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Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar

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ABSTRACT: The inoculation of rice straw biochar with PAH-degrading *Mycobacterium gilvum* ($1.27\times10^{11}\pm1.24\times10^{10}$ cell g^{-1}), and the subsequent amendment of this composite material to PAHs contaminated (677 mg kg^{-1}) coke plant soil, was conducted in order to investigate if would enhance PAHs biodegradation in soils. The microbe-biochar composite showed superior degradation capacity for phenanthrene, fluoranthene and pyrene. Phenanthrene loss in the microbe-biochar composite, free cell alone and biochar alone treatments was, respectively, 62.6±3.2%, 47.3±4.1% and non-significant ($P>0.05$); whereas for fluoranthene loss it was 52.1±2.3%; non-significant ($P>0.05$) and non-significant ($P>0.05$); and for pyrene loss it was 62.1±0.9%; 19.7±6.5% and 13.5±2.8%. It was hypothesized that the improved remediation was underpinned by i) biochar enhanced mass transfer of PAHs from the soil to the carbonaceous biochar “sink”, and ii) the subsequent degradation of the PAHs by the immobilized *M. gilvum*. To test this mechanism, a surfactant (Brij 30; 20 mg g^{-1} soil), was added to impede PAHs mass transfer to biochar and sorption. The surfactant increased solution phase PAH concentrations and significantly ($P<0.05$) reduced PAH degradation in the biochar immobilized *M. gilvum* treatments; indicating the enhanced degradation occurred between the immobilized *M. gilvum* and biochar sorbed PAHs.

Keywords:

PAHs

Biochar

Microbe immobilization

Soil

Degradation

24 **Abbreviations**

25 PAHs[1], AC[2], BC[3]

26 HA[4], DOC[5], CP soil[6]

27 BET[7], *M. gilvum*[8], MM[9]

28 LB[10], SEM[11], qPCR[12]

29 CPD[13], EPS[14], DLVO[15]

30 CSH[16], PHE[17], FLA[18], PYR[19]

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[1] Polycyclic aromatic hydrocarbons
[2] Activated carbon
[3] Biochar
[4] Humic acids
[5] Dissolved organic carbon
[6] Beijing coking plant soil
[7] Brunauer-Emmett-Teller
[8] *Mycobacterium gilvum*
[9] Minimal medium
[10] Lysogeny Broth
[11] Scanning electron microscopy
[12] Real time quantitative PCR
[13] Critical-point drying
[14] Extracellular polymeric substances
[15] Derjaguin–Landau–Verwey–Overbeek theory
[16] Cell surface hydrophobicity
[17] Phenanthrene
[18] Fluoranthene
[19] Pyrene

38 **1. Introduction**

39 Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic and mutagenic
40 compounds (Mastrangelo, Fadda et al. 1996, Goldman, Enewold et al. 2001). Their
41 ubiquitous occurrence (Samanta, Singh et al. 2002), intrinsic toxicity (Mastrangelo,
42 Fadda et al. 1996, Goldman, Enewold et al. 2001) and bioaccumulation potential
43 (Baussant, Sanni et al. 2001) raise significant concerns for human and ecosystem health,
44 wherever PAHs are present at elevated concentrations, such as those found in urban or
45 industrial soils (Wagrowski and Hites 1996, Bakker, Casado et al. 2000, Tang, Tang et
46 al. 2005).

47 Developing cost-effective methods to clean up PAHs from contaminated land
48 remains a technological challenge (Gan, Lau et al. 2009). One approach is to lower the
49 bioavailability of PAHs through the introduction of sorbent amendments, to strongly
50 bind the PAHs, and thereby reduce exposure and associated risks (Ghosh, Luthy et al.
51 2011). The most extensively studied amending agents for such purposes, being a
52 sorption "sink" for PAHs, are carbonaceous materials such as activated carbon (AC)
53 and biochars (Beesley, Moreno-Jiménez et al. 2011, Hale, Hanley et al. 2011, Lehmann
54 and Joseph 2012, Oleszczuk, Hale et al. 2012). Such carbonaceous materials have been
55 observed to sorb PAHs up to 10-1000 times stronger (per unit mass) than other types of
56 soil organic carbon (Accardi-Dey and Gschwend 2003, Cornelissen and Gustafsson
57 2004). When a strong sorbent, like biochar or AC, is introduced to a contaminated soil,
58 the contaminants are transferred from the aqueous phase and weaker sorption (fast-
59 desorption) sites of the contaminated soil to the strong sorption site of the added sorbent
60 (Werner, Ghosh et al. 2006, Rhodes, Carlin et al. 2008, Cho, Werner et al. 2012,
61 Oleszczuk, Hale et al. 2012, Liu, Chen et al. 2015). As an example, the uptake of
62 polychlorinated biphenyls in passive samplers decreased up to 73% with amendment

63 of 3.7 % of AC after 5 years (Cho, Werner et al. 2012). Though AC can sorb PAHs
64 more effectively than biochars (Cornelissen, Breedveld et al. 2006, Gomez-Eyles,
65 Yupanqui et al. 2013), biochars offer other advantageous traits, such as lower cost, the
66 ability to be made from organic waste materials, and reduced (or even negative) CO_2
67 emissions associated with their production (Lehmann 2007, Lehmann and Joseph 2015).

68 One significant disadvantage of sorbent amendments is that they generally lead to
69 reduced biodegradation of PAHs because the sorbed PAHs have a reduced microbial
70 bioaccessibility and cannot, therefore, be degraded (Rhodes, Carlin et al. 2008, Rhodes,
71 McAllister et al. 2010, Cho, Werner et al. 2012, Arp, Lundstedt et al. 2014). For
72 example, the mineralization extent of phenanthrene (PHE) decreased by up to 50% with
73 amendment of 5% BC (Rhodes, Carlin et al. 2008); whereas for AC amendment, it
74 declined from 87.2% (in absence of AC) to 0.4% (5% AC) (Rhodes, McAllister et al.
75 2010). This is potentially problematic as many regulatory standards, and site-specific
76 remediation targets, are based on total concentrations, and not bioavailable
77 concentrations (Latawiec, Swindell et al. 2010, Ortega-Calvo, Harmsen et al. 2015).
78 Therefore, establishing technologies that both lower total soil concentration (via
79 biodegradation) and bioavailability through sorption and entrapment would be
80 advantageous.

81 These conflicting goals might be reconciled through the impregnation of sorbent
82 amendments with PAH-degrading microorganisms (Wick, Springael et al. 2001, Wick,
83 De Munain et al. 2002, Uyttebroek, Ortega-Calvo et al. 2006). Through such an
84 approach the mass transfer of PAHs from contaminated soil to the degrading microbial
85 community might be enhanced, and in addition there would be an enrichment of
86 degrading bacteria and biofilm formation on the strong sorbing materials (Bastiaens,
87 Springael et al. 2000, Wick, Springael et al. 2001, Uyttebroek, Ortega-Calvo et al. 2006),

88 which could provide to stimulate the biodegradation of PAHs (Liu, Chen et al. 2015).
89 Supporting this reasoning, additions of humic acids (HA) (Smith, Thullner et al. 2009),
90 model synthetic amberlite sorbents (Uyttebroek, Ortega-Calvo et al. 2006), clay
91 (Ortega-Calvo and Saiz-Jimenez 1998), and dissolved organic carbon (DOC) (Haftka,
92 Parsons et al. 2008) have been shown to promote both PAH sorption and degradation.
93 Particularly, a 4.8- and 9.5-folds increase in PHE degradation rates was respectively
94 observed when in the presence of HA (Smith, Thullner et al. 2009) and model synthetic
95 amberlite sorbents (Uyttebroek, Ortega-Calvo et al. 2006). It is important in this context
96 to note that biochars, with high surface area and porosity, make good candidates for
97 microbial habitats (Saito 1990, Pietikäinen, Kiikkilä et al. 2000, Hale, Luth et al. 2014).
98 Microbe-AC/biochar composites have been employed in wastewater treatment process,
99 to provide protection to microbes, and/or to increase contaminant removal performance
100 (Morsen and Rehm 1990, Song, Edwards et al. 2006, Lin, Donghui et al. 2010). For
101 example, a 2-times higher pyridine-degradation rate constant (0.12 mg $g_{carrier}^{-1}$ h^{-1}) was
102 observed when *Paracoccus sp.* strain KT-5 was pre-immobilzed on bamboo-carbon
103 (Lin, Donghui et al. 2010).

104 Elevated metabolic activities resulting from biochar amendment to soil have also
105 been observed (Steinbeiss, Gleixner et al. 2009), but studies focus on the application of
106 microbe-biochar composite in the remediation of contaminated soils are rare. Therefore
107 there is a research gap regarding the effectivity of such approaches. In order to evaluate
108 the potential for bacteria-inoculated biochar to remediate PAH contaminated soil, we
109 investigated the influence of pre-immobilizing the actinobacteria cells, of *M. gilvum*,
110 on biochar, and quantified its ability to sorb and biodegrade PAHs in a real-world,
111 historically contaminated soil.

112 ## 2. Materials and methods

113 ### 2.1. Reagents and Chemicals

114 Hexane, cyclohexane, acetone, and dichloromethane (all HPLC-spectro grade) were
115 purchased from Tedia (TC, USA). Internal standard hexamethylbenzene (99% purity)
116 was acquired from Dr·Ehrenstorfer (Augsburg, Germany). The surrogate standard mix
117 was acquired from AccuStandard (CT, USA), and contained naphthalene-d_{10},
118 acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12} and perylene-d_{12} (4 mg ml^{-1}). A
119 standard solution of 16 US EPA PAHs, and the non-ionic surfactant Brij 30
120 [$CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_nOH$, were obtained from Sigma-Aldrich (St. Louis, MO).

121 ### 2.2. Soil

122 A real-world, PAH contaminated soil was collected from a former Coking Plant (CP
123 soil) (N 39°51′ 0.42″, E 116°31′ 38.83″) in Beijing, China. The soil samples were
124 stored in dark glass containers at -80°C until use. The total concentrations of 16 US
125 EPA PAHs in this soil, indicating heavy contamination, were 677 mg kg^{-1}. Physical and
126 chemical properties of CP soil include: pH 8.37, total organic matter 10%, total N
127 0.231%, total C 12.92%, total S 0.593%, clay 2.39%, silt 15.4%, and sand 82.2% (see
128 the Supporting Information (SI) for quantification details).

129 ### 2.3. Biochar

130 Rice straw (*Oryza Sativa*), sewage sludge, and pig manure were applied as raw stocks
131 to produce separate biochars by pyrolysis at 500°C (see the SI for more information
132 about selection of pyrolysis temperature) for 4 h in a muffle furnace under limited
133 oxygen conditions. Characteristics of the produced biochars were analyzed (see the SI

134 for quantification details and results). Rice straw derived biochar was ultimately
135 selected to study as the inoculum carrier, as the largest specific surface area (68.1 m^2 g^{-1}), pore volume (0.17 cm^3 g^{-1}) and surface basic groups (0.172 mmol g^{-1}) were observed
137 in this biochar (Table S1 & S2, SI), indicating a higher bacterial adsorption capability
138 (Krisdhasima, Vinaraphong et al. 1993, Hale, Luth et al. 2015). Moreover, rice straw is
139 a practical biomass-waste feedstock for biochar production (Wu, Yang et al. 2012).
140 Other physical and chemical properties of this biochar were: pH 10.14, total N 1.73%,
141 total C 48.6%, ash content 29.3%. The total concentration of 16 US EPA PAHs was
142 4.35 mg kg^{-1} (see the SI for quantification details).

143 2.4. Bacteria strain, isolation, culture conditions, and preparation of cell 144 suspensions

145 *Mycobacteria* have been reported to be adept degraders of gasoline components and
146 sorbed PAHs (Kim, Kweon et al. 2010). As an indigenous bacteria in the tested soil, *M.*
147 *gilvum* was employed. *M. gilvum* was isolated from the CP soil via a classical shaken
148 liquid medium enrichment method as described elsewhere (Bastiaens, Springael et al.
149 2000). *M. gilvum* was confirmed, in preliminary tests, to be capable of degrading
150 naphthalene, fluorene, phenanthrene, anthracene, fluoranthene (FLU), and pyrene
151 (PYR) effectively in minimal medium (MM) solutions (described in the SI Table S3)
152 (see the SI Figure S2). *M. gilvum* isolate was maintained using pyrene as the sole carbon
153 and energy source on MM agar plates. For the preparation of cell suspensions, one loop
154 of isolate was picked up and inoculated into a liquid Lysogeny Broth (LB, no PAHs
155 were added). After 1-week incubation on a rotary shaker at 30°C, 180 rpm, cell growth
156 approached a steady state (OD_{600}=1.6) and cells were then harvested. The cell culture
157 was centrifuged at 3500 r min^{-1} for 10 min, the supernatant was discarded and sterilized

158 fresh LB was added to re-suspend the cells; thereby, the cell suspension was condensed
159 and prepared for further inoculations.

160 **2.5. Immobilization of *M. gilvum* cells on biochar**

161 To immobilize *M. gilvum* cells on rice straw biochar, the biochar was firstly grounded
162 by a wood roller in valve bag and then passed through a 2-mm sieve, and 0.25 g biochar
163 (dry weight) was then soaked with fresh LB (1:20, w/v) in 50 ml-flasks. Subsequently,
164 the flasks were closed with Teflon-lined stoppers and sterilized twice at 121°C for 30
165 min. Cell suspensions were introduced to the flasks, after they had cooled, with each
166 flask receiving 2.5 ml of condensed cell suspension, containing 8.45×10^{10} cells
167 (confirmed by plate counting). The flask contents were then incubated on a rotary
168 shaker at 30°C, 80 rpm for 48 h. The mixtures were separated with a 75-µm sieve and
169 rinsed with de-ionized water thrice to remove the planktonic cells. Obtained *M. gilvum*-
170 composite should be collected and stored at 4°C if immediate inoculation into soil is
171 not possible (Lin, Wu et al. 2015). All operations were performed under strict aseptic
172 conditions.

173 The accumulated biomass of *M. gilvum* on rice straw biochar was evaluated by real-
174 time PCR assays. To test the immobilization durability, biochar inoculated with *M.*
175 *gilvum* cells, following 48-h of culturing, was reintroduced to fresh LB and then
176 incubated on a rotary shaker (30°C, 180 rpm min^{-1}) for 30 days. Samples incubated for
177 4, 8, 12, 16, 18, 22, 26, and 30 day periods were collected, with three flasks being
178 sacrificed at each sampling event. Parts of the samples were used for scanning electron
179 microscopy (SEM) imaging, and the remainder was stored at -80 °C until DNA
180 extraction.

181 **2.6. SEM Observation**

182 Biochars inoculated with *M. gilvum* cells were imaged using SEM. Samples were
183 prepared by chemical fixation and critical-point drying (CPD) (Karcz, Bernas et al.
184 2012). Briefly, samples were fixed in 2.5% glutaraldehyde in 0.1 M PB buffer (pH 7.2)
185 for 2 h. The samples were then rinsed by 0.1 M PB buffer twice, and dehydration was
186 carried out in a graded ethanol/water series of 30, 50, 70, 90, and 100%, at 20 min for
187 each concentration. Dehydrated samples were then dried to the critical-point with
188 carbon dioxide in a Pelco CPD2 apparatus. A 5-nm gold film was sputter-coated on the
189 samples. Images were then recorded by SEM (Hitachi S4800) operated at 5 kV.

190 **2.7. Biodegradation**

191 A batch biodegradation experiment was conducted in 150-ml flasks. CP soil (5 grams
192 dry weight) and 30 ml MM solutions were mixed and incubated for 72 hours to revive
193 the soil microbes. Four different treatments were then prepared. These were: (i) raw CP
194 soil only (the control), (ii) 5 g of raw CP soil inoculated with 2.5 ml of condensed *M.*
195 *gilvum* cell suspension (circa $1.69x10^{10}$ CFU/$g_{dw\ soil}$), (iii) 5 g of raw CP soil with 0.25
196 g dry weight of sterile rice straw biochar (at a ratio of 0.05 $g_{dw\ biochar}$/$g_{dw\ soil}$), and (iv) 5
197 g of raw CP soil added with 0.25 g dry weight of biochar inoculated with 1.27×10^{11}
198 cells/$g_{dw\ biochar}$, resulting in a cell density in the CP soil of $6.43x10^{9}$ cells/$g_{dw\ soil}$.
199 Unavoidably, less *M. gilvum* cells were introduced in this treatment compared with
200 direct inoculation of free cells (due to incomplete transfer of *M. gilvum* cells from the
201 loading solution onto the biochar). Ultimately, all flasks were supplemented with more
202 MM solutions to obtain a final volume of 50 ml (final soil to water ratio was 1:10, w/v).
203 For each treatment, independent biodegradation assays were performed in triplicate,
204 and all flasks were randomly placed on a rotary shaker (180 rpm) in the dark at 30°C,

205 for 18 days. Sterile MM solution was supplemented every 2 days to keep the
206 suspensions at a fixed volume. Slurries with a volume of 10 ml were taken from all
207 treatments for DNA extraction at day 0 and day 18.

208 In order to explore the hypothesized mechanism, that biochar would promote mass
209 transfer of PAHs to the biochar and the immobilized cells would then degrade the
210 transferred PAHs, a further set of flasks were prepared. These were identical to those
211 described above but to each flask the anionic surfactant Brij 30 was added (20 mg g^{-1}
212 soil; thereby achieving an experimental concentration of 2 g L^{-1}). Brij 30 has
213 demonstrated high PAH solubilizing ability that results in a considerable reduction of
214 PAH sorption to biochars (53%) (Ahn, Kim et al. 2008).

215 **2.8. DNA Extraction and Real-Time PCR**

216 DNA extraction was achieved using FastDNA SPIN kit for soil (MoBio Laboraories,
217 Carlsbad, CA, USA), in accordance with the manufacturer's instructions. FastDNA
218 SPIN kits have previously been used in DNA extraction from biochar amended soil
219 (Quilliam, Marsden et al. 2012, Leite, Balieiro et al. 2014). Real time quantitative
220 TaqMan PCR assays were conducted, targeting *Mycobacterium nidA*. Primer and probe
221 sets target conserved regions determined from a multiple alignment of *nidA* obtained
222 from several PAH-degrading *Mycobacterium* (DeBruyn, Chewning et al. 2007). The
223 TaqMan probe sequences were 5′ -FAMTCCTACCCGTCGCCGGTACA-BHQ1,
224 forward and reverse primer sequences were 5′ -TTCCCGAGTACGAGGGATAC and
225 5′ -TCACGTTGATGAACGACAAA, respectively.

226 Quantitative PCR assays were performed using a real-time quantitative PCR
227 detection system (Roche 480, Roche, Indianapolis, IN, US., Light Cycler FastStart
228 DNA Master Hybridization Probes (Roche) was used for qPCR reactions. Reaction

229 conditions were as follows: 50ºC for 5 min, 95ºC for 15 min, then 40 cycles of
230 denaturing at 94ºC for 30 s, annealing at 56ºC for 30 s, lengthening at 72ºC for 30 s.
231 For each sample, independent quantitative assays were carried out in triplicate. In every
232 amplification reaction, 10-fold diluted standard plasmid containing *nidA* genes were
233 amplified with the primers to obtain standard curves, and negative controls were also
234 included. Efficiencies of amplification were 90% to 110% for all samples.

235 **2.9. Analysis of PAHs**

236 PAHs in the soil phase and aqueous phases were analyzed as follows. Biodegradation
237 assay flasks were allowed to settle for 1 h, then the supernatant solution was pipetted
238 out and stored in 80-ml K-D tubes. The remaining soils were freeze-dried, and the 16
239 US EPA PAHs were analyzed using a method previously reported for quantifying PAHs
240 in biochars and biochar amended soils (Fabbri, Rombolà et al. 2013) (see the SI).
241 Analysis of aqueous PAHs in samples without Brij 30 amendment was carried out
242 following a method described elsewhere (Ahn, Werner et al. 2008). Briefly, aluminum
243 sulfate was added to the K-D tubes to flocculate suspended solids, and then
244 hydrochloric acid was added to adjust the pH to 7. The K-D tubes were capped and then
245 shaken by hand to enhance flocculation for 3 min, and subsequently centrifuged for 15
246 min at 2000 rpm twice to remove flocs. The clear supernatant was transferred to 250-
247 ml size screw-capped flasks, hexane (15 ml) was added to each flask, and the flasks
248 were then shaken on a rotary shaker at 180 rpm for 30 min. The extracts were then
249 separated using separatory funnels. Extraction of each batch of supernatant was
250 performed three times to ensure full PAH recovery. Subsequent up-concentration and
251 clean-up steps of the extracts are described in the SI.
252 The presence of Brij 30 in the aqueous phase of the surfactant dosed treatments

253 precluded back extraction of the aqueous supernatant into hexane (as the surfactant
254 causes demulsification of the mixture), and so, in these treatments, aluminum sulfate
255 was added and the samples were shaken and centrifuged (as described above) to obtain
256 a clear supernatant. Thereafter, PAHs were quantified using high-performance liquid
257 chromatography (HPLC) with direct injection of the clear supernatant (Zhu and Aitken
258 2010).

259 **2.10. Statistical analysis**

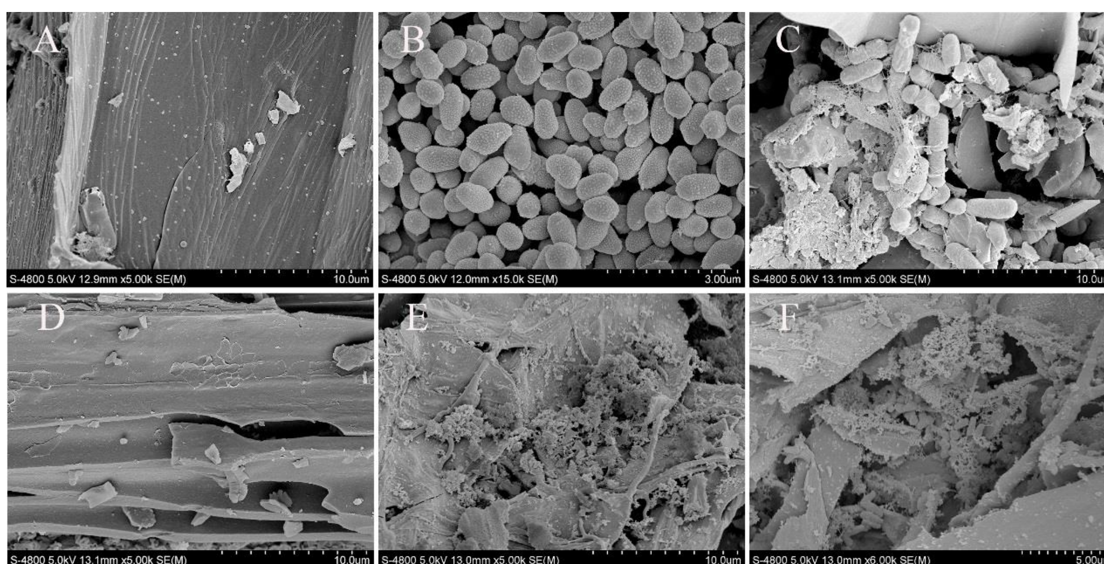
260 Microsoft Excel software (version 2013) was used for data processing. SPSS (version
261 22.0) was used to verify normality of data with Shapiro-Wilk's test. Means were then
262 compared using one-way ANOVA followed by either the LSD test or Dunnett's T3 test ,
263 depending on whether equal variances were or were not assumed, respectively, to
264 compare differences between multiple groups.

265 **3. Results**

266 **3.1. Immobilization of *M. gilvum* on Biochar**

267 The presence of *M. gilvum* cells on rice straw biochars after the initial 48-h culturing
268 or 18-day of incubation in LB after culturing was imaged using SEM, alongside images
269 of the sterile biochar and free *M. gilvum* cells (Figure 1). After 48-h of culturing, the
270 adhesion of *M. gilvum* cells on rice straw biochar was observed (Figure 1C). Cells were
271 observed to frequently colonize the surface and pore entrances of the rice straw biochar.
272 As evident from Figure 1C, the cells appear grouped together as cell aggregates,
273 additionally extracellular polymeric substances (EPS) were observed. After 18 days,
274 the EPS was further pronounced, as observed in Figure 1E and Figure 1F, and formed

275 network-like structures extending over and in between the *M. gilvum* cells (Figure 1F).



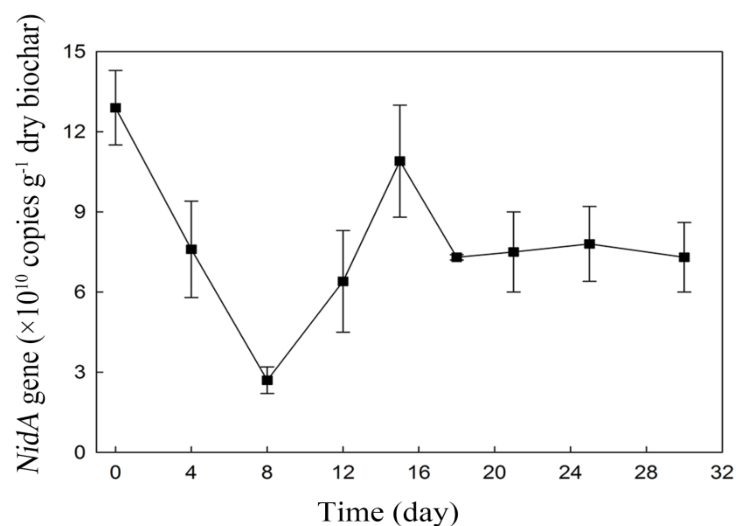
276

277 **Figure 1.** SEM images of samples collected after 48 h (A-C) and 18 days (D-F). (A)
278 Surface structure of sterile rice straw biochar (×5000). (B) Free *M. gilvum* cells with
279 rough and raised cell walls (×15000). (C) Cell aggregates of *M. gilvum* isolate attached
280 on rice straw biochar after two-day culturing, and the net-like extracellular polymeric
281 substances (EPS) formed around the cells (×5000). (D) Surface of biochar soaked in
282 LB for 18 days without bacteria inoculation (×5000). (E) Biochar inoculated with *M.*
283 *gilvum* cells (×5000). (F) Same as E but showing biofilm arranged in mesh-like
284 aggregates, and EPS visible as network-like structures extending over and in between
285 the *M. gilvum* cells (×6000).

286

287 The accumulated biomass of *M. gilvum* isolate on biochars was assessed by
288 quantitative PCR assays (Figure 2). The initial amount of *nidA* copies after 48-h
289 inoculation was $1.27\text{x}10^{11}\pm1.24\times10^{10}$ copies/$g_{\text{dw biochar}}$. Afterwards, the *nidA* copies on
290 biochars varied during the incubation period from $2.7\times10^{10}\pm9.26\times10^{9}$ to
291 $1.1\text{x}10^{11}\pm5.3\times10^{9}$ copies/$g_{\text{dw biochar}}$ (respectively, representing a 5-folds decreased to a
292 10-folds increase). A dynamic fluctuation of *nidA* copies was evident by a sharp
293 decrease in the first eight days, followed by a rapid increase over the next 10 days.
294 Ultimately, a stationary biomass (approximated $7.0\times10^{10}\pm1.5\times10^{9}$copies/$g_{\text{dw biochar}}$) was
295 maintained at 18 days. This combination of SEM imaging and qPCR assays revealed
296 that the immobilization of *M. gilvum* cells on rice straw biochar were effective and
297 durable in LB.

298



299 **Figure 2.** *NidA* copies on biochar for 30 days of incubation

300 **3.2. Biodegradation of PAHs**

301 A preliminary test using *M. gilvum* cells inoculated biochar indicated 98% removal
302 of pyrene within 5 days when 0.5 g of inoculated biochar was placed in a 50 ml MM
303 solution with 50 mg L^{-1} of pyrene (Figure S3, SI). It is anticipated that the presence of
304 soil and other PAHs may slow down the removal rate compared to a single PAH. The
305 residual PAHs in CP soil with the different treatments mentioned above were analyzed
306 after 18 days (Table S4, SI). Recoveries of the 6 surrogate standards ranged from 78%
307 to 99% for all samples (i.e. for acenaphthene-d_{10}, phenanthrene-d_{10} and chrysene-d_{12},
308 they were 78±4%, 88±2%, 86±5% in soils without any treatment, and 74±3%, 89±7%,
309 82±2% in soils with 5% biochar amendment, respectively). No significant difference in
310 recoveries between raw soil and soil with 5% of biochar amendment was observed
311 (P=0.257, 0.906, 0.319 for acenaphthene-d_{10}, phenanthrene-d_{10} and chrysene-d_{12}
312 respectively). No corrections for recovery were made. Residual PAH concentrations in
313 the different treatments established after 18 days are reported in Table S4 (SI).

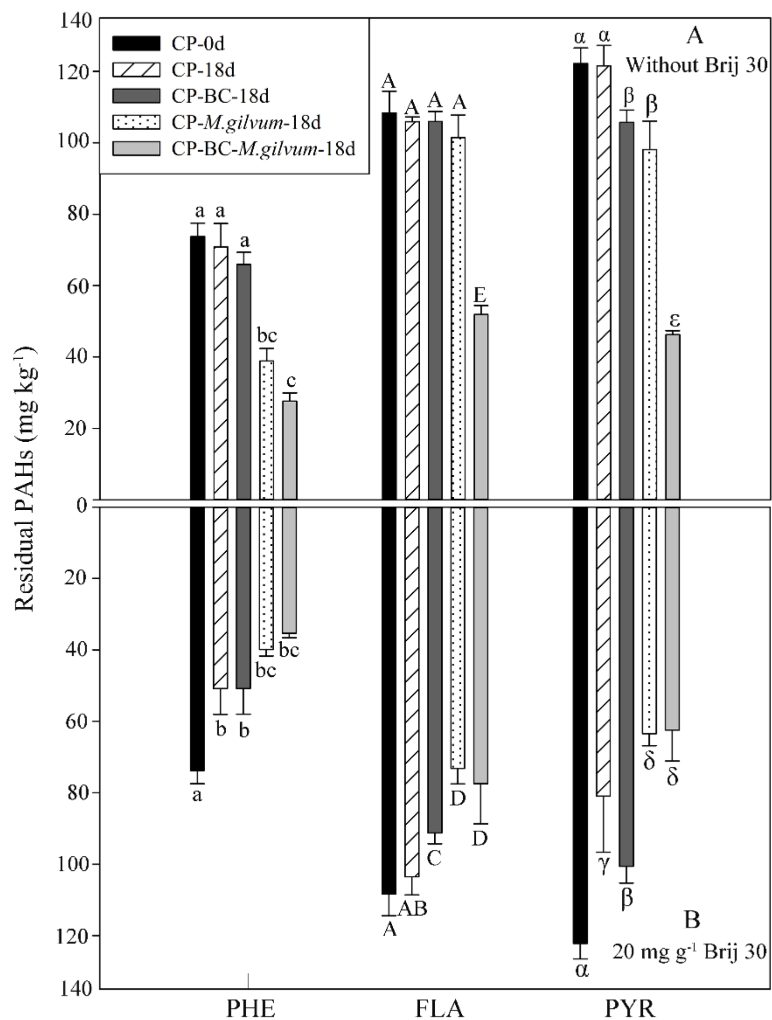
314 PHE, FLA, and PYR concentrations were observed to decrease most extensively
315 (Table S4). A preliminary experiment with PAHs in solution with *M. gilvum* cells

316 (Figure S2) showed marked decreases in these three PAHs, as well as naphthalene,
317 fluorene and anthracene, but not other PAHs. Naphthalene, fluorene and anthracene
318 were degraded to a lesser extent in CP soil in comparison to PHE, PLA and PYR (Table
319 S4). The difference in the extent of degradation for the different PAHs are suggested to
320 relate to lower bioaccessibility of naphthalene, fluorene and anthracene in the CP soil
321 as a consequence of native bacteria degrading these compounds in the field. This
322 reasoning is supported by the relatively low concentrations of these three PAHs
323 (naphthalene at 0.6%, fluorene at 1.0% and anthracene at 2.5% of the total 16 US EPA
324 PAHs concentrations (Table S4)). By contrast, PHE, FLA and PYR, respectively,
325 contributed 11.2, 16.2 and 18.3% of the total 16 US EPA PAHs to the CP soil. Given
326 the greater prevalence and marked reduction in their concentrations, further discussion
327 in this study is directed towards PHE, FLA and PYR.

328 The loss of PHE, FLA, and PYR in the control treatments was minimal over 18 days
329 (< 4.0%±3.5%) (Figure 3A); while biochar amendment resulted in a small amount of
330 PHE, FLA, and PYR loss compared to the control, of 7.2%, 2.8% and 13.2%,
331 respectively. In contrast, the rice straw biochar inoculated with *M. gilvum* cells
332 exhibited the highest removal ability. Specifically, losses of PHE, FLA, and PYR,
333 within 18 days, were 62.6±3.2%, 52.1±2.3%, and 62.1±0.9%, respectively, or compared
334 to the control, 58.6%, 49.9%, and 61.6%, respectively. In comparison, free *M. gilvum*
335 cells resulted in 43.3%, 4.1%, and 19.2% losses of PHE, FLA, and PYR when compared
336 to the control. Thus, biochar inoculated with *M. gilvum* cells resulted in 15.5% (PHE),
337 45.5% (FLA), and 42.6% (PYR) more degradation than just the free *M. gilvum* cells, at
338 18 days of incubation.

339 Changes in PHE, FLA, and PYR concentrations in the presence of 20 mg g^{-1} Brij 30
340 are presented in Figure 3B and Table S4. The surfactant, on its own, or in the presence

341 of the biochar or free cells amendment, enhanced biodegradation of PHE, FLA and
342 PYR compared to the treatments without surfactant. However, for the inoculated
343 biochar amendments, the degradation assays with surfactant showed reduced
344 biodegradation compared to assays without surfactant (Figure 3). Specifically, for
345 treatments containing inoculated biochar, the degradation for PHE, FLA, and PYR,
346 decreased in the presence of surfactant by 10.5%, 13.3%, and 23.6%, respectively, when
347 compared to the surfactant-free treatments amended with the inoculated biochar. Thus,
348 while the surfactant enhanced biodegradation in the presence of the free microbes;
349 presumably on account of improved soil to solution mass transfer of PAHs (making
350 them more bioavailable), the surfactant was effective in impeding onward mass transfer
351 of PAHs to the biochar and their subsequent degradation by the *M. gilvum* cells.



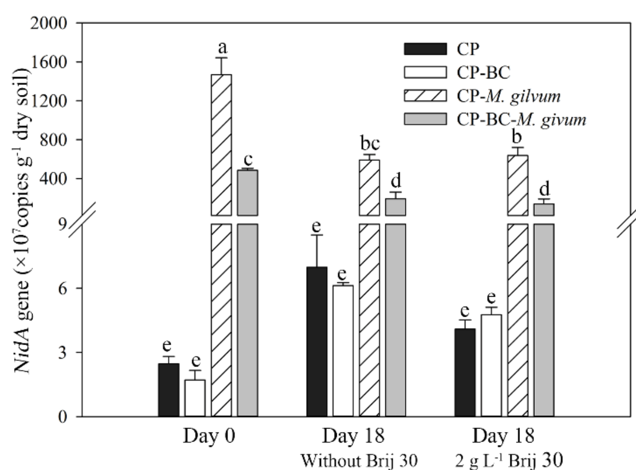
352

353 **Figure 3.** Residual phenanthrene (PHE), fluoranthene (FLA), and pyrene (PYR)
354 concentrations in coke plant soil in the absence of Brij 30 (panel A) and presence of
355 Brij 30 (panel B) while incubated in a mixed-media solution, comparing the initial
356 concentration (CP-0d), with concentrations after 18 days following natural removal
357 with no amendment (CP-18d), biochar-only amendment (CP-BC-18d), *M. gilvum* free
358 cells amendment (CP-*M. gilvum*-18d), and biochar containing immobilized *M. gilvum*
359 amendment (CP-BC-*M. gilvium*-18d) inoculation. Error bars represent the standard
360 deviation (n=3). Different letters indicate the mean difference is significant between
361 treatments at the 0.05 level.

362 **3.3. Abundance Monitoring of *M. gilvum* in CP Soil with Different Treatments**

363 The change in *M. gilvum* cell numbers in the CP soil with different treatments at day
364 0 and day 18 were monitored by Taqman PCR assays, targeting the PAH-degrading
365 *mycobacteria nidA* dioxygenase gene. The indigenous *nidA* copies in CP soil was
366 $2.5x10^7±3.4x10^6$ copies/$g_{dw\ soil}$, and the initial inoculation of free and biochar-
367 immobilized cells increased *nidA* copies to $1.5×10^{10}±1.8x10^9$ and $4.9×10^9±1 ×$
368 10^8copies/$g_{dw\ soil}$ (i.e. by factors of 622 and 288) respectively (Figure 4). Evidently,
369 fewer *M. gilvum* cells were introduced to the treatments via the inoculated biochar
370 amendment than the free cell addition, due to incomplete immobilization or biomass
371 loss during the inoculation process. Figure 4 shows that, after 18-days of incubation,
372 the *nidA* copies in the CP, Brij 30, and biochar amended soil was approximately 1.7 to
373 3.6 greater than those observed on day 0, but no significant difference in *nidA* copies
374 among these treatments (P>0.05) was observed. However, in the case of free-cell and
375 biochar-inoculated *M. gilvum* treatments, *nidA* copies all decreased at day 18 to about
376 60% of the day 0 numbers (down to $5.9×10^9±5.7×10^8$, $1.9×10^9±6.8×10^8$ copies/$g_{dw\ soil}$,
377 respectively). At day 18, *nidA* copies on the inoculate-biochar amended soils remained
378 lower than the free cell inoculations (P<0.01). The presence of 20 mg g^{-1} Brij 30 had
379 little influence on *nidA* copies for each of these two treatments (both P>0.05 for free
380 cell treatment and inoculated-biochar treatment).

381



382 **Figure 4.** *NidA* copies in CP soil with different treatments at day 0 and day 18,
383 including natural removal with no amendments (CP-0d and CP-18d), biochar-only
384 amendment (CP-BC-0d and CP-BC-18d), *M. gilvum* free cells amendment (CP -*M.*
385 *gilvum*-0d and CP -*M. gilvum*-18d), and biochar containing immobilized *M. gilvum*
386 amendment (CP-BC-*M. gilvum*-0d and CP-BC-*M.gilvum*-18d). Different lower-case
387 letters indicate the mean is significantly different between treatments at the 0.05 level.

388 ## 4. Discussion

389 ### 4.1. Immobilization of *M. gilvum* on biochar

390 SEM images and *nidA* copies presented in Figures 1 and 2 highlight the potential for
391 biochar to provide a suitable habitat for microbial colonization (Saito 1990, Pietikäinen,
392 Kiikkilä et al. 2000, Thies and Rillig 2009). Previous authors have proposed that two
393 stages are involved in the immobilization of microbes on biochar (Klein and Ziehr
394 1990); the initial stage being adsorption of microbes onto biochars, which could be
395 interpreted by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid
396 stability (Hermansson 1999), and the second stage being biofilm formation. The
397 accumulated biomass on biochar has been previously related to specific intrinsic
398 properties of biochar, which vary significantly based on the preparation temperature
399 and feedstock of bioochar (Tang, Zhu et al. 2013). In particular, pore size distribution,
400 pore volume, surface area and surface properties (hydrophobicity, metallic oxides and
401 functional groups) of chars, are critical parameters that affect the ability of biochar to

402 serve as carrier for introducing bacteria to soils (Messing and Oppermann 1979,
403 Rivera‐Utrilla, Bautista‐Toledo et al. 2001, Yamamoto, Nakakoshi et al. 2001,
404 Upadhyayula, Deng et al. 2009, Hale, Luth et al. 2015). Messing et. al found that pore
405 size distribution governed the optimum loading of bacteria, and that the maximum
406 accumulation of stable biomass occurred when the pore diameters were in the range of
407 one to five times the major dimension of the bacteria (Messing and Oppermann 1979).
408 The existence of such pores within the rice straw biochar was evident in the SEM
409 images (Figure S1(A)). The microbial adsorption capacity of chars has also been
410 commonly observed to increase with the specific surface area, surface hydrophobicity
411 and the amount of macropores (Krisdhasima, McGuire et al. 1992, Krisdhasima,
412 Vinaraphong et al. 1993, Rivera‐Utrilla, Bautista‐Toledo et al. 2001). Metallic
413 oxides and oxygen functional groups on the surface of chars are excellent adsorbents
414 of microbes and thereby will increase the accumulation biomass (Rivera‐Utrilla,
415 Bautista‐Toledo et al. 2001, Upadhyayula, Deng et al. 2009). Modification of AC with
416 cations of Fe^{3+}, Ca^{2+}, Mg^{2+}, respectively, has been found to lead to 87.8%, 54.7% and
417 24.8% increases in the microbial biomass accumulation (Rivera‐Utrilla, Bautista‐
418 Toledo et al. 2001). While the development of inoculant after incorperation into soil is
419 more closely associated with the physical features of biochar, including surface area,
420 pore opening diameter and water-filled pore spaces, which might play a significant role
421 in protecting pre-immobilized colonies from predation (Hale, Luth et al. 2015).
422 On the other hand, microbes in themselves are expected to play a role in cell
423 immobilization on biochar, particularly the surface hydrophobicity (CSH) of cells, as
424 hydrophobic bacteria are favorably attached to abiotic/hydrophobic surfaces
425 (Krasowska and Sigler 2014). Genus of *Mycobacterium* has been reported to have
426 extremely hydrophobic cell envelops (Hartmans, de Bont et al. 2006). Overall,

427 hydrophobic areas of the right pore size therefore likely serve as centers for clustering
428 of *M. gilvum* cells and biofilm, and the large surface area and pore volume (BET surface
429 68.06 m^2 g^{-1}, pore volume 0.17 cm^3 g^{-1}) support the potential for successful *M. gilvum*
430 cell immobilization (Bastiaens, Springael et al. 2000, Uyttebroek, Breugelmans et al.
431 2006).

432 **4.2. PAH degradation after immobilization of *M. gilvum* cells on biochar**

433 PAH degradation depends on environmental conditions, the number and type of
434 microorganisms, as well as the properties and chemical structure of the compound being
435 degraded (Haritash and Kaushik 2009). The lower *nidA* copies detected in the biochar-
436 inoculated cell treatment compared to the free cell treatment, which underwent more
437 biodegradation in CP soil, indicates that increased cell numbers alone is not the driver
438 of increased PAH biodegradation (Figures 3 & 4). The aqueous concentrations of PHE,
439 FLA, and PYR after 18 days (Figure S4, panel A, SI) were similar in all treatments
440 (without surfactant). This indicates that neither the presence of biochar nor *M. gilvum*
441 cells significantly affected the aqueous concentration of PAHs (based on the
442 quantification method used).

443 Other researchers have reported increased PAH biodegradation is related to the
444 increased presence of certain sorbing matrices (Ortega-Calvo and Saiz-Jimenez 1998,
445 Uyttebroek, Ortega-Calvo et al. 2006, Mayer, Fernqvist et al. 2007, Smith, Thullner et
446 al. 2009). Uyttebroek and co-workers observed the final degradation extent of PHE in
447 porous synthetic amberlite sorbent (IRC50) (53-62%) was significantly higher than in
448 absence of IRC50 (18-52%) for all tested *Mycobacterium* strains; these results were
449 interpreted as preferential degradation of PHE sorbed to IRC50 (Uyttebroek, Ortega-
450 Calvo et al. 2006). Furthermore, in the presence of IRC50, the maximum PHE

451 mineralization rate (1.1-1.9 ng ml^{-1} h^{-1}) for all tested *Mycobacteria* were significantly
452 higher than the initial abiotic desorption rate (0.2 ng ml^{-1} h^{-1}), suggesting that the
453 bacterial utilized sorbed PHE with a higher rate than can be explained by abiotic
454 desorption. Smith et al. observed sorption to humic acids increased degradation rates of
455 PHE by factors up to 4.8 while leaving the water-dissolved PAH concentrations
456 unchanged; proposing that additional humic acids-mediated transport was the
457 responsible mechanism for enhanced PHE degradation (Smith, Thullner et al. 2009).
458 Ortaga-Calvo and co-workers detected a shortened biodegradation acclimation phase
459 and higher PHE mineralization rates in the presence of 100 μg ml^{-1} humic acid and 10
460 g L^{-1} clay, both separately and in combination. They proposed that the enrichment of
461 PHE and degrading cells on these soil components resulted in the improved utilization
462 of PHE, and thus the total degradation was increased (Ortega-Calvo and Saiz-Jimenez
463 1998). These studies all indicate that both dissolved and solid sorbing matrices can
464 facilitate PAH degradation by assisting in PAH mass transfer to degrading cells in a
465 sorbent-amended system. We propose, in our system, that biochar may act in a similar
466 way, playing a role in the supply of PAHs to sorbed *M. gilvum* cells.
467 Reduced PAH degradation by immobilized bacteria, in the presence of surfactant Brij
468 30 (Figure 3), further supports this mechanism. The toxicity of Brij 30 to *M. gilvum*
469 cells can be excluded as the reason of reduced PAH loss, because i) in our study
470 increased biodegradation was exhibited in other parallel treatments in the presence of
471 Brij 30 (Figure 3), without notable changes in *nidA* copies; and ii) Brij 30 exhibits low
472 microbial toxicity below 1.5 g L^{-1} (Kim, Park et al. 2001). Several studies have reported
473 that organic contaminant adsorption onto ACs, and especially biochars, would be
474 considerably restrained by surfactants (Ahn, Kim et al. 2008, Han, Liu et al. 2013). Ahn
475 et al. found that the amount of PHE adsorbed onto AC and biochar substantially

476 decreased in the presence of Brij 30, likely due to the high solubilizing ability of the
477 Brij 30 surfactant (Ahn, Kim et al. 2008). Aqueous concentrations for PHE, FLA, and
478 PYR in the Brij 30 amended system after 18 days, were generally greater by a factor of
479 8.3, 6.1 and 4.5, respectively (SI-Figure S4B, Table S5). Thus, the lower biodegradation
480 observed in the Brij 30 systems is likely due to less sorption to both biochar and *M.*
481 *gilvum* cells.

482 **4.3. Effective biodegradation of biochar-sorbed PAHs**

483 Biodegradation of sorbed PAHs has been reported by various pathways, in particular:
484 (1) high-affinity uptake systems of degrading cells (Wick, Springael et al. 2001, Wick,
485 De Munain et al. 2002), (2) adhesion/biofilm formation on sorbed-PAHs (Wick, De
486 Munain et al. 2002, Johnsen and Karlson 2004, Uyttebroek, Breugelmans et al. 2006),
487 and (3) biosurfactant excretion (Deziel, Paquette et al. 1996, Willumsen and Karlson
488 1996).

489 *M. gilvum* is one of the most effective PAH-degrading *Mycobacteria* (Kim, Kweon
490 et al. 2010). This genus has been reported to have a high specific substrate affinity
491 towards PAHs, well adapted to degrade sorbed PAHs (Guerin and Boyd 1992, Bastiaens,
492 Springael et al. 2000, Derz, Klinner et al. 2004, Hartmans, de Bont et al. 2006,
493 Uyttebroek, Ortega-Calvo et al. 2006). *Mycobacteria* have complex and extremely
494 hydrophobic rigid cell envelopes, rich in mycolic acids (Hartmans, de Bont et al. 2006),
495 which are important for the interaction with or uptake of hydrophobic compounds
496 (Rijnaarts, Norde et al. 1992). Particularly, several studies reported that the mycolic
497 acid wall monolayer in *Mycobacteria* forms a hydrophobic surface, which may enhance
498 the specific substrate efficiency of PAHs (Bastiaens, Springael et al. 2000, Wick, De
499 Munain et al. 2002, Wick, Pasche et al. 2003). Concentrations of PAHs could be

500 effectively reduced at the *M. gilvum* cells surface; hence, creating a steep concentration
501 gradient between cell surface and biochar-binding PAHs, which could lead to
502 continuously uptake-driven desorption. In the case of biochar-inoculated with *M.*
503 *gilvum* cells, biochar acts as an effective sink, to increase PAH flux more than just the
504 freely suspended *M. gilvum* cells.

505 *M. gilvum* cells may also experience advantages associated with sorbed-PAHs as a
506 substrate (Wick, De Munain et al. 2002, Uyttebroek, Breugelmans et al. 2006). Our
507 durability test data showed appreciable *M. gilvum* biomass was steadily maintained on
508 rice straw biochar (approximately 7.0×10^{10}copies/$g_{dw\ biochar}$) on the 18th day after
509 immobilization (Figure 2). The enrichment of PAHs on the biochar, as a substrate, not
510 only increases contact opportunity with PAHs, it also increases concentration gradients
511 between PAHs sorbed to biochar surfaces and cell-surfaces across short distances, as
512 was observed elsewhere for 3-chlorodibenzofuran (Harms and Zehnder 1995). Mayer
513 et. al reported that the direct contact between a digesting gut and sediment matrix
514 resulted in a ~230 times increase in the PHE mass transfer coefficient (Mayer, Fernqvist
515 et al. 2007), indicating that efficient contact between *M. gilvum* cells and PAHs on
516 biochar would be beneficial to the PAH mineralization.

517 An additional explanation to account for the quicker degradation kinetics is biofilm
518 formation on biochar. Wick et al. reported the attachment and biofilm formation of
519 *Mycobacterium* sp. LB501T on solid anthracene surfaces using SEM (Wick, Ruiz et al.
520 2002). In our study, biofilm flocs and EPS on the surface of biochar immobilized *M.*
521 *gilvum* cells were commonly recorded during SEM imaging (Figure 1 (E) & (F)).
522 Biofilm and EPS formation are conducive to the mass transfer or substrate uptake of
523 PAHs, and the biofilm from the *Mycobacterium* is likely unique to other cell-interface

524 process occurring in the CP soil. On the other hand, biochar in itself can harbor both
525 hydrophilic and hydrophobic functional groups (SI, Table S2), particularly, comparably
526 higher surface basic (hydrophobic) groups in rice straw biochar would be advantageous
527 for the adsorption of hydrophobic organic contaminants. Thus, several plausible
528 mechanisms may explain why *M. gilvum* cells immobilized on biochar degraded PAHs
529 more rapidly than free *M. gilvum* cells.

530 **5. Conclusions**

531 Indigenous PAH-degrading microbes (*M. gilvum*) were immobilized on rice straw
532 biochar, with high abundance and durability. The *M. gilvum*-biochar composite showed
533 superior degradation capacity for phenanthrene, fluoranthene and pyrene, with a
534 62.6±3.2%, 52.1±2.3% and 62.1±0.9% of removal from the historically contaminated
535 soil, respectively. It was proposed that the improved remediation was attributed to the
536 targeted degradation of biochar sorbed PAHs. Biochar in itself was a carbonaceous 'sink'
537 both for pollutants and degrading cells. Such simultaneous enrichment provided a
538 means to reduce pollutants and degrading microbes being spatial isolated from one and
539 other. The proposed mechanism was further supported by observing a significantly
540 decrease in the degradation of PAHs in a biochar-*M. gilvum* composite system when
541 the surfactant Brij 30 was added to impede the PAH mass transfer to biochar. Further
542 research to investigate the delivery of PAHs towards the biochar surfaces could provide
543 better understanding of the underlying mechanisms of this enhanced PAH-degradation
544 soil system. Whether similar results would occur in the field as observed in this lab-
545 study is uncertain, and would need to be investigated as a follow-up study.

546 Devolopment of inoculant on biochar is also expected to be investigated for a better
547 understanding of biochar-microbe interactions.

548 **Supporting Information.**

549 Additional methods, data tables and figures can be founded in the Supporting
550 Information.

551 **Acknowledgment**

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Supporting Information

Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar cells

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S1. Supporting Methodology

pH was measured in a suspension of soil/biochar and 0.01 M $CaCl_2$ (1:2.5).

Total C, N and S in soil, biochar were measured by dry combustion using a macroelementor (VarioMax CNS, Germany). Soil (400 mg) and biochar (300 mg) samples were processed using combustion temperature (H1) 1140°C, post combustion (H2) 800°C and reduction (H3) 850°C, while He flow was 680 ml/min as mentioned in the operational manual. Soil particle size was measured using the Mastersizer 2000 (Malvern Instruments Ltd, UK)

Soil organic matter (SOM) was determined by oxidation with potassium dichromate–titration with $FeSO_4$.

Soil particle size distribution was measured using the micro-pipette method.(Miller and Miller 1987)

Selection of **biochar pyrolysis temperature**. Rice straw was pyrolized at 300°C, 400°C, 500°C, 600°C to produce separate biochars. Characterization of these biochars showed that their specific surface area, pore volume and mesopore volume increased with an increase of pyrolysis temperature. Specifically, the specific surface area of biochar produced at 300°C, 400°C, 500°C, 600°C was 9.45, 11.36, 68.06 and 76.14 m^2 g^{-1}, respectively; whereas for pore volume, it was 0.0114, 0.0159, 0.1732 and 0.1862 cm^3 g^{-1}, respectively; and for the mesopore volume, it was 6.4, 9.5 15.7 and 17.3 m^2 g^{-1}, respectively. However, biochar yield decreased substantially with an increase of pyrolysis temperature, with a yield of 42.23%, 35.52%, 34.23% and 25.94%, respectively. Higher specific surface area, pore volume and mesopore volume are advantageous for bacterial adsorption. Considering these fetures of biochar prepared at 500°C and 600°C were similar, ultimately we selected pyrolysis temperature of 500°C

for a higher yield of biochar.

The Brunauer-Emmett-Teller method of multiple points was employed for **surface area** calculation, while four data points, with relative pressures of 0.05 to 0.3, were used to construct the monolayer adsorption capacity.

Ash content was determined by ASTM proximate analysis method for biochars (ASTM D1762-84, reapproved 2007). Detailedly, ash is determined as the residue after burning to constant weight at 750°C.

The titration method proposed by Boehm (Boehm 2002) was used to estimate the number of **oxygenated surface groups**. Rice straw biochar (0.5 g) was respectively added with 25 ml of the following solutions: 0.1 M HCl, 0.1M NaOH, 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ in 50-ml centrifuge tube. All solutions were then incubated on a roatary shaker at 200 r min^{-1} for 30 min. After that solutions were equilibrated in a constant temperature incubator at 25°C for 24 h. After the equilibration process, samples were filtered through the polycarbonate filter. A volume of 10 ml of each solution obtained by the filtration process was transferred into 150-ml flasks and diluted with 40-ml distilled water. A drop (200 µl) of the indicator (phenothalin/methyl red) was added to each of the test flask which were subsequently stirred at the vortex. Then the all prepared solutions were used to the acid–base titration by 0.1 M NaOH or 0.1 M HCl.

PAH analysis in CP soil. PAHs in CP soil with different treatments were extracted using a previously established method for quantifying the 16 US EPA PAHs in biochars and biochar amended soils.(Fabbri, Rombolà et al. 2013) Briefly, 1 g_{dw} soil was extracted in a

cellulose thimble in 200 ml of acetone:cyclohexane (1:1, v/v) in a Soxhlet for 48 h. Twenty-five µl of surrogate standard mix at a concentration of 400 µg ml^{-1} were added prior to extraction. Soxhlet extracts were concentrated to approximately 1 ml by rotary vacuum evaporation at 39ºC. Then 15 ml of hexane were added and further concentrated to 1 ml to accomplish the exchange of solvent before clean-up, following the US EPA Method 3630C, and thereafter exchanged back to hexane and diluted by a factor of 100 (to quantify the abundant PAHs in the CP soil). Fifty µl of internal standard hexamethylbenzene (10 µg ml^{-1} in acetone) were added before analysis by gas chromatography-mass spectroscopy.

S2. Supplementary Data Tables

Table S1 Characteristics of biochar derived from different feedstocks

Biochar \ Characteristics	Elemental analysis (%)		Ash content (%)	pH	BET SSA ($m^2 \cdot g^{-1}$)	Pore volume ($cm^3 \cdot g^{-1}$)
	C	N				
Rice straw biochar	48.6	1.7	29.3	10.14	68.06	0.173
Sewage sludge biochar	27.1	3.4	70.4	10.00	5.42	0.014
Pig manure biochar	63.9	4.9	70.4	9.52	10.48	0.020

Table S2 Results of Boehm titrations of different biochars

Biochar \ Surface oxides	Acidic surface functions (mmol g^{-1})			Basic groups (mmol g^{-1})	Total surface oxides (mmol g^{-1})
	Carboxyl groups	Lactones	Hydroxyl group of phenolic character		
Rice straw biochar	0.167	0.156	0.228	0.172	0.723
Sewage sludge biochar	0.272	0.172	0.611	0.122	1.177
Pig manure biochar	0.161	0.056	0.278	0.133	0.628

Table S3 Formula of minimal medium (MM) solutions and plate

One liter MM solution contained K_2HPO_4 (6.0 g), KH_2PO_4 (5.5 g), Na_2SO_4 (2.0 g), KCL (2 g), 1 ml of trace element solution.

Trace element solution:

Nitrilotriacetic acid	1.500 g
$MgSO_4 \times 7\ H_2O$	3.000 g
$MnSO_4 \times\ H_2O$	0.500 g
NaCl	1.000 g
$FeSO_4 \times 7\ H_2O$	0.100 g
$CoSO_4 \times 7\ H_2O$	0.018 g
$CaCl_2 \times 2\ H_2O$	0.100 g
$ZnSO_4 \times 7\ H_2O$	0.180 g
$CuSO_4 \times 5\ H_2O$	0.010 g
$KAl(SO4)_2 \times 12\ H_2O$	0.020 g
$H_3BO_3 \times\ H_2O$	0.010 g
$Na_2MoO_4 \times 2\ H_2O$	0.010 g
$NiCl_2 \times 6\ H_2O$	0.025 g
$Na_2SeO_3 \times 5\ H_2O$	0.300 mg
Distilled water	1000.000 ml

Extra 15 g of agar was added in one liter MM solution during the preparation of MM plate.

Table S4 Residual PAH in CP soil with different treatments for 18 days

PAH	CP-0d (mg kg^{-1})	residual PAH (mg kg^{-1}) without Brij 30 CP-18d	without Brij 30 CP-BC-18d	without Brij 30 CP-*M. gilvum* -18d	without Brij 30 CP-BC-*M. gilvum*-18d	2 mg g^{-1} Brij 30 CP-18d	2 mg g^{-1} Brij 30 CP-BC-18d	2 mg g^{-1} Brij 30 CP-*M. gilvum* -18d	2 mg g^{-1} Brij 30 CP-BC-*M. gilvum*-18d
Naphthalene	4.01±0.39	3.84±0.53	4.07 ±0.33	4.07±0.33	2.42±1.47	4.48±0.74	4.50±0.73	4.34±0.01	3.37±1.15
Acenaphthylene	9.81 ±0.60	9.28±0.34	9.90±0.83	9.91±0.81	8.90±0.63	9.58±0.28	8.99±0.59	9.20±0.01	8.18±0.83
Acenaphthene	2.69 ±1.05	2.94±0.87	2.26±0.18	2.26±0.18	1.91±0.85	2.86±0.69	2.85±0.68	2.26±0.28	2.15±0.65
Fluorene	6.67 ±1.87	6.23±1.74	6.37±0.51	6.35±0.51	4.93±1.31	7.00±0.50	6.99±0.50	6.49±0.08	5.90±0.68
Phenanthrene	73.74 ±3.75	70.81±6.59	65.89±3.46	38.86±3.47	27.59±17.33	50.84±7.21	50.82±7.21	39.87±1.84	35.36±1.21
Anthracene	16.89 ±0.46	17.67±6.83	14.00±1.00	13.87±1.00	11.13±2.73	15.09±1.54	15.06±1.60	13.04±0.45	13.25±2.00
Fluoranthene	108.34±6.09	105.94±1.31	105.97±2.82	101.46±6.31	51.91±2.51	103.56±5.05	91.27±3.01	73.22±4.31	77.44±11.29
Pyrene	122.20±4.34	121.58±5.69	105.75±3.46	98.08±7.97	46.26±1.09	80.99±15.69	100.63±4.68	63.48±3.35	62.47±8.67
Benzo(a)anthracene	49.52 ±2.19	47.88±6.08	45.73±4.65	45.94±4.70	44.87±0.92	50.42±1.06	45.02±1.60	46.18±1.39	49.47±5.10
Chrysene	66.59 ±2.23	60.56±6.77	62.80±8.50	62.99±8.48	62.86±1.55	58.15±0.58	61.69±0.22	60.45±1.69	62.12±8.08
Benzo(b)fluorene	46.55 ±2.31	50.4±2.42	50.71±3.48	50.97±3.52	29.71±2.47	48.55±3.81	46.91±2.79	49.28±3.06	47.22±5.62
Benzo (k)fluorene	42.87 ±2.46	41.3±4.54	42.82±1.83	42.91±1.85	41.32±7.90	41.17±4.37	37.96±4.87	42.86±1.04	39.45±10.55
Benzo(a)pyrene	53.92 ±8.72	49.82±8.44	54.73±7.82	54.93±7.81	47.07±3.32	50.27±9.23	52.07±9.05	48.73±2.26	47.45±4.84
Indeno(1,2,3-cd)pyrene	10.66 ±0.65	10.83±0.13	11.22±0.43	11.24±0.39	10.94±1.43	12.56±0.26	11.11±0.19	11.62±0.15	11.37±0.62
Dibenzo(a,h)anthracen	19.40 ±0.11	16.43±2.8	20.25±1.95	20.25±1.87	19.22±0.36	20.79±0.58	18.95±0.35	17.40±0.32	17.33±0.60
Benzo(g,h,i)perylene	34.10 ±1.51	35.38±0.65	33.47±2.64	35.03±0.09	36.85±2.13	35.56±0.10	33.37±1.97	35.24±0.03	35.39±0.06
Σ16 PAHs	667.96±10.87	644.88±13.25	555.46±18.60	596.13±28.85	359.90±37.39	581.88±20.53	515.81±10.66	488.65±6.23	486.92±56.37

Table S5 Aqueous PAH insuspensions separated from soils with different treatments for 18 days

PAH	**Aqueous** PAH (μg L^{-1})							
	without Brij 30				2 g L^{-1} Brij 30			
	CP-18d	CP-BC-18d	CP-*M. gilvum* -18d	CP-BC-*M. gilvum*-18d	CP-18d	CP-BC-18d	CP-*M. gilvum* -18d	CP-BC-*M. gilvum*-18d
Naphthalene	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthylene	27.1±3.6	25.1±2.7	20±2.4	22.5±1.9	127.1±10.3	125.1±9.4	115±11.9	128.5±15.6
Acenaphthene	20.5±3.4	14.5±2.6	16.3±1.6	15.8±1.3	87.1±4.8	91.2±6.4	85.3±10.2	90.6±6.8
Fluorene	14±2.9	12.2±1.6	10.2±1.8	13.7±2.0	140.8±14.2	125.2±12.6	130.71±14.9	139.7±19.3
Phenanthrene	376.8±21.1	358.1±58.3	358.8±24.6	391.4±9.2	3168.1±105.7	2981.5±292.1	3288.9±112.9	3014. 8±45.7
Anthracene	17.21±3.1	12.1±2.3	15.9±3.1	13.4±1.2	157.2±20.5	102.1±6.7	145.1±16.8	123.2±13.3
Fluoranthene	31.14±7.5	33.1±3.2	35.7±7.6	35.1±3.1	211.4±23.4	231.29±17.4	257.57±16.0	251.29±23.6
Pyrene	75.4±15.9	81.8±1.6	68.4±8.1	81.8±1.5	354.2±23.2	318.7±5.8	384.9±24.6	328.7±9.2
Benzo(a)anthracene	14.6±4.2	ND	ND	ND	93.7±19.6	84.2±14.6	69.1±13.3	77.3±10.7
Chrysene	6.9±2.8	4.2±1.1	11.4±3.5	8.2±3.1	27.1±4.6	22.2±2.6	25.8±5.1	28.6±7.0
Benzo(b)fluorene	49.2±1.3	29.7±3.6	38.2±4.6	37.8±5.2	46.2±4.5	49.7±8.9	52.2±5.4	57.4±6.1
Benzo (k)fluorene	ND	ND	ND	ND	33.1±5.7	37.1±7.8	34.9±6.1	38.6±5.2
Benzo(a)pyrene	ND	ND	ND	ND	57.2±4.7	60.4±4.2	55.8±3.7	62.16±6.8
Indeno(1,2,3-cd)pyrene	ND	ND	ND	ND	18.7±3.7	18.1±4.1	17.7±4.6	19.5±3.5
Dibenzo(a,h)anthracen	13.6±2.3	10.5±3.8	12.4±3.3	14.9±3.7	33.6±4.9	36.4±5.8	32.2±7.1	34.7±4.6
Benzo(g,h,i)perylene	ND	ND	ND	ND	23.4±1.4	22.7±5.3	19.6±4.4	24.7±3.2
Σ 16 PAHs	652.9±35.1	581.3±24.6	567.36±40.6	634.6±29.7	4578.8±106.9	4305.8±112.3	4714.8±136.4	4419.7±125.8

S3. Supplementary Figures

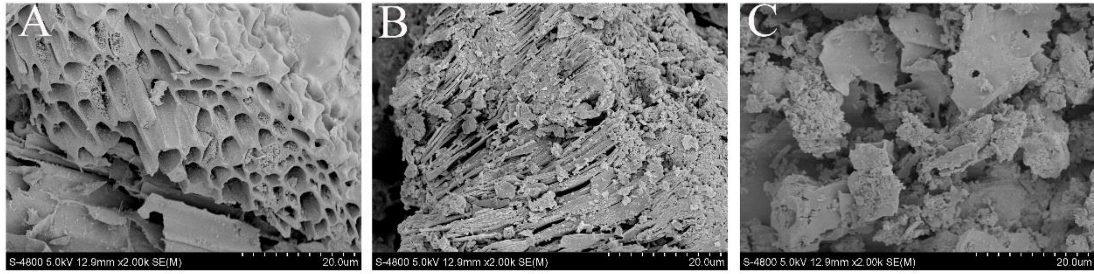
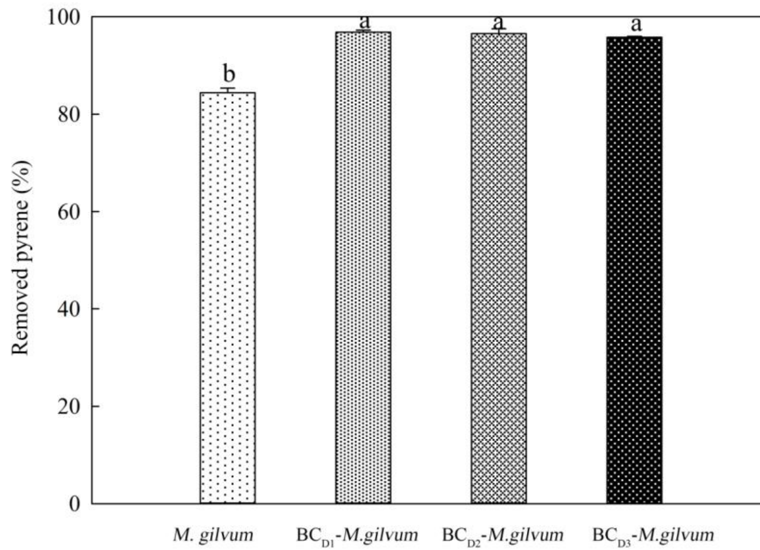
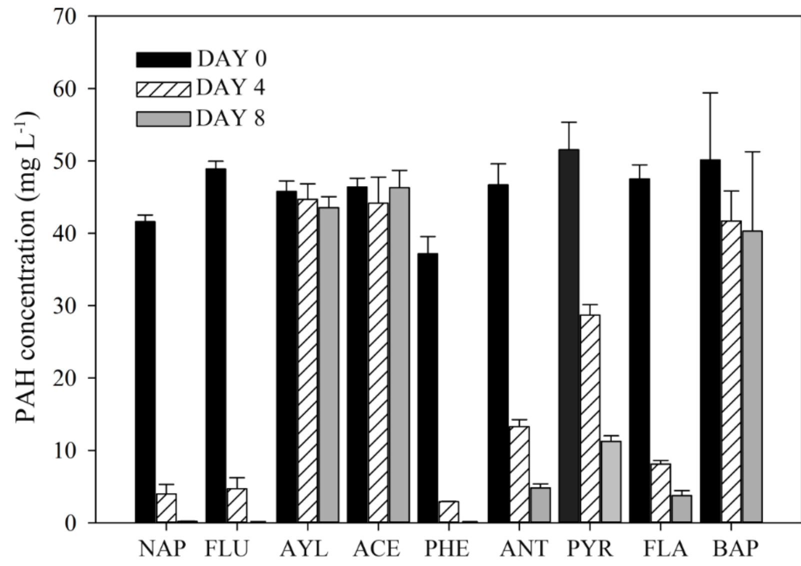


Figure S1. SEM image of biochar derived from different feedstocks: (A), rice straw biochar (×2000); (2) sewage sludge biochar (×2000); and (3) pig manure biochar (×2000). As evident in Figure S1, the surface structure of three biochars were varied greatly: porous structure was commonly observed in rice straw biochar; whereas on sewage sludge and pig manure biochar, it was faultage and bulk aggregates. The existence of suitable pores within the rice straw biochar was evident in the SEM image of Figure S1 (A), indicating that rice straw biochar was more suitable carrier for microbial immobilization than sewage sludge and pig manure biochar.



13 Figure S2. **Biodegradation of 9 PAHs by *M.***
14 ***gilvum* in MM solutions. *M. gilvum* was**
15 **confirmed to be capable of degrading**
16 **naphthalene (NAP), fluorene (FLU),**
17 **phenanthrene (PHE), anthracene (ANT),**
18 **pyrene (PYR), and fluoranthene (FLA).**
19 **Benzo(a)pyrene (BAP) was partially**
20 **degraded. No significant difference in**
21 **acenaphthylene (AYL) and acenaphthene**
22 **(ACE) concentrations was observed for 8-**
23 **day incubation (P=0.964, 1.000 for AYL and**
24 **ACE respectively), indicating *M. gilvum* was**
25 **not able to use both AYL and ACE as solo**
26 **sources of carbon and energy. These results**
27 **agree with other reports.(Kim, Park et al.**
28 **2001)**

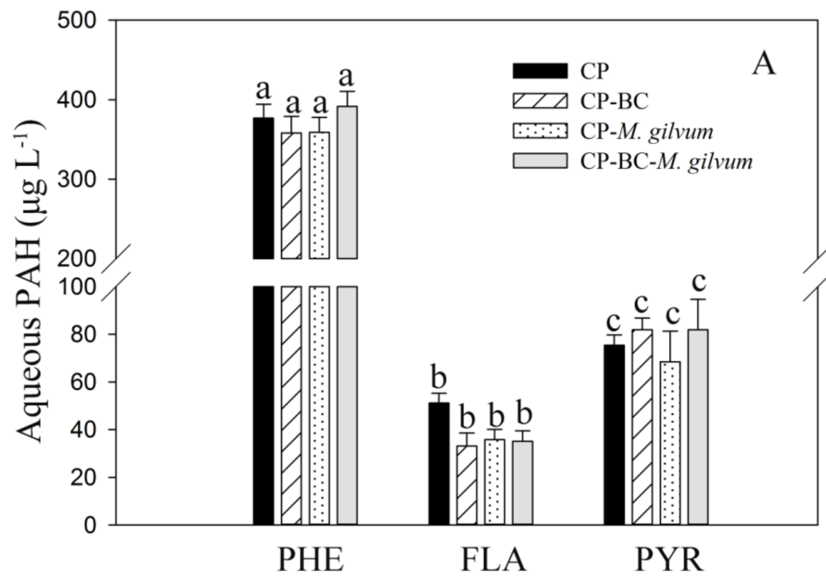
29



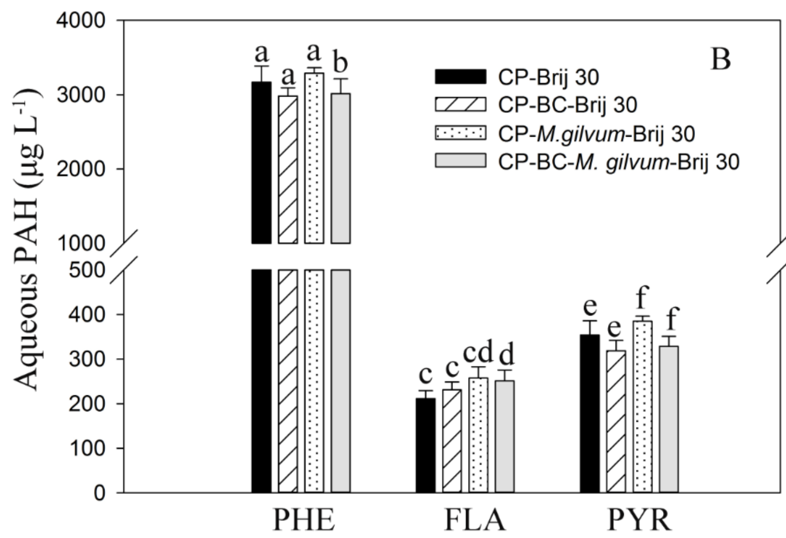
30

Figure S3. Removal of pyrene in minimal medium solution with different treatments **for 5 days. Removal of pyrene was significantly increased in the presence of biochar immobilized with *M. gilvum* cells (P<0.05), regardless of biochar particle size. Abbreviations: *M. gilvum*—free *Mycobacterium gilvum*, BC$_{D1\text{-}D3}$-*M. gilvum*—*Mycobacterium gilvum* cells immobilized on biochar with particle size ranging from 0.25mm-2mm (D1), 0.15mm~0.25mm (D2), and < 0.15mm (D3) respectively. Sample numbers n = 3, error bar = 1 standard deviation. Different lower-case letters indicate the mean difference is significant between treatments at the 0.05 level.**

48



49



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51 **Figure S4.** Aqueous PAH concentration in
52 suspensions separated from soils with **different**
53 **treatments for 18 days. Panel A: Treatments**
54 **without Brij 30 amendment. Panel B:**
55 **treatments with 2 mg g_{soil}^{-1} Brij 30.**
56 **Abbreviations: CP—raw CP soil only; CP-**
57 **BC—CP soil with sterile rice straw biochar.**
58 **CP-*M. gilvum*— CP soil with free**
59 ***Mycobacterium gilvum* cells inoculation. CP-**
60 **BC-*M. gilvum*—CP soil with biochar**
61 **impregnated *M. gilvum* amendment**

62 **Extended Discussion of Figure S4**

63 In Figure S4A, similar water-dissolved PAH concentrations were detected in
64 solutions separated from soils with four different treatments. Mean porewater solution
65 concentrations of PHE, FLA, and PYR were 371.33±8.38, 38.79±6.13, 76.91±6.39 μg
66 L^{-1}. Those values were a little higher than concentrations of porewater solutions of
67 PAHs reported in analogous studies (Beesley, Moreno-Jiménez et al. 2010). This may
68 be attributed the presence of dissolved organic carbon (DOC), such as originating from
69 extracellular polymeric substances, increases the number of dissolved sorption sites for
70 PAHs, enhancing the fraction of PAHs into the aqueous phase (and lowering apparent
71 K_D values based on total aqueous concentration).

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73 **S4. References to the Supplementary Information Section**

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