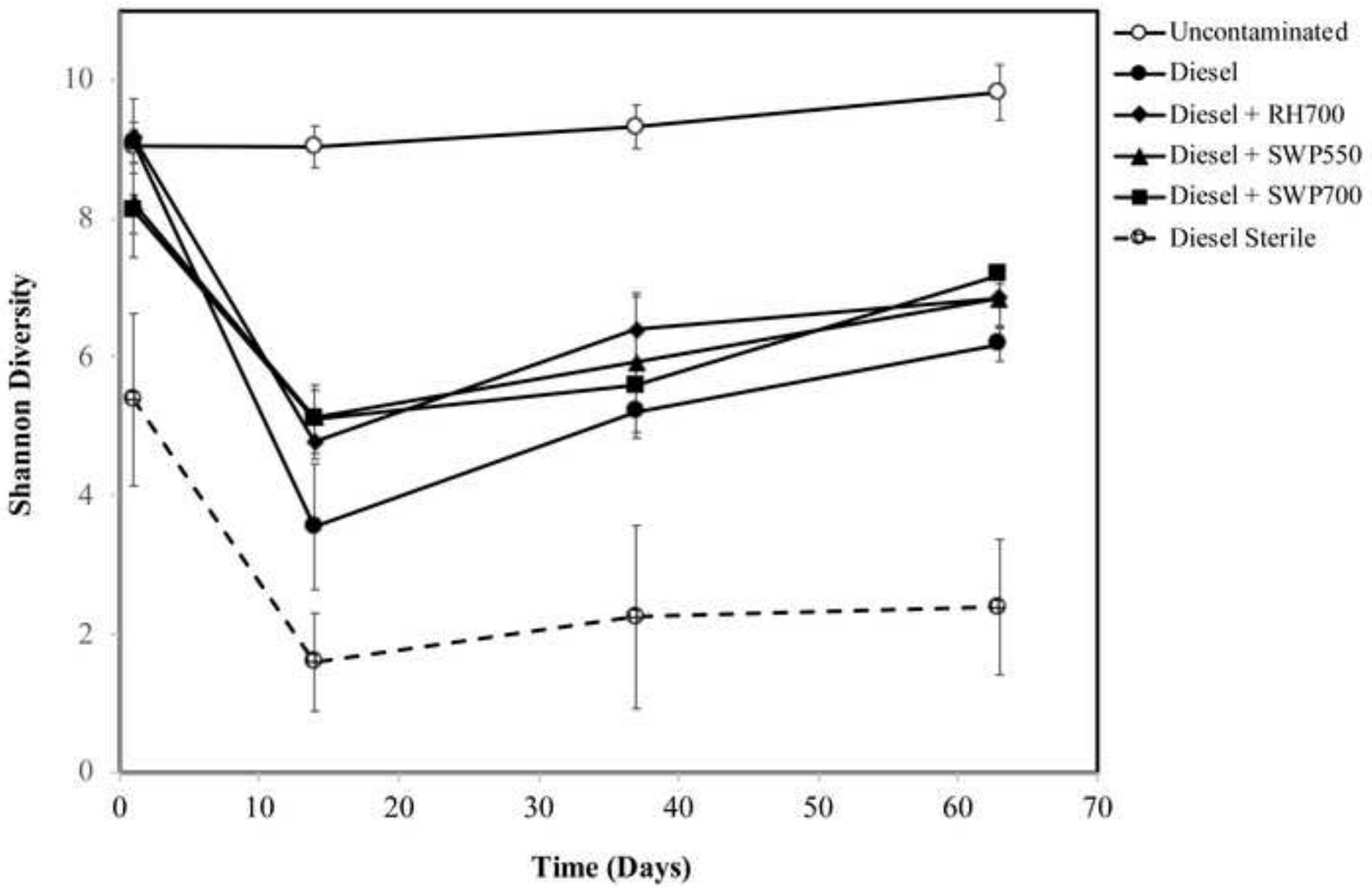
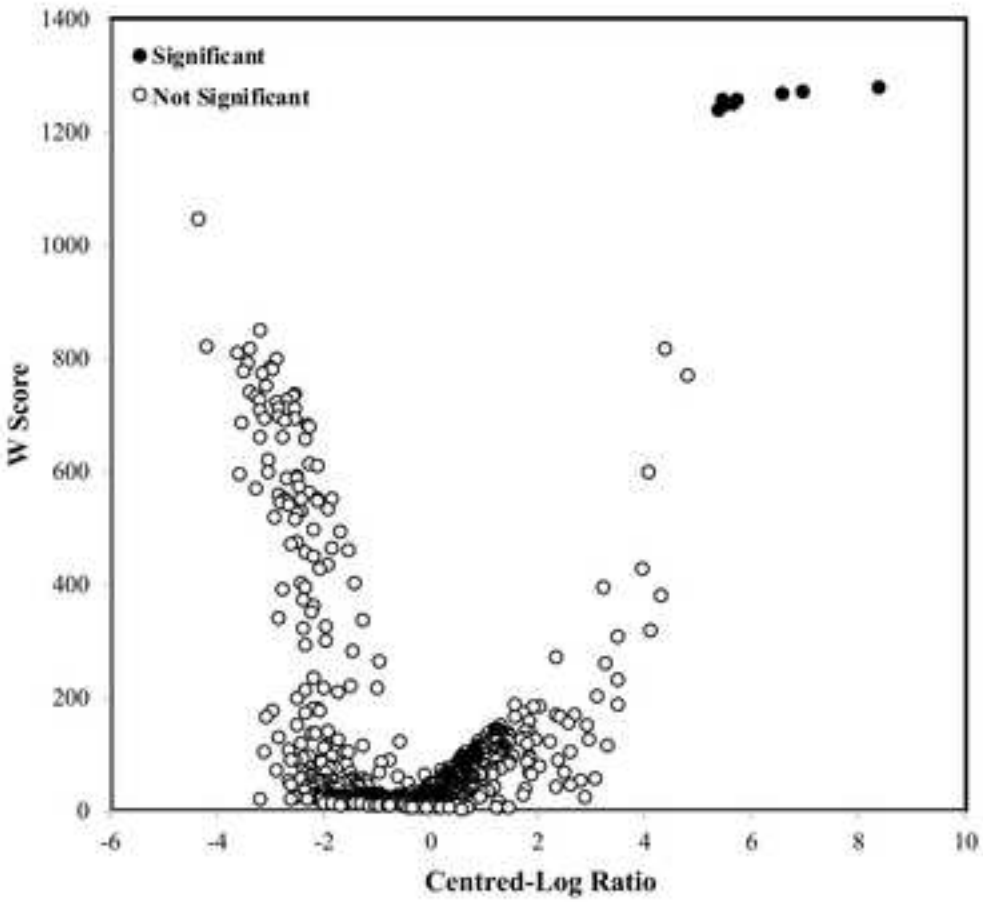


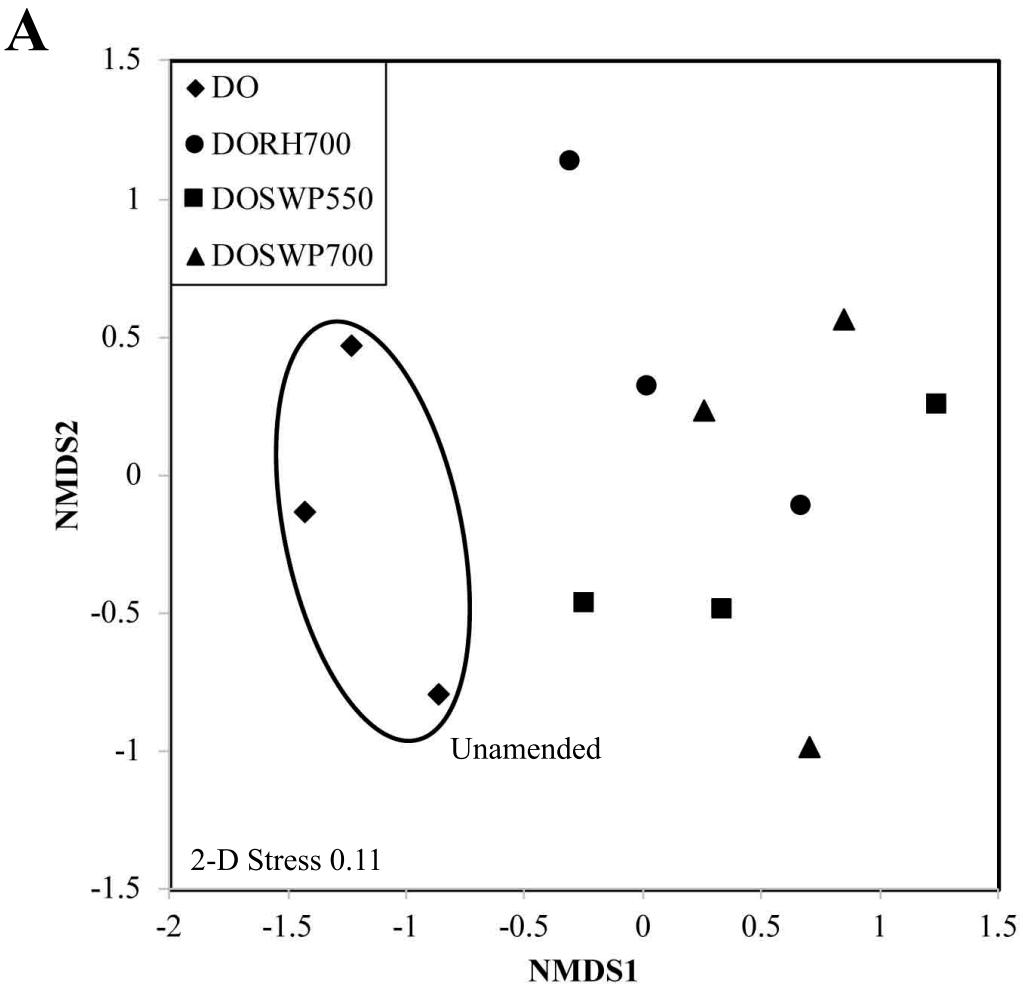
Figure 2

A



B Details of discriminant genera.

Genus	Mean % (diesel)	Mean % (uncont)	CLR	ANCOM W Score
Aquabacterium	25.4	0.216	8.39	1274
Nocardia	4.56	0.022	6.98	1268
Cavicella	12.2	0.082	6.62	1264
Methylophilaceae MM2	0.495	0	7.75	1255
Sphingopyxis	0.972	0.013	5.49	1252
Rhodococcus	8.83	0.071	5.67	1247
Caulobacter	2.82	0.037	5.47	1244
uncultured *Rhodospirillales*	1.64	0.011	5.42	1235
unclassified *Sphingomonadaceae*	0.006	0.230	-4.30	1043*
Parafilimonas	0	0.074	-3.17	847*
Nitrosospira	0.026	0.413	-4.17	818*


A
DO
DORH700
DOSWP550
DOSWP700
Unamended
2-D Stress 0.11
NMDS1
NMDS2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5

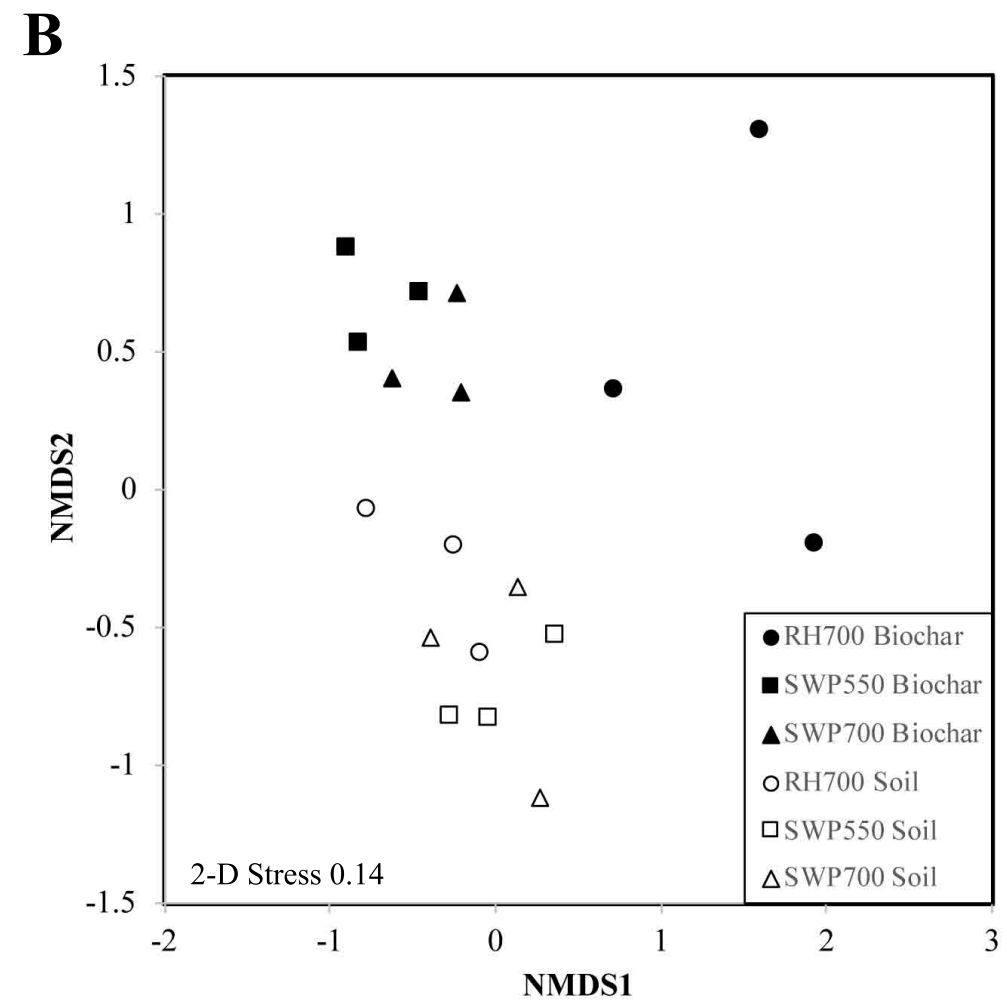
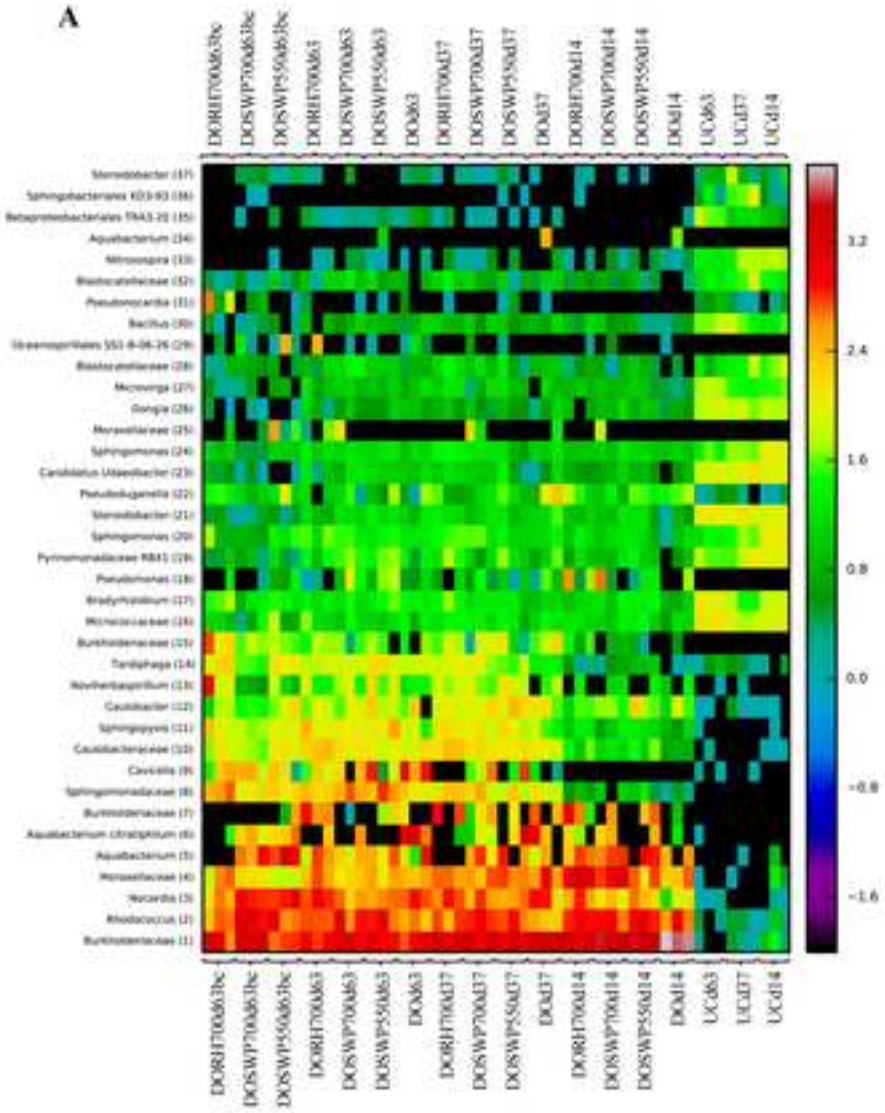

B
RH700 Biochar
SWP550 Biochar
SWP700 Biochar
RH700 Soil
SWP550 Soil
SWP700 Soil
2-D Stress 0.14
NMDS1
NMDS2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-1
0
1
2
3

Figure 4

Click here to access/download;Figure;Figure 4_Assil.tif



B Differential abundances of the top 13 diesel-selected ASVs

Taxonomy	RH700 CLR	SWP550 CLR	SWP700 CLR	No.
Aquabacterium	-0.133	0.033	0.864	1
Rhodococcus	-0.418	0.225	0.984	2
Aquabacterium	-3.31	0.414	0.859	3
Aquabacterium	-1.77	1.48	1.26	4
Cavicella	-0.346	-0.573	0.316	5
Nocardia	-0.868	1.26	1.42	6
Aquabacterium citratiphilum	-0.668	5.50	3.72	7
Sphingomonas	0.439	-0.466	-0.497	8
Cavicella	0.127	0.153	2.70	9
Caulobacter	-0.785	0.491	-0.154	10
Sphingopyxis	-0.160	-0.181	-0.057	11
Noviherbaspirillum	1.33	-0.371	-0.550	12
Noviherbaspirillum	1.48	0.579	0.469	13

Figure 5

Click here to access/download;Figure;Figure 5_Assil.tif

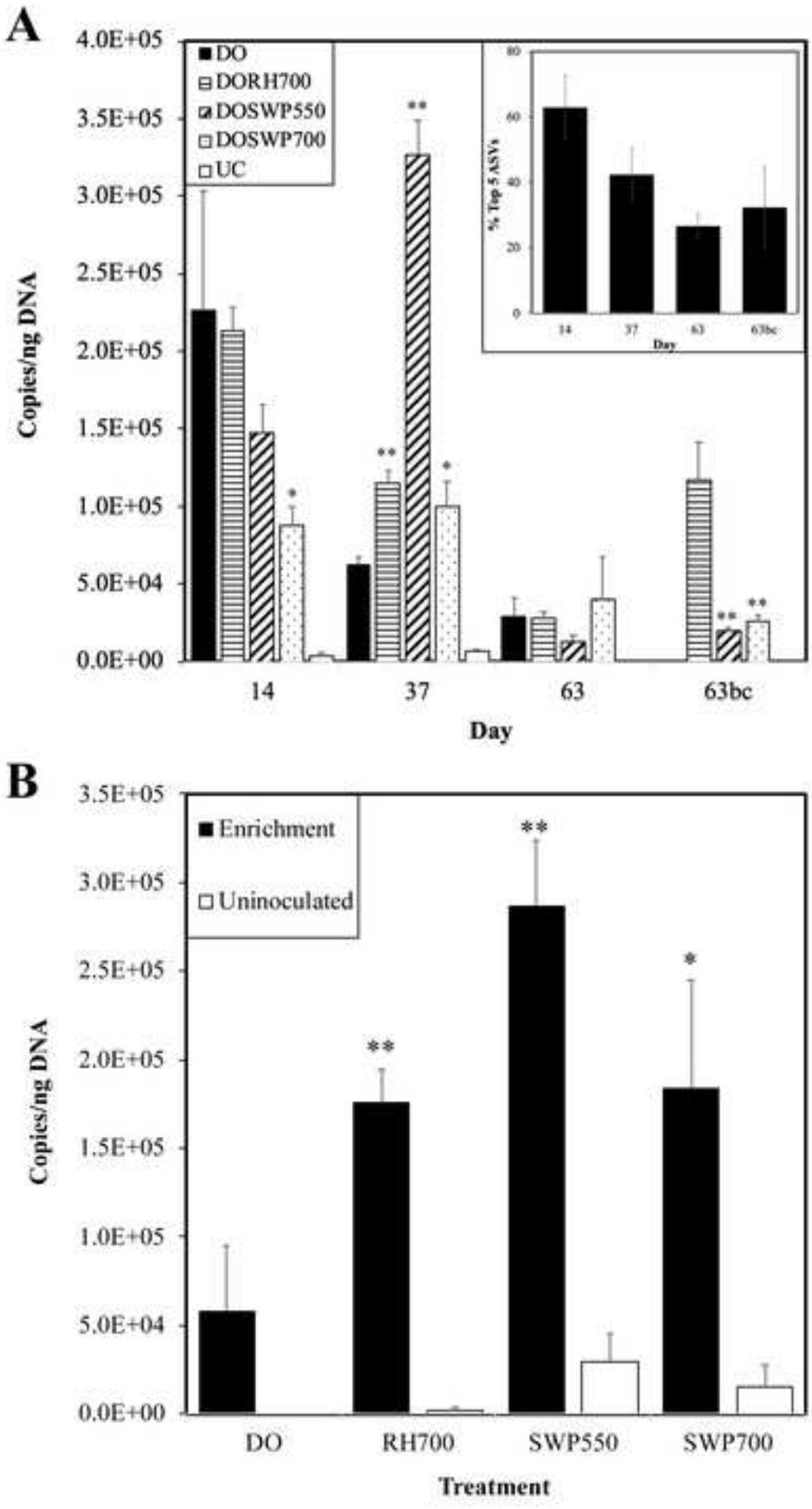
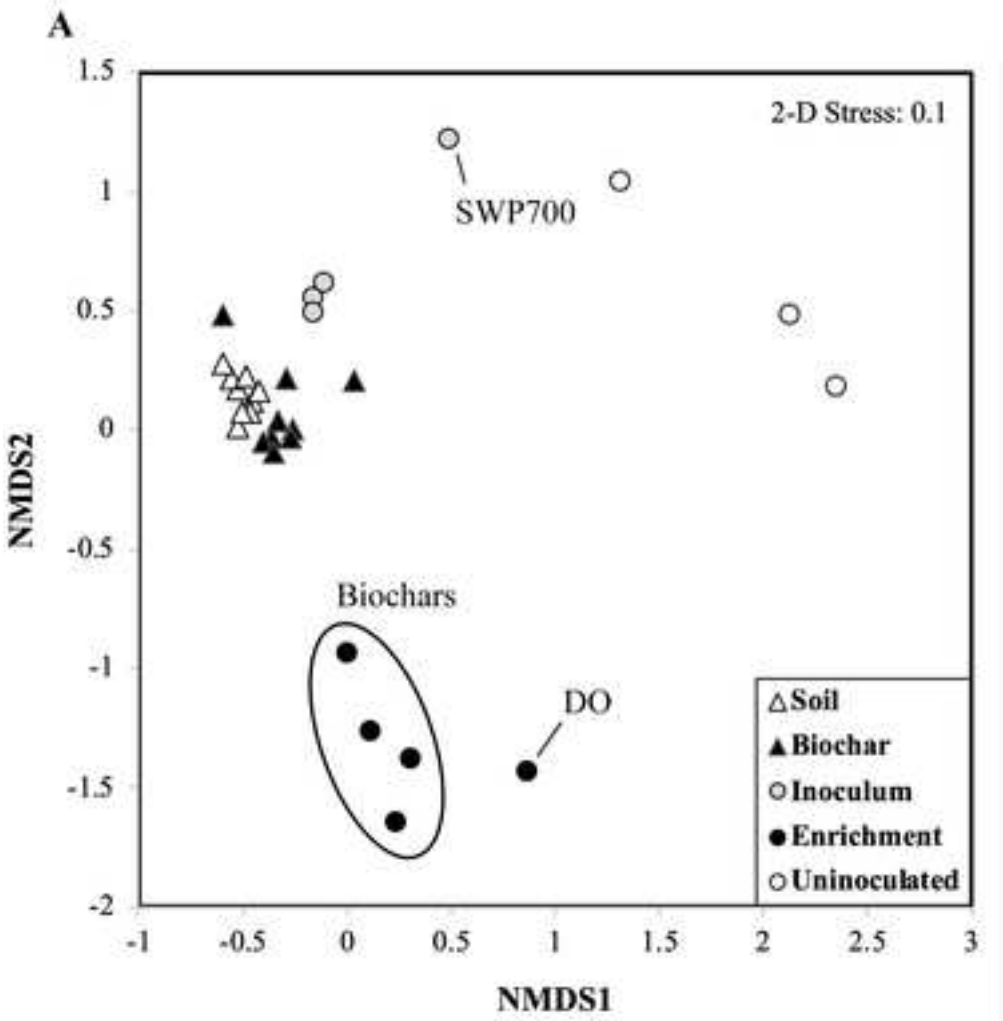


Figure 6



B Differential abundances of the top 10 ASVs in the enrichment experiment.

Genus	Mean % (enrichment)	Mean % (inoculum)	CLR	ID
Rhodococcus	18.0	0.944	4.03	a (2)
Aquabacterium citratiphilum	8.69	0.868	3.12	b (7)
Acinetobacter	16.0	0.100	5.98	c
Pseudarthrobacter	0.119	13.5	-3.26	d (19)
Aquabacterium	3.27	6.35	-2.72	e (1)
Pseudomonas	2.51	6.80	-3.98	f
Parvibaculum	3.21	0.148	4.55	g
Cervicella	0.594	2.24	-4.34	h (9)
Sphingomonas	0	2.88	-5.56	i (8)
Cervicella	0.021	2.64	-5.03	j (5)