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Specific enrichment of hydrocarbonclastic bacteria from diesel-amended soil on biochar particles

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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

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

27

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

30

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

33

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,

is attributed to its physical and chemical adsorptive abilities, resulting in removal of toxic compounds from the hydrophobic or aqueous phase (Ahmad et al., 2014). Optimal adsorption for specific compounds is affected by both feedstock and pyrolysis temperature, and includes the removal of hydrocarbons (Kong et al., 2018; Silvani et al., 2017; Tang et al., 2018). In general, higher-temperature biochars show greater adsorption due to higher specific surface area and stronger π - π bonding (Ni et al., 2020). However, while greatest for aromatic hydrocarbons, sorption to biochar does not seem to compromise biodegradation of short-chain normal (*n*) alkanes in short-term experiments in a sandy soil (Bushnaf et al., 2011). Moreover, the porous structure of biochar forms an excellent habitat for microbial biofilms, where colonizing organisms gain access to water, air and nutrients, potentially including adsorbed compounds (Schnee et al., 2016; Zhu et al., 2017). The taxonomic composition of biochar surface biofilms is affected by the feedstock used for pyrolysis, and can include enrichment of hydrocarbonoclastic bacteria when their substrates are present (Kong et al., 2018). However, immobilisation of some nutrients, including contaminants, on biochar surfaces may limit their bioavailability (Joško et al., 2013), and high biochar pH (>9 in some cases; (Mašek et al., 2018)) may also limit hydrocarbon biodegradation.

Biochar also has the potential to be used as a microbial carrier (biochar augmented with microorganisms), protecting the microbes within its matrix from both physicochemical stresses and biological competition (Partovinia and Rasekh, 2018). However, somewhat equivocal results have been obtained from attempts to bioaugment hydrocarbon remediation systems with inoculated biochars: although faster removal of petroleum hydrocarbons from liquid medium has been shown using biochar-attached *Acinetobacter* or *Corynebacterium* species (Chen et al., 2016; Zhang et al., 2016), and biochar-associated *Mycobacterium gilvum* has been shown 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 NH₄NO₃, 10.85 g/l K₂HPO₄) 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 MgSO₄, 0.02 g/l CaCl₂, 1 g/l KH₂PO₄, 1 g/l K₂HPO₄, 1 g/l NH₄NO₃, 0.05 g/l FeCl₃, 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

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

Variable V4 regions of bacterial and archaeal 16S rRNA genes were amplified using a modified, barcoded 515F-Y/806R primer set (Caporaso et al., 2012), where the forward primer contained the modification described by Parada *et al.* to remove biases against certain archaeal taxa (Parada et al., 2016). PCR-grade water (Sigma), Taq polymerase buffer and MgCl₂ (Roche) were pre-treated with UV in a PCR cabinet [15 W UV light (λ = 254 nm); 15 mins] prior to setting up reactions containing 1 x buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 250 µM each primer and 0.05 U/µl Taq DNA polymerase (Roche). DNA (typically 1-10 ng) or PCR-grade water (negative controls) were added for a final reaction volume of 25 µl. Cycling conditions were 94 °C for 3 min followed by 35 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. Entire PCR reactions were separated by 1% (w/v) TAE-agarose gel electrophoresis, visualized with ethidium bromide staining and amplicons excised under UV light. DNA was purified from the gel fragments using a Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. DNA concentrations were determined using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and amplicons were pooled in equimolar concentrations for Illumina MiSeq 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, 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 MgCl₂ 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

260

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

274

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

3.2. Effect of diesel oil on soil community composition

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

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

Not unexpectedly, this may reflect the ability of some microorganisms to survive even the harshest treatments (Otte et al., 2018), as discussed in more detail later (section 4.3).

Because metataxonomic datasets of this type exhibiting widespread changes of community relative abundance are highly sensitive to the compositionality of the data (Gloor et al., 2017), we employed the compositionality-insensitive ANCOM method (Mandal et al., 2015) to detect genera which showed significantly altered abundance in diesel-treated microcosms without biochar amendment compared to untreated controls between days 14 to 63. Of the >1,200 genera within the soil community dataset, ANCOM detected 8 genera which were significantly elevated in diesel-treated microcosms (Fig. 2). The plot of ANCOM W score against Centred Log Ratio (CLR) revealed that these genera were highly enriched in the presence of diesel oil, while many other genera were moderately enriched in uncontaminated soil and, to a lesser extent in diesel-contaminated soil.

The significantly enriched genera (Fig. 2) included abundant *Aquabacterium*, *Nocardia*, *Cavicella* and *Rhodococcus*, and lower-abundance *Caulobacter* and uncultured *Rhodospirillales* sequences. Collectively, these genera accounted for >55% of the microbial community abundance in this set of microcosms, while all of them had a mean abundance of <1% in untreated microcosms. Their enrichment accounts for the increased abundance of the classes *Gammaproteobacteria* and *Actinobacteria* in oil-treated microcosms seen in Fig. S1. In contrast, no taxa elevated in uncontaminated microcosms were detected as significant by ANCOM, although three low-abundance genera, unclassified *Sphingomonadaceae*, *Parafilimonas* and *Nitrosospira*, depleted by oil treatment fell just below the significance threshold (Fig. 2). These data indicate that diesel oil causes a highly-specific enrichment of a

limited set of genera to high relative abundance, while at the same time depleting or eliminating a large number of other genera within the soil community.

3.3. Microbial communities of biochar-amended soil and biochar-associated taxa

To test the effect of biochar amendment of soil microbial communities in the presence of diesel oil, community (Bray-Curtis) similarity was compared between amended and unamended soils from days 14 to 63. An NMDS plot revealed that, while all communities had similar composition at day 14, they diverged over time (Fig. S2A). In addition to the statistically-strong effect of time (PERMANOVA: Pseudo-F = 7.85, $p < 0.001$), biochar amendment also had a significant effect on composition (PERMANOVA: Pseudo-F = 2.12, $p = 0.034$). Although the NMDS plot suggested some clustering of samples from soils amended with individual biochar types, this was not significant except for the comparison of rice husk 700°C biochar-amended soil to unamended soil (pairwise PERMANOVA: $t = 0.80$, $p = 0.044$). To visualise the effects of biochar amendment more clearly, day 63 samples, taken after biochar had been present in the soil for the maximum length of time, were considered in isolation. NMDS ordination (Fig. 3A) showed a clear clustering of unamended soils separately from those supplemented with biochar (PERMANOVA: Pseudo-F = 2.63, $p = 0.002$), as well as an effect of biochar type on community composition (PERMANOVA: Pseudo-F = 1.68, $p = 0.005$).

We then examined the microbial communities associated specifically with biochar particles isolated from the microcosms, as compared to those in bulk soil. There was a clear separation between soil microcosm communities at day 63 and those associated with biochar particles isolated at the same time (Fig. 3B), with strong statistical support (PERMANOVA:

Pseudo-F = 3.22, $p < 0.001$). There was some evidence of clustering of biochar-associated communities by biochar type, which was clearest when these communities were considered in isolation (Fig. S2B). Again, this was supported by a PERMANOVA test (Pseudo-F = 3.14, $p = 0.002$). As might be expected, alpha (Shannon) diversity at day 63 (Fig. S2C) was also significantly lower in biochar-associated communities than in the corresponding soil communities (Kruskal-Wallis test: $H = 9.28$, $p = 0.002$). While there appeared to be slightly different levels of Shannon diversity between different biochar-associated communities, this had no overall or pairwise significance, although RH700-associated communities had significantly higher Pielou's evenness (Kruskal-Wallis $H = 5.96$, $p = 0.05$).

To exploit the resolution of ASVs, we performed analysis of the ASVs which were enriched in diesel-treated microcosms over time and which were selected in the presence of different types of biochar. Extraction of the top 15 most abundant ASVs for each sample at days 14 to 63 (including uncontaminated microcosms) yielded a subset of 119 ASVs which represented $76.9 \pm 8.9\%$ of the total sequences in contaminated samples, but only $20.9 \pm 2.6\%$ of the sequences in uncontaminated samples. This disparity illustrates again the highly-specific enrichment of a limited set of taxa in the presence of diesel oil. To simplify the data further for detailed comparison, we restricted our analysis to the top 5 most abundant ASVs in each sample, a set of 33 features representing $10.2 \pm 1.4\%$ of total sequences in uncontaminated samples, but $60.6 \pm 12.4\%$ of the sequences in contaminated samples. Thirteen of these ASVs, assigned to the genera *Aquabacterium*, *Rhodococcus*, *Cavicella*, *Nocardia*, *Sphingomonas*, *Sphingopyxis*, *Caulobacter* and *Noviherbaspirillum* were highly abundant in most or all diesel-treated samples, but rare or undetectable in uncontaminated samples, as illustrated by a heatmap (Fig. 4; features 1-13). The remarkable overlap of these taxonomic assignments with the genera found to be significantly enriched in contaminated samples by ANCOM (Fig. 2)

suggests that in most cases, individual species are specifically selected in the presence of oil. Moreover, beta-diversity analysis based solely on the distribution of these 13 ASVs reproduced closely the patterns seen in Fig. 3 based on day after treatment, biochar amendment, sample type (soil vs. biochar) and biochar type ($\rho = 0.843-0.947$; $p < 0.001$ in all cases). Hence these 13 species are fundamental to the soil community response to diesel contamination and biochar amendment.

To analyse the ability of these highly-selected species to colonise the surfaces of different types of biochar particles, we calculated the centred-log ratios of the ASV abundances on biochar samples isolated from contaminated microcosms at day 63, compared to bulk soil isolated from the same microcosms (Fig. 4). In general, the CLR values were close to zero, consistent with the observation that the abundant diesel-selected taxa were found in both types of sample. However, the three most abundant *Aquabacterium* ASVs (i.e. ASVs 1, 3 and 4) were noticeably less abundant on RH700 biochar particles, compared to slight enrichment on SWP biochar particles, whereas ASV-7 (*Aquabacterium citratiphilum*) was strongly enriched on both types of SWP biochar, but slightly depleted on RH700 biochar. ASV 9 (*Cavicella*) was specifically enriched on SWP700 biochar compared to the other types. Across all of these 13 ASVs, the mean CLR was positive for SWP550 and SWP700 biochars (0.68 and 0.87 respectively), but negative for RH700 biochar (-0.39). Considering all ASVs in the dataset, enrichment on SWP550 and SWP700 biochars was significantly correlated ($r = 0.78$, $p = 0.009$), but enrichment patterns between RH700 biochar and either of the SWP biochars were negatively correlated, albeit below the significance threshold (Fig. S3). These data suggest that distinct properties of the RH biochar inhibit its colonisation by representative soil microorganisms compared to SWP-derived chars, and this applies to some of the highly-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*-C₁₇ and *n*-C₁₈ *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*-C₁₇ and *n*-C₁₈, was

preferentially adsorbed to the biochar surface and became resistant to further degradation on this timescale.

To correlate these biochar effects on hydrocarbons with those on hydrocarbonoclastic bacteria, we performed qPCR for the alkane 1-monooxygenase (*alkB*) gene in biochar-amended soils throughout the experiment, and on biochar particles isolated on day 63. Compared to unamended, diesel-treated soil, soils amended with biochars showed reduced copies of *alkB* at day 14, though the difference was only significant for SWP700 biochar amendment (Fig. 5A). In contrast, biochar-amended soils showed significantly higher levels of *alkB* at day 37, especially for low-temperature biochar (SWP550). By day 63, copies of the gene had declined in all soil samples, whereas uncontaminated soil contained $<7 \times 10^3$ copies/ng DNA throughout the experiment. These results are consistent with a delayed response by *alkB*-containing soil bacteria to diesel oil in the presence of biochar. Final SWP biochar particles themselves contained similar levels of *alkB* to bulk soil at day 63, although RH biochar retained significantly more copies of the gene (Fig. 5A).

3.5. Enrichment of hydrocarbon degraders extracted from contaminated microcosms in the presence of biochar

The microcosm data suggest that biochar can act as a sorbent for hydrocarbon components of diesel oil, and may also be a habitat for hydrocarbon-degrading soil microorganisms, which are enriched upon diesel contamination. Therefore, we sought to test whether these selected species could be further enriched by culture in minimal medium supplemented with diesel oil, with or without biochar amendment. Microorganisms were 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 $H = 7.385$; $p = 0.007$),

indicating that enrichment in the presence of diesel oil was highly selective for a subset of taxa. Interestingly, enrichments in the presence of biochar contained different microbial compositions from enrichment in medium plus oil alone (Fig. 6), with higher levels of *Rhodococcus* species which were only moderately enriched in liquid medium (Fig. S6). This suggests that members of this genus preferentially associate with biochar surfaces. There was also a noticeably distinct composition of species associated with SWP-derived biochars (richer in *Aquabacterium*) compared to the RH700 biochar (enriched in *Acinetobacter*). Although the SWP700 inoculum exhibited a divergent taxonomic composition, inoculating a Bushnell-Hass medium-SWP700 biochar enrichment with the inoculum derived from unamended microcosms instead resulted in a similar final enrichment composition, although this enrichment contained almost 50% *Rhodococcus* (Fig. S6). Supporting the selectivity of enrichment in the presence of biochar, char-amended enrichments tended to have lower Shannon diversity than enrichment in liquid medium. These enrichments also contained significantly higher levels of the *alkB* gene as determined by qPCR (Fig. 5B); lower levels of *alkB* were also detectable in SWP biochar-amended uninoculated enrichments.

Finally, we constructed an ASV-level heatmap of the top 5 most abundant features for each sample in the enrichment experiment (not including uninoculated cultures). These 22 features comprised $49 \pm 12\%$ of all sequences in inoculum samples, but $74 \pm 10\%$ of all sequences in enrichment samples, highlighting again the selectivity of enrichment. Of the 10 most abundant ASVs in the enrichment experiment dataset (Fig. 6), 7 were also present in the set of 33 most abundant ASVs from the microcosm experiment (Fig. 4; indicated by numbering in Fig. S7), with ASV nos. 2 (a; *Rhodococcus*), 7 (b; *Aquabacterium citratiphilum*) and 1 (e; *Aquabacterium*) highly abundant in some or all enrichments. The first two of these ASVs, along with *Acinetobacter* and *Parvibaculum* sequences (c and g) rare in the microcosms, were

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

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-

enriched population. Finally, we have shown that it is possible to enrich a subset of these species from contaminated soil on biochar particles in liquid medium containing oil, which suggests a possible strategy for bioaugmentation of contaminated soils using endogenous microorganisms.

4.1. Taxa specifically selected by diesel contamination

Key taxa selected in the contaminated microcosms and enrichment cultures are detailed in Table 1. The most abundant ASV in contaminated microcosms (1 in Fig. 4) is assigned to the genus *Aquabacterium* and reaches a mean abundance of 20% in all contaminated samples. However, it makes up part of the rare community (mean 0.1%) in uncontaminated microcosms, and is undetectable in the starting soil sample. While not quite as striking as the >60% relative abundance of *Alkanindiges* sp. observed in diesel-contaminated microcosms by Fuentes *et al.* (Fuentes et al., 2016), this is still a remarkable and consistent level of selection by oil exposure. A second *Aquabacterium* ASV (ASV-3; Fig. 4) is almost universal (mean 5.3%) in contaminated microcosms and undetectable in both bulk soil and uncontaminated microcosms. The closest match to both of these sequences in the NCBI 16S rRNA sequence database is *Aquabacterium commune* strain B8 (Table 1). There are two other diesel-selected ASVs assigned to the genus *Aquabacterium* in the dataset (ASV-4 and ASV-7 in Fig. 4) which are almost universal in contaminated samples (means 5.2% and 2.5% respectively), and these are very sporadically detected or undetectable in uncontaminated microcosms and bulk soil. The first of these sequences is 98.8% identical to *Aquabacterium commune* B8, while the second is 100% identical to *Aquabacterium citratiphilum* strain B4 (Table 1). Finally, an *Aquabacterium* 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

relative abundance over time and the abundance of the alkane 1-monooxygenase (*alkB*) gene as determined by qPCR (Fig. 5A).

4.2. Taxa specifically selected by enrichment on diesel oil as a carbon source

Consistent with their enrichment in diesel-contaminated soil, the *Rhodococcus* and *Aquabacterium* ASVs -1(e), -2(a) and -7(b) were also abundant in enrichments containing diesel oil as the sole carbon source (Figs. 7 and S7). This illustrates the potential for using enriched extracts from a given soil type to inoculate biochar particles in the laboratory, prior to application of this inoculated biochar for bioremediation *in situ*. However, other abundant ASVs from the oil-selected set of 13 observed in soil microcosms were not abundant in the enrichments, and while in some cases this was probably due to their poor survival in the soil extracts, the *Sphingomonas* and *Cavicella* species ASV-5(j), ASV-8(l) and ASV-9(h) had low abundance in enrichment cultures despite being abundant in the extracts. This may reflect poor culturability under the enrichment conditions, although ASV-9 did grow in one enrichment culture. One other species with lower abundance in the original microcosms, *Pseudarthrobacter* ASV-19(d), was highly abundant in soil extracts but reduced in the enrichment cultures, although it remained universally present (Fig. S7). This sequence is 100% identical to *Pseudarthrobacter phenanthrenivorans* sp. nov. (Table 1), a phenanthrene-degrader isolated from creosote-contaminated soil (Kallimanis et al., 2009).

As might be expected, there are some other taxa which are rare or undetectable in the inoculum samples, but abundant in the enrichment cultures (Fig. S7). One of these, *Acinetobacter* ASV-c, was abundant in most enrichments (mean 13.5%), but occurred 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

cultures compared to those containing SWP biochar (Fig. S7). RH700 char has a 50-fold higher ash content than the SWP chars (Mašek et al., 2018), has a high silica content and is slightly hydrophilic, which may affect oil adsorption. Likewise, RH700 has ~2-fold lower carbon content (47 wt%, compared to 85-90 wt% for SWP chars). However, there is no indication that it affects alkane degradation differently from the SWP biochars (Fig. 5). The pH of the RH biochar is also strongly alkaline (9.8, compared to 7.9-8.4 for the SWP biochars), and it contains 3-fold more potassium than the SWP chars (Cabeza et al., 2018; Mašek et al., 2018), which may impose selection against specific bacterial species. Biochar can also influence microbial community composition by interfering with bacterial cell-cell communication (Gao et al., 2016). However, in enrichment cultures, all three types of biochar show greater copy numbers of the *alkB* gene involved in hydrocarbon degradation, compared to unamended culture (Fig. 5).

Overall, the two SWP biochars showed similar enrichment of ASVs from diesel-contaminated soil (Figs. 3, 4 and S3), suggesting that pyrolysis temperature does not make a significant difference to colonisation by these taxa for this feedstock. SWP700 has the highest BET surface area of the three chars (162 m²/g, compared to 26.4 m²/g for SWP550 and 42 m²/g for RH700), which may enhance oil adsorption, although this is due to the presence of micropores of ~10 Å size (Wong et al., 2019), which will be too small for bacteria to colonise. However, differences in oil adsorption may affect microbial diversity indirectly. SWP700 char particles isolated from soil microcosms at day 63 exhibited the lowest Shannon diversity of the three types, while RH700 biochar showed the highest diversity and significantly greater evenness (Fig. S2), despite its lower C content. Our overall conclusion is that SWP biochars show better colonisation by the main potential hydrocarbon degraders in this soil type, while RH700 biochar shows more general microbial association and may select against

Aquabacterium spp. Hence SWP biochars would be preferred as a matrix for bioaugmentation in this context.

4.4. Species dynamics and hydrocarbon degradation in sterilised microcosms

Analysis of hydrocarbon degradation in sterilised soil microcosms suggested a slow rate of *n*-alkane degradation (Fig. 5), although the changes over time were below the significance threshold and much smaller than those in unsterilised soil. Likewise, taxonomic changes occurred in the sterilised microcosms over time compared to sterilised bulk soil, suggestive of residual microbial activity (Fig. S1). Although repeated autoclaving is an effective sterilisation strategy, hydrocarbon degradation and other microbial activity have been observed in autoclaved soils and sediments over similar time periods (Lu et al., 2019; Otte et al., 2018), suggesting that abiotic controls in such studies are not completely free of microbial activity. Prolonged autoclaving has also been shown to allow limited microbial survival and PAH degradation in soils, in comparison to treatment with toxic HgCl₂ (Wang et al., 2011a).

Major taxa (>5% mean abundance) in the sterilised microcosms were assigned to the genera *Luteibacter*, *Massilia* and *Pseudomonas* and to the family *Micrococcaceae*, and while undetectable in the starting sterilised soil, were also detected in unsterilised microcosms, especially those contaminated with diesel oil: this suggests survival and outgrowth of these organisms in the presence of hydrocarbons. In support of this hypothesis, the genera *Luteibacter*, *Massilia* and *Pseudomonas* include members with reported hydrocarbon-degrading abilities (Gutierrez et al., 2015; Lu et al., 2019; Mahjoubi et al., 2013). In contrast, uninoculated enrichment cultures were rich in *Dietzia* spp. and *Mycobacterium* spp. which were undetectable in soil and microcosms; this suggests that these organisms may have been

present in the diesel oil itself, or possibly on added biochar particles. *Dietzia* and *Mycobacterium* species have both been shown to have hydrocarbon degradation ability (Kim et al., 2015; Wang et al., 2011b), and have been observed at high abundance in hydrocarbon-contaminated soils amended with biochar (Gielnik et al., 2019).

5. Conclusions

In conclusion, by using exact amplicon sequence variant analysis methods, our study shows the extreme species selectivity of soil community responses to hydrocarbon contamination. Just 13 highly-selected ASVs, mapping to 8 known hydrocarbon-degrading genera, characterise the oil-, time- and biochar-dependent changes in the structure of the highly diverse soil community. These conditionally-rare taxa are likely to be soil type-specific, making them prime candidates for bioaugmentation into similar types of soil to bioremediate hydrocarbon contamination. Similar specificity is seen in the interactions of these enriched species with biochar particles, with softwood pellet-derived biochars enriching the majority of these species further, while rice husk-derived biochar is particularly counter-selective for *Aquabacterium* spp. Enrichment experiments with diesel oil as the sole carbon source showed that softwood pellet biochar could be used to further enrich *Aquabacterium* and *Rhodococcus* species isolated from the contaminated soil, and was more effective than liquid enrichment alone in increasing copy numbers of the *alkB* gene.

Our work suggests a bioremediation strategy in which autochthonous conditionally-rare taxa could be isolated from a given soil type, enriched on biochar particles in a liquid-based culture system, and then used to carry out bioaugmentation for *in situ* bioremediation of the soil. This would allow application of hydrocarbon degraders likely to thrive within the target environment, especially in cases of acute contamination where the endogenous

770 community may not be able to respond sufficiently. Such an approach takes account of both
771 the composition of the endogenous community, to ensure enrichment with compatible taxa
772 likely to persist in the environment, and the appropriate type of standard biochar to achieve
773 their enrichment and stable delivery. It would therefore be expected to have advantages over
774 the application of exogenous species, which may not become stabilised in the environment, or
775 of biochar alone, which would facilitate only biostimulation and adsorption unless colonised
776 *in situ*. Key challenges for such an approach are an ability to culture the necessary species in
777 an enrichment, preparation of sufficient amounts of inoculant for a field site, and delivering the
778 inoculated biochar particles at scale. Important next steps for this research are to test the
779 effectiveness of pre-inoculated biochar particles - compared to uninoculated biochar, biochar
780 inoculated with exogenous hydrocarbon degraders and liquid inoculum without biochar - in
781 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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807

808 **Appendix A. Supplementary data**

809

810 Supplementary data to this article can be found online.

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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	<i>Aquabacterium commune</i> B8	NR_024875	99.2
2/a	<i>Rhodococcus erythropolis</i> ATCC 4277	NR_119125	100
3	<i>Aquabacterium commune</i> B8	NR_024875	99.6
4	<i>Aquabacterium commune</i> B8	NR_024875	98.8
5/j	<i>Cavicella subterranea</i> W2.09-231	NR_145637	97.2
6	<i>Nocardia ignorata</i> DSM 44496	NR_117326	100
7/b	<i>Aquabacterium citratiphilum</i> B4	NR_024871	100
8/l	<i>Sphingomonas insulae</i> DS-28	NR_044187	99.2
9/h	<i>Cavicella subterranea</i> W2.09-231	NR_145637	100
19/d	<i>Pseudarthrobacter phenanthrenivorans</i> sp. nov.	NR_042469	100
24	<i>Aquabacterium parvum</i> B6	NR_024874	99.2
c	<i>Acinetobacter oleivorans</i> NR1	NR_102814	100
g	<i>Parvibaculum indicum</i> 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 $\pm$ 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

B Details of discriminant genera.

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

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

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

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