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► **To cite this version:**

Fu Chen, Xiaoxiao Li, Qianlin Zhu, Jing Ma, Huping Hou, et al. Bioremediation of petroleum-contaminated soil enhanced by aged refuse. *Chemosphere*, 2019, 222, pp.98-105. 10.1016/j.chemosphere.2019.01.122 . hal-02132897

HAL Id: hal-02132897

<https://hal.science/hal-02132897>

Submitted on 21 Oct 2021

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1 **Bioremediation of petroleum-contaminated soil enhanced by aged refuse**

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22 **Abstract**

23 In this study, the effect of aged refuse on biodegradation of total petroleum
24 hydrocarbons (TPH), microbial counts, soil ecotoxicity, dehydrogenase activity and
25 microbial community compositions were investigated in solid phase reactors during a
26 30-week period. The results demonstrate that the removal efficiency of TPH was
27 significantly higher in the soil supplemented with aged refuse than in the soil without
28 aged refuse. After 30 weeks, the removal efficiencies of TPH in soils were 29.3%,
29 82.1%, 63.7% and 90.2% in the cases of natural attenuation, nutrient addition (with
30 NH_4NO_3 and K_2HPO_4), supplement with 20% (w/w, dry weight basis) of aged refuse
31 and the combination of nutrient and aged refuse. Nutrient plus aged refuse made the
32 TPH concentration decrease to below the threshold level of commercial use required for
33 Chinese soil quality for TPH (< 3000 mg/kg) in 30 weeks. It was also found that
34 dehydrogenase activity, bacterial counts and degrader abundance in the soil were
35 remarkably enhanced by the addition of aged refuse (20%,w/w). Total organic carbon
36 analysis demonstrates that large amounts of hydrocarbon intermediates occurred in the
37 soil after bioremediation.

38 **Keywords:** aged refuse; bioremediation; solid phase bioreactor; dehydrogenase;
39 biostimulation

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43 **1. Introduction**

44 Remediation of petroleum-contaminated soil (PCS) is a hot topic in the field of
45 environmental science and ecological reservation (Lim et al., 2016). Various
46 physiochemical (such as soil washing, vapor extraction, flushing, thermal desorption,
47 etc) and biological approaches (such as landfarming, biopiles, bioslurry systems,
48 bioventing, etc) have been used to remediate petroleum contaminations (Khan et al.
49 2004). Among them, bioremediation methods have drawn extensive research interests
50 due to the low energy consumption, low operating costs, and no secondary pollution
51 (Dindar et al., 2013). Bioremediation has also its limitations including the production of
52 toxic metabolites, long duration, deep preliminary study, and microbial variation.

53 Bioremediation methods mainly include bio-aeration, bio-composting,
54 prefabricated beds and bioreactors. The power consumption of bio-aeration is high;
55 bio-composting needs a long duration, whereas prefabricated beds are characterized by
56 high costs of transportation and operation (Dindar et al., 2013). In contrast, the
57 bioreactor method has been widely applied and studied for soil remediation due to its
58 good effect and short reaction time (Rizzo et al., 2010; Safdari et al., 2018). The basic
59 principle of this technology is that using efficient homogenization (such as rotation and
60 slurrification) and aeration systems to enhance biodegradation rates and extent.
61 Nevertheless, conventional bioreactors have some limitations such as high operating
62 cost, difficulty in obtaining highly efficient microbial strains, and the vulnerability of
63 inoculants to environmental impact. Thus, it is necessary to develop bioremediation

64 technology that permits the easy obtaining of highly efficient microbial strains and low
65 cost.

66 Refuse in landfills and dumping sites becomes aged and stabilized after 8–10 years
67 of placement, and the resultant partly or fully stabilized refuse is referred to as aged
68 refuse (Zhang et al., 2012). Aged refuse contains various microbial species which exist
69 in the original refuse system. Aged refuse is a good microbial carrier due to the high
70 specific surface areas and porosity, excellent physical-chemical properties and hydraulic
71 properties (Zhu et al., 2012). During the long-term biodegradation process, the surface
72 of aged refuse is adhered by large numbers of microbial communities (Zhao et al., 2017).
73 These microbes have been naturally acclimatized for a long time under the harsh
74 environmental conditions of landfills, which renders the microbes have strong
75 decomposition capability for both biodegradable and recalcitrant organic pollutants
76 (Zhao et al., 2017). Thus, using aged refuse to remediate contaminated soils not only
77 achieves the purpose of disposing waste with waste, but also reduces the treatment cost;
78 meanwhile, the excavation of aged refuse can reuse landfill space and recover
79 recyclable materials (Zhang et al., 2012; Zhu et al., 2012; Zhao et al., 2017).

80 The aim of this work was to perform an assessment of PCS bioremediation
81 approach in a solid phase bioreactor amended with aged refuse. A series of laboratory
82 scale experiments with different experimental conditions were carried out. The
83 degradation mechanism, ecotoxicity, enzyme activity, and microbial community
84 changes were also discussed in this study.

85 **2. Material and methods**

86 2.1. Contaminated soil

87 The PCS used in this study was collected from a crude oil spill site in Shengli
88 Oilfield, Shandong Province, China. It is not feasible to determine the particle size
89 distribution of the contaminated soil because of its adhesive structure and high
90 hydrocarbon content. Thereupon, surface litter and stones were removed manually and
91 the soil sample was air-dried and sieved through a 2-mm mesh sieve, homogenized by
92 hand with shovels, and then stored at 4 °C in the dark until used. The sieved clay loam
93 soil contained 28% sand, 43% silt, and 29% clay. The physiochemical characteristics of
94 the soil are as follows: bulk density, 1.8 g/cm³; water holding capacity, 26%; pH, 6.9;
95 total nitrogen, 1.1 g/kg; total phosphorous, 34 mg/kg; total petroleum hydrocarbons
96 (TPH), 26300 mg/kg; total organic carbon (TOC), 4.3%.

97 2.2. Aged refuse

98 Aged refuse was excavated from one closed chamber of Xuzhou City Municipal
99 Solid Waste landfill that had been covered for 10 years. The refuse was screened
100 through a 40-mm mesh to remove large litter and then ground by a grinder to obtain a
101 fine powder. Key physical, chemical, and biological characteristics of the screened aged
102 refuse are listed in Table 1.

103 2.3. Experimental design

104 Experiments were carried out in a series of identical plexiglass columns. Each has
105 10 cm inner diameter and 30 cm height with a working volume of 1.5 L. A 5-cm layer

106 of pebbles was placed at the bottom of column to create vent holes. A side-opening
107 polyvinyl chloride (PVC) pipe (10 mm diameter) was vertically installed at the center of
108 column. The height of solid phase was about 20 cm when operated.

109 Five treatment modes were applied including (1) abiotic control (AC: soil was
110 supplemented with NaN_3 (0.5 wt.%) and HgCl_2 (2 wt.%) to account for abiotic loss of
111 pollutants; (2) natural attenuation (NA: no nutrient addition and no inoculation); (3)
112 biostimulation (BS: nutrient addition, without soil inoculation); (4) refuse spiked (RS:
113 soil inoculation with 20% (w/w, dry weight basis) of aged refuse but without nutrient
114 addition; (5) combination of biostimulation and refuse spiked (BS-RS: nutrient addition
115 coupled with aged refuse inoculation (20%, w/w, dry weight basis). Each treatment was
116 carried out in triplicate. The soil and aged refuse were homogenized thoroughly before
117 being loaded into the bioreactors. At a 1-week interval, NH_4NO_3 and K_2HPO_4 in
118 solution form were added stepwise to treatments BS and BS-RS to give a final
119 carbon:nitrogen:phosphorus (C:N:P) ratio of 100:10:5 (Gong 2012). Soil moisture was
120 maintained at 18–20% by the regular addition of distilled water. The content of each
121 bioreactor was thoroughly homogenized every alternate day to allow good aeration. The
122 trials were performed in a greenhouse at 25–30 °C.

123 2.4. Hydrocarbon analysis

124 Quantification of TPH in the PCS sample was conducted by gas
125 chromatography/mass spectrometry (GC-MS) (Agilent Technologies 7890/5975c
126 GC/MS system) equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm)

127 according to the modified EPA 8015B method, and soil extraction was done following
128 the EPA 3550B method (USEPA 1996). The Super Flash Alumina Neutral columns
129 (Agilent Technologies) were used to separate alkanes and polycyclic aromatic
130 hydrocarbons (PAHs) by eluting the columns with hexane and dichloromethane,
131 respectively. The concentrations of alkanes and PAHs were analyzed using GC-MS.

132 2.5. Soil analysis

133 The basic soil properties were determined using the conventional laboratory
134 methods described by Lu (2000). Briefly, soil pH was determined using a pH meter with
135 a soil:water ratio of 1:2.5. Moisture content was determined by drying a pre-weighed
136 soil sample in an oven at 105 °C for 24 h, and calculating mass loss. Cation exchange
137 capacity was determined by extraction with 1 M ammoniumacetate at pH 7, flushing
138 three times with isopropyl alcohol followed by extraction with 2 M KCl. Total nitrogen
139 (TN) was measured by semi-micro Kjeldahl digestion. Total phosphorus was
140 determined colorimetrically after perchloric/sulfuric acid digestion, by using inductively
141 coupled plasma atomic emission spectrometry (ICP-AES) (Seiko Instruments, Chiba,
142 Japan). TOC was determined by a TOC analyzer (Shimadzu TOC-VCPH, Japan). The
143 soil extract obtained by the EPA 3550B method (USEPA 1996) was weighed after
144 evaporating the solvent under N₂, and this dry residue was called total extractable
145 organics (TEO).

146 2.6. Biochemical analyses

147 Total heterotrophic aerobic bacteria (HAB) were enumerated over nutrient agar

148 plates by using the plate spread method (Lu and Zhang, 2014). The nutrient agar had the
149 following composition: peptone 5 g/L, yeast extract 3 g/L, agar 15 g/L, and NaCl 5 g/L.
150 The hydrocarbon-degrading bacteria (HDB) were enumerated on Bushnell–Haas
151 medium (BHM) agar plates with 0.5% filter-sterilized diesel oil as the sole carbon
152 source (Fan et al., 2014). The BHM consisted of (g/L): KH_2PO_4 , 1.0; K_2HPO_4 , 1.0;
153 NH_4NO_3 , 1.0; FeCl_3 , 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. A 10-fold serial
154 dilution factor (from 10^{-1} to 10^{-8} dilution) was prepared for each retrieved soil sample.
155 An aliquot of 0.1 mL diluted culture was spread over plates, and each dilution had three
156 replicates. Results were expressed as colony forming units (CFU)/g soil.

157 Microbial dehydrogenase activity (DHA) in soil samples was determined as
158 described previously (Lu et al., 2009), and results were expressed as $\mu\text{g TPF}/(\text{g soil} \cdot 6$
159 h).

160 The soil extract was determined by using the microtox® bioassay (Lu et al., 2010).
161 The toxicity values of the soil extract were expressed as EC_{50} and defined as the
162 effective concentration of pollutants, which reduced the luminescence of
163 *Photobacterium phosphoreum* by 50%.

164 2.7. Pyrosequencing and data analysis

165 High-molecular-weight DNA from the soil and aged refuse was extracted with a
166 commercially available kit (Beyotime Biotechnology, China) according to
167 manufacturer's protocol. Fragments of 16S rRNA genes containing variable V4-V5
168 regions were amplified by polymerase chain reaction (PCR) in a GeneAmp PCR system

169 9600 (Applied Biosystems, CA), using the forward primer 563F
170 (5'-AYTGGGYDTAAAGNG-3') at the 50-end (*E. coli* positions 563–578) of V4 region,
171 and a cocktail of four equally mixed reverse primers, that is, R1
172 (5'-TACCRGGGTHCTAATCC-3'), R2 (5'-TACCAGAGTATCTAATTC-3'), R3
173 (5'-CTACDSRGGTMTCTAATC-3') and R4 (5'-TACNVGGGTATCTAATC-3'), at the
174 30-end of the V4 region (*E. coli* positions 785–802) (Murphy et al., 2010). Then DNA
175 samples with different barcodes were mixed in equal concentration and sequenced by a
176 Roche 454 FLX Titanium sequencer (Roche, Nutley, NJ, USA) at the Beijing Genomics
177 Institute (Shenzhen, China). The pyrosequencing methodology used was identical to
178 that reported by Davis et al. (2011).

179 Raw sequences were sorted by barcode, and fusion primers were removed. The raw
180 reads were treated with the Pyrosequencing Pipeline Initial Process (Cole et al., 2009)
181 of the Ribosomal Database Project (RDP), (1) to sort those exactly matching the
182 specific barcodes into different samples, (2) to trim off the adapters, barcodes and
183 primers using the default parameters, and (3) to remove sequences containing
184 ambiguous 'N' or shorter than 150 bps (Claesson et al., 2009). The reads selected above
185 were defined as 'raw reads' for each soil sample.

186 Sequences were aligned by MUSCLE 3.5 (Multiple sequence comparison by
187 logexpectation) (<http://www.drive5.com/muscle/>) (Edgar, 2004) and were classified
188 using RDP II classifier with a 50% bootstrap confidence. To calculate richness and
189 diversity indices of the microbial community, 1100 aligned sequences were randomly

190 selected from each sample and clustered by RDP's complete-linkage clustering tool
191 (Cole et al., 2009).

192 2.8. Data analysis

193 In this study, all experiments were performed in triplicate to get reliable data, and
194 results were reported as means \pm standard deviations on the basis of dry weight.
195 Statistical significance was evaluated using SPSS package (version 11.0) with two-way
196 ANOVA and least significant difference (LSD) was applied to test for significance at p
197 < 0.05 between the means.

198 **3. Results and discussion**

199 3.1. TPH dissipation

200 The average TPH content in the tested soil determined by analyzing three
201 replicates of a composite sample was 26300 ± 250 mg/kg. This value was deemed as the
202 initial TPH concentration for each soil pile. Apparently, the initial TPH content
203 exceeded substantially the intervention threshold for residential use (<1000 mg/kg),
204 commercial use (<3000 mg/kg) and industrial use (<5000 mg/kg), set by the China
205 Environment Protection Ministry (GB 15618-2008), thus this soil needed a thorough
206 remediation. In general, autochthonous TPH-degrading microbes can evolve into
207 dominant species over time in the contaminated soils as a response to hydrocarbon
208 stresses. Therefore, these microbial communities contribute to a natural attenuation of
209 petroleum hydrocarbons (Guarino et al., 2017). In practice, hydrocarbons
210 biodegradation by indigenous microorganisms can be accelerated by some factor such

211 as aeration and nutrients (Gong, 2012; Fan et al., 2014).

212 Figure 1 shows TPH variation during 30 weeks of study for the five modes. After
213 30 weeks, TPH concentration decreased from initial concentration of 26300 mg/kg to
214 24500, 18600, 4720, 9560 and 2580 mg mg/kg for AC, NA, BS, RS and BS-RS modes,
215 respectively. The total removal efficiencies of TPH were 6.8%, 29.3%, 82.1%, 63.7%
216 and 90.2% for AC, NA, BS, RS and BS-RS modes, respectively. The AC mode showed
217 low TPH reduction during 30 weeks. Thus, TPH reductions in the biotic modes are
218 mainly due to biodegradation. The BS-RS mode showed the maximum TPH removal
219 percentage among the five cases (Figure 1). This result indicates that the combined
220 application of nutrients and aged refuse could produce highest biodegradation efficiency.
221 The removal ratio was higher in the BS mode than in the RS mode, which demonstrates
222 that nutrient addition can have a greater impact on TPH biodegradation than the
223 addition of aged refuse. It is assumed that TPH has a nominal molecular formula $(CH_2)_n$,
224 then the organic carbon content of 26300 mg/kg of TPH is about 22500 mg/kg.
225 According to the generally used and recommended molar C:N:P ratio of 100:10:1 for
226 soil bioremediation (Maddela et al., 2016; Wu et al., 2017), 2.63 g/kg of N and 263
227 mg/kg of P was required. Thus, the initial contents of N (1.1 g/kg) and P (34 mg/kg) in
228 the PCS were far below the above standards, namely the PCS was lack of N and P
229 nutrients. When the PCS was supplemented with 20wt.% aged refuse, the initial
230 contents of N and P were 1.52 and 1.08 g/kg, respectively, and N was still a limiting
231 nutrient factor for good biodegradation of TPH in the soil. It is known that nutrient

232 status is a key factor for soil bioremediation (Gong, 2012; Maddela et al., 216; Wu et al.,
233 2017). The deficiency of available nitrogen and/or phosphorus nutrients would lessen
234 biodegradation performance of organic pollutants.

235 In general, the biodegradation rate of petroleum hydrocarbons in soils is rapid
236 during the initial stage, then gradually slows down and reaches a plateau phase finally
237 (Alexander, 1995). In the present study, the most rapid removal of TPH across various
238 biotic modes was obtained during the initial 10 weeks of bioremediation, followed by a
239 gradual decrease of removal rate over time (Figure 1). The addition of both nutrients
240 and aged refuse accelerated TPH removal during the initial period (Figure 1). A
241 degradation plateau was observed at the end of the experiments in this study. It is known
242 that petroleum hydrocarbons are composed of thousands of organic components
243 (Alexander, 1995). Some components are strongly absorbed into soil particles and soil
244 organic matter, leading to difficult accessibility of microbes to these compounds. In
245 addition, petroleum metabolites such as fatty acids, naphthenic acids, and oxygenated
246 polycyclic aromatic hydrocarbons can be formed and further biodegradation may be
247 inhibited in the presence of these compounds due to the suppressed microbial
248 degradative activity (Lu et al., 2010). These toxic metabolites can be
249 destroyed/transformed by chemical oxidation and the subsequent biodegradation may
250 proceed, which has been successfully tested by Lu et al. (2010).

251 3.2. Removal of hydrocarbon fractions

252 TPH consists of three components: saturated (or aliphatic), aromatic, and polar

253 fractions. The saturated fraction was divided into C5-C8, C9-C12, C13-C16, C17-C20,
254 and C20+ groups. Figure 2 demonstrates the removal percentages of saturated, aromatic
255 hydrocarbons and polar fraction for each mode after 30 weeks of bioremediation. It can
256 be observed that the removal efficiency decreased with the increase in carbon numbers.
257 C5-C8 fraction was the most susceptible compounds in this study, and its removal ratio
258 even reached 36.3% in the AC mode (Figure 2). This is understandable since they have
259 high volatility and lower molecular weights. Short-chain saturated hydrocarbons were
260 more biodegradable than long-chains. The saturated hydrocarbons with a carbon
261 number of less than C12 were lost in BS and BS-RS modes (Figure 2). Removal
262 percentages of saturated hydrocarbons during 30 weeks of bioremediation were 13.5%,
263 38.5%, 89.5%, 72.4% and 96.3% for AC, NA, BS, RS and BS-RS modes, respectively.

264 The molecular structure and biodegradation mechanism of aromatic hydrocarbons
265 are far more complex than saturated ones (Haritash and Kaushik, 2009). Thus, removal
266 efficiencies of aromatics during 30 weeks of bioremediation were 3.2%, 15.5%, 37.6%,
267 29.4% and 52.2% for AC, NA, BS, RS and BS-RS modes, which were much less than
268 that of saturated hydrocarbons.

269 Polar fraction (mainly resins and asphaltenes) shows accumulation in the NA and
270 BS modes but abatement in the RS and BS-RS modes (Figure 2). The increased content
271 of polar fraction could be ascribed to the accumulation of metabolic byproducts. Polar
272 fractions in crude oil are partially or completely resistant to biodegradation (Fan et al.,
273 2014). Nevertheless, a 30% biodegradation of polar compounds was reported by

274 Chaillan et al. (2006). In this study, the concentration of polar fraction decreased by
275 21.4% in the BS-RS mode. This suggests that the application of nutrients and aged
276 refuse could promote the biodegradation of not only saturated/aromatic hydrocarbons
277 but also of the polar fraction.

278 3.3. TOC content change

279 Figure 3 shows the variations in the TOC and TN content of soils before and after
280 30 weeks of bioremediation. After 30 weeks, at the final stage of the experiment, the
281 TOC content decreased in the biotic modes (Figure 3). Obviously, nutrients combined
282 with aged refuse (BS-RS) led to a better and faster mineralization of organic matter both
283 from the soil/refuse and petroleum hydrocarbons, with correspondent reduction of TOC
284 values (Figure 3).

285 The main effect of nutrient addition was to create and maintain, on the entire
286 experimental time period, appropriate levels of nutrients available to both those natural
287 inhabitants and inoculants, thus they are able to multiply and breakdown the petroleum
288 hydrocarbons. It is found that the removal percentages of TOC were lower than those of
289 TPH in respective modes. For example, 33.5%, 28.6% and 37.3% of TOC was removed
290 for BS, RS and BS-RS modes, respectively, with the corresponding TPH removal of
291 82.1%, 63.7% and 90.2%, respectively. This result indicates that there existed large
292 amounts of hydrocarbon intermediates in the soils after bioremediation. It is known that
293 petroleum hydrocarbons cannot be completely mineralized by microbes to CO₂ and H₂O,
294 and always leaves more or less complex residues (mainly recalcitrant compounds and

295 metabolites) (Atlas, 1995). These components often become increasingly less
296 bioavailable with the passing of time due to their low solubility in water and their
297 sequestration by soils (Alexander, 1995).

298 3.4. Microbial counts

299 During the 30-week incubation, the numbers of HAB and HDB were determined
300 by the plate spread method and the results are shown in Figure 4. The number of
301 bacteria in the AC mode was lower than 1000 CFU/g soil, thus these data were not
302 included in Figure 4. The count of HAB increased with time during the initial period
303 (Figure 4a). The increased HAB count in NA mode can be attributed to the water
304 addition and aeration in the reactor, since no nutrient supplement was made. As
305 expected, nutrient addition offered a nutrient source for microbes and thus strongly
306 improved the initial development of HAB (Figure 4a). Indeed, in the treatment without
307 nutrient amendment (NA mode), the HAB count was about two orders of magnitude
308 lower. The initial HAB count was 5.30×10^6 CFU/g in the PCS. In the contaminated soil,
309 the pattern of microbial counts was characterized by an initial increase and then gradual
310 decrease with time (Figure 4a). The maximum HAB counts were 4.22×10^7 , 2.65×10^9 ,
311 8.43×10^8 , and 5.50×10^9 CFU/g for NA, BS, RS and BS-RS modes, respectively. The
312 final HAB counts at 30 weeks were 1.10×10^7 , 7.52×10^8 , 5.68×10^8 , and 1.76×10^9 CFU/g
313 for NA, BS, RS and BS-RS modes, respectively. Apparently, the hybrid application of
314 nutrients and aged refuse could produce the highest bacterial density in the soil. This is
315 due to nutritional stimulation, on the other hand is due to the high specific surface areas

316 and porosity of aged refuse (Zhu et al., 2012).

317 The initial HDB count was 2.55×10^6 CFU/g in the PCS. The initial HDB counts of
318 RS and BS-RS modes were lower than that of NA and BS modes, which was due to the
319 dilution effect of refuse spiking and the relatively low HDB density (4.20×10^5 CFU/g)
320 in aged refuse. Like the case of HAB, the counts of HDB increased significantly after
321 nutrient and/or refuse spiking (Figure 4b). The final count populations of HDB at 30
322 weeks were 5.42×10^6 , 4.35×10^7 , 7.28×10^7 , and 2.50×10^8 CFU/g for NA, BS, RS and
323 BS-RS modes, respectively. These results demonstrate that the amendment of aged
324 refuse overall was beneficial for microbial growth and gathering, and the high HDB
325 density contributed to the high TPH degradation in the soil.

326 3.5. Dehydrogenase activity

327 Soil DHA is an index for overall microbial activity including total oxidative, which
328 can indicate whether stimulation or inhibition of the microbial communities is present
329 (Lu et al., 2009). The initial DHA values were $52.4 \mu\text{g TPF}/(\text{g soil} \cdot 6 \text{ h})$ for NA and BS
330 modes and $46.5 \mu\text{g TPF}/(\text{g soil} \cdot 6 \text{ h})$ for RS and BS-RS modes (Figure 5). Soil DHA
331 increased substantially after incubation in each mode. The maximum DHA was found at
332 week 20 in NA mode [$84.5 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$], week 10 in BS mode [$238 \text{ TPF}/(\text{g soil} \cdot 6$
333 h)], and week 15 in RS mode [$185 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$], when the greatest activity occurred
334 at week 10 in BS-RS mode [$320 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$] (Figure 5). From then on, DHA
335 continuously declined with time.

336 In the present study, in general, the variation tendency of soil DHA was similar to

337 that of bacterial densities. However, at the end of the experiment, the highest DHA was
338 observed in RS mode, though BS-RS mode had the maximum bacterial densities
339 (Figure 5). The observed increase in DHA after startup can be related to the increased
340 substrate conversion and mineralization due to nutrient stimulation. In the present study,
341 the reduction of DHA at the later stage could be due to the accumulation of toxic
342 intermediates and the reduced levels of nutrients and bacterial densities. DHA indicates
343 the onset of biodegradation but decreases rapidly after the biodegradation rate has
344 declined (Lu et al., 2009). DHA is correlated with microbial counts, but it could be
345 suppressed by toxic intermediates and lacks in propagation factors during
346 biodegradation, despite of high microbial counts (Margesin et al., 2000). The decrement
347 of DHA matched well with the decreased removal rate of TPH (Figures 1 and 5),
348 suggesting that DHA could be used as a monitoring parameter for the bioremediation
349 process (Margesin et al., 2000).

350 3.6. Microtoxicity analysis

351 Microtox® analysis was conducted over the course of the experiment to monitor
352 microtoxicity changes in the incubated soils and the results are shown in Figure 6. After
353 start-up, the toxicity first increased and then started to decrease, but increased again for
354 BS, RS and BS-RS modes. Nevertheless, the final toxicity levels of various modes were
355 lower than their initial values (Figure 6), indicating bioremediation could efficiently
356 reduce the microtoxicity of PCS. In the present study, the varying tendency of
357 microtoxicity differed with that of bacterial counts and DHA. According to the

358 determination method of microtoxicity (Lu et al. 2010), only water-soluble components
359 can act on the bacterial reagent (*Photobacterium phosphoreum*). Thus, the microtoxicity
360 of PCS is correlated with water-soluble portions of hydrocarbons and their intermediary
361 metabolites in the soil. Hydrocarbon intermediates such as aldehydes and fatty acids are
362 typically more hydrophilic than hydrocarbons and, therefore, more efficiently extracted
363 in aqueous solution (Vuorinen et al., 2006). During bioremediation, on the one hand,
364 these intermediates were produced by biodegradation process; to the other hand they
365 could be further degraded/transformed by microbes under soil conditions. Thereupon,
366 the soil microtoxicity underwent complicated dynamic changes. The Microtox® test
367 was found sensitive to toxic components of crude oil and was used successfully to
368 monitor oil residues toxicity during bioremediation (Kang et al., 2014; Brakstad et al.,
369 2018).

370 3.7. Evolution of microbial community structure and diversity

371 Bacterial community structures at the phylum and genus level are shown in Figure
372 7. The major phylum groups in aged refuse were *Proteobacteria* (31.3%), *Tenericutes*
373 (22.6%), *Firmicutes* (16.1%), *Bacteroidetes* (6.5%), *Acidobacteria* (4.3%),
374 *Synergistetes* (2.2%), and *Spirochaetes* (1.5%) (Figure 7a). The top six predominant
375 phyla in the PCS were *Actinobacteria* (45.2%), *Proteobacteria* (18.6%), *Bacteroidetes*
376 (10.3%), *Firmicutes* (7.5%), *Acidobacteria* (5.8%), and *Verrucomicrobia* (3.4%) (Figure
377 7a). The dominant genus groups in aged refuse were *Pseudomonas* (46.3%, affiliated
378 with *Proteobacteria*), *Acholeplasma* (15.3%, affiliated with *Tenericutes*), *Balneola*

379 (11.5%, affiliated with *Bacteroidetes*) and *Fluviicola* (8.6%, belonging to *Bacteroidetes*)
380 (Figure 7b). The dominant seven genera in the PCS were *Nocardioides* (14.5%),
381 *Pseudomonas* (21.4%), *Saccharibacteria* (12.3%), *Dietzia* (8.7%), *Acinetobacter*
382 (7.4%), *Microcella* (3.6%), *Bacillus* (5.3%), and *Mycobacterium* (1.7%).

383 After 15 weeks of incubation, the bacterial community structures of soil samples
384 changed apparently (Figure 7). The abundance of several bacterial phyla, like
385 *Proteobacteria*, *Sphingomonadales* and *Alphaproteobacteria*, increased significantly,
386 whereas the abundance of *Actinobacteria* decreased apparently (Figure 7a). The
387 dominant genera at week 15 were *Bacillus*, *Pseudomonas*, *Microcella*, *Mycobacterium*
388 and *Alkanibacter* (Figure 7b). *Bacillus*, *Pseudomonas*, and *Mycobacterium* are
389 well-known hydrocarbon degraders (Fuentes et al., 2014), which were more
390 predominant in BS-RS mode compared to NA, BS and RS modes in the present study.
391 The results indicate that aged refuse not only improved TPH degradation efficiency, but
392 also promoted the generation of TPH-degrading species in the contaminated soil.

393 **4. Conclusions**

394 This work demonstrates the feasibility of a bioremediation process using biostimulation
395 with aged refuse amendment for petroleum-contaminated soil. The results of solid phase
396 bioreactor experiments show that the soil amended with both nutrients and aged refuse
397 resulted in higher pollutant removal compared with natural attenuation, biostimulation,
398 or aged refuse addition alone. In the case of aged refuse–biostimulation, the TPH level
399 decreased to below the threshold level of commercial use required for Chinese soil

400 quality for TPH (< 3000 mg/kg dry weight) in 30 weeks. The results show the
401 applicability in use of aged refuse for remediation of petroleum-contaminated soil.

402 **Acknowledgments**

403 This work was supported by the Major Project in the Fundamental Research Funds for
404 the Central Universities under No. 2017XKZD14.

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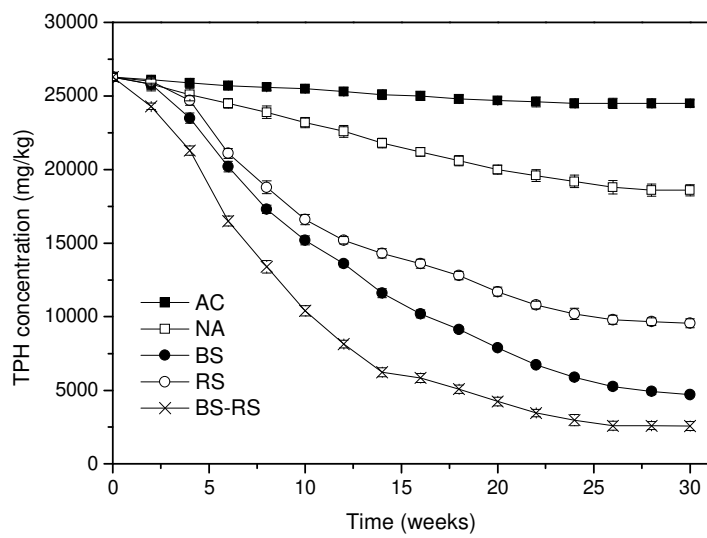


Figure 1. Changes in concentrations of TPH during bioremediation. The TPH concentrations in RS and BS-RS modes have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments (n = 3).

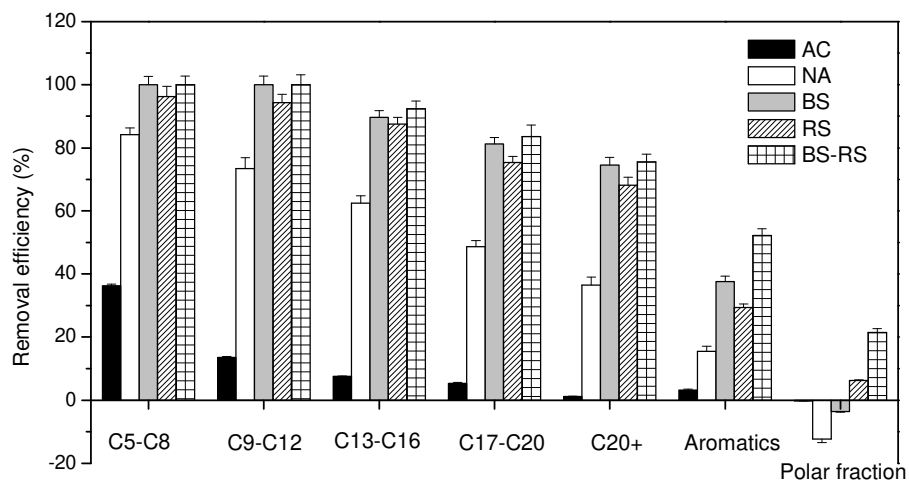


Figure 2. Removal efficiency of individual hydrocarbons at the end of experiments. The hydrocarbon concentrations in RS and BS-RS modes have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments ($n = 3$).

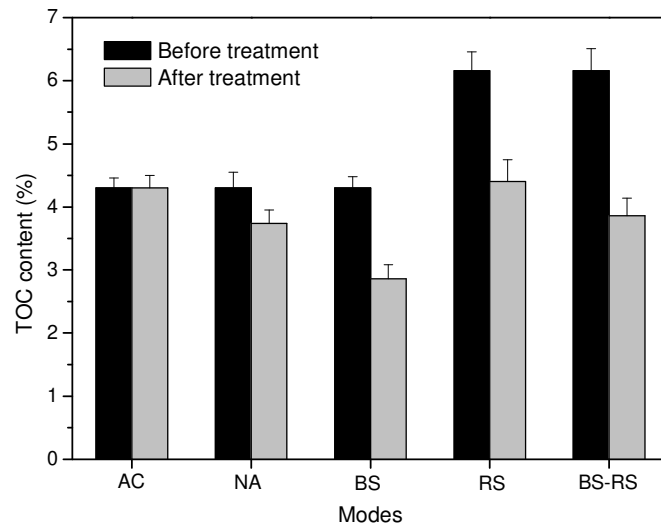


Figure 3. TOC content before and after 30 weeks of bioremediation. The TOC concentrations in the soils have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments (n = 3).

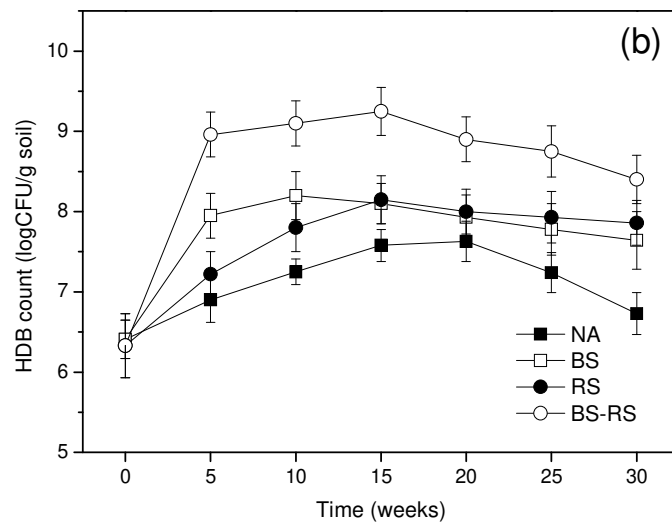
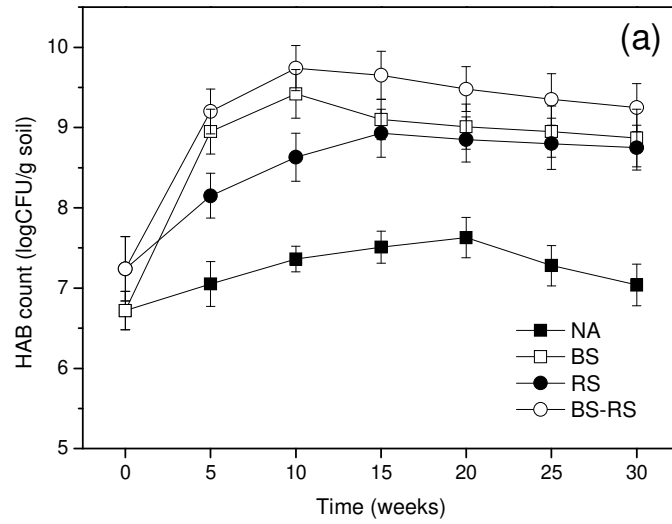


Figure 4. Evolution of (a) HAB and (b) HDB counts in the soil during 30 weeks of incubation.

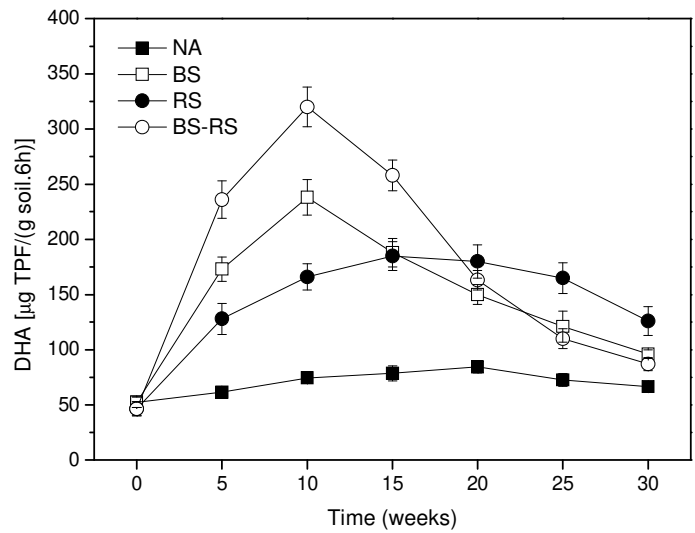


Figure 5. Evolution of soil DHA in the soil during 30 weeks of incubation.

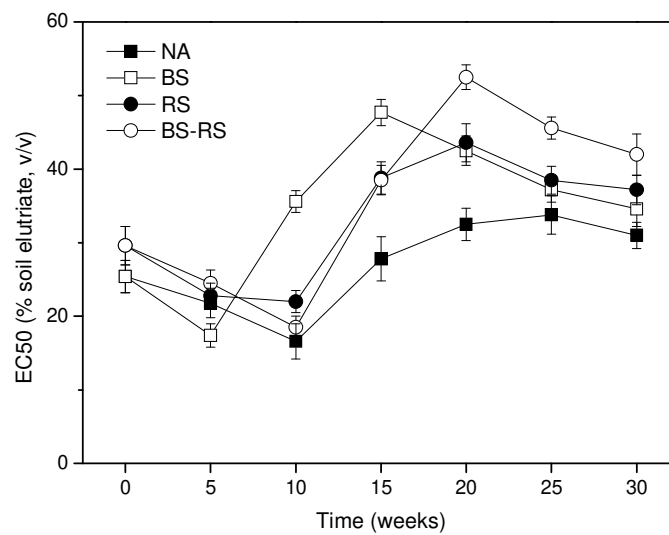


Figure 6. Time course of EC₅₀ value in the soil during 30 weeks of incubation. Data represent EC₅₀ values for single mode with error bars corresponding to 95% confidence intervals calculated using Microtox Data Capture and Reporting software (Ver. 7.8). The higher EC₅₀ value, the lower microtoxicity.

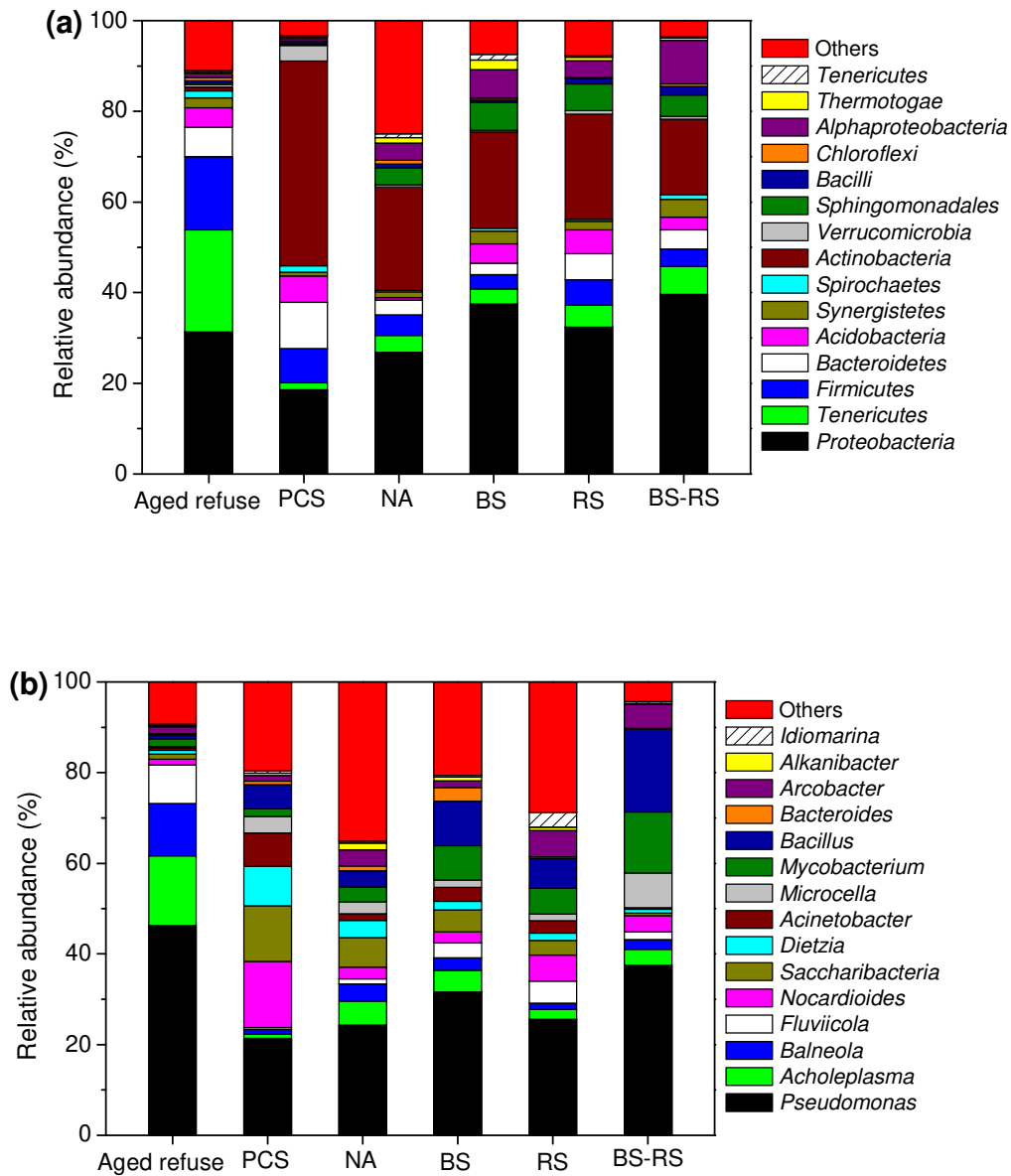


Figure 7. Relative abundance of bacterial phyla (a) and genera (b) in soils before and after 15 weeks. Except for PCS and aged refuse samples, other soils were collected at week 15. The relative abundance was displayed in terms of percentage in total effective sequences.

Table 1 Physical, chemical and biological characteristics of screened aged refuse (less than 40 mm) (sample no = 3).

Parameter	Value
Moisture content (%)	35.3
Porosity (%)	39.6
Permeability coefficient (cm/min)	2.58
Water specific retention (%)	5.73
Specific weight (g/cm ³)	1.45
Bulk density (g/cm ³)	0.98
pH	7.9
Total organic carbon (%)	13.6
Cation exchange capacity (mmol/100 g dry refuse)	18.3
Conductivity (μS/cm)	950
Total nitrogen (g/kg)	3.21
Total phosphorus (g/kg)	5.26
Total potassium (g/kg)	12.6
Total bacteria count (CFU/g)	6.7×10 ⁷