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

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## **Abstract**

In this study, the effect of aged refuse on biodegradation of total petroleum hydrocarbons (TPH), microbial counts, soil ecotoxicity, dehydrogenase activity and microbial community compositions were investigated in solid phase reactors during a 30-week period. The results demonstrate that the removal efficiency of TPH was significantly higher in the soil supplemented with aged refuse than in the soil without aged refuse. After 30 weeks, the removal efficiencies of TPH in soils were 29.3%, 82.1%, 63.7% and 90.2% in the cases of natural attenuation, nutrient addition (with  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$ ), supplement with 20% (w/w, dry weight basis) of aged refuse and the combination of nutrient and aged refuse. Nutrient plus aged refuse made the TPH concentration decrease to below the threshold level of commercial use required for Chinese soil quality for TPH ( $< 3000 \text{ mg/kg}$ ) in 30 weeks. It was also found that dehydrogenase activity, bacterial counts and degrader abundance in the soil were remarkably enhanced by the addition of aged refuse (20%,w/w). Total organic carbon analysis demonstrates that large amounts of hydrocarbon intermediates occurred in the soil after bioremediation.

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

## **1. Introduction**

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

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

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

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

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

## 2. Material and methods

### 2.1. Contaminated soil

The PCS used in this study was collected from a crude oil spill site in Shengli Oilfield, Shandong Province, China. It is not feasible to determine the particle size distribution of the contaminated soil because of its adhesive structure and high hydrocarbon content. Thereupon, surface litter and stones were removed manually and the soil sample was air-dried and sieved through a 2-mm mesh sieve, homogenized by hand with shovels, and then stored at 4 °C in the dark until used. The sieved clay loam soil contained 28% sand, 43% silt, and 29% clay. The physiochemical characteristics of the soil are as follows: bulk density, 1.8 g/cm<sup>3</sup>; water holding capacity, 26%; pH, 6.9; total nitrogen, 1.1 g/kg; total phosphorous, 34 mg/kg; total petroleum hydrocarbons (TPH), 26300 mg/kg; total organic carbon (TOC), 4.3%.

### 2.2. Aged refuse

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

### 2.3. Experimental design

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

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

Five treatment modes were applied including (1) abiotic control (AC: soil was supplemented with  $\text{NaN}_3$  (0.5 wt.%) and  $\text{HgCl}_2$  (2 wt.%) to account for abiotic loss of pollutants; (2) natural attenuation (NA: no nutrient addition and no inoculation); (3) biostimulation (BS: nutrient addition, without soil inoculation); (4) refuse spiked (RS: soil inoculation with 20% (w/w, dry weight basis) of aged refuse but without nutrient addition; (5) combination of biostimulation and refuse spiked (BS-RS: nutrient addition coupled with aged refuse inoculation (20%, w/w, dry weight basis). Each treatment was carried out in triplicate. The soil and aged refuse were homogenized thoroughly before being loaded into the bioreactors. At a 1-week interval,  $\text{NH}_4\text{NO}_3$  and  $\text{K}_2\text{HPO}_4$  in solution form were added stepwise to treatments BS and BS-RS to give a final carbon:nitrogen:phosphorus (C:N:P) ratio of 100:10:5 (Gong 2012). Soil moisture was maintained at 18–20% by the regular addition of distilled water. The content of each bioreactor was thoroughly homogenized every alternate day to allow good aeration. The trials were performed in a greenhouse at 25–30 °C.

#### 2.4. Hydrocarbon analysis

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

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

## 2.5. Soil analysis

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

## 2.6. Biochemical analyses

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



plates by using the plate spread method (Lu and Zhang, 2014). The nutrient agar had the following composition: peptone 5 g/L, yeast extract 3 g/L, agar 15 g/L, and NaCl 5 g/L. The hydrocarbon-degrading bacteria (HDB) were enumerated on Bushnell–Haas medium (BHM) agar plates with 0.5% filter-sterilized diesel oil as the sole carbon source (Fan et al., 2014). The BHM consisted of (g/L):  $\text{KH}_2\text{PO}_4$ , 1.0;  $\text{K}_2\text{HPO}_4$ , 1.0;  $\text{NH}_4\text{NO}_3$ , 1.0;  $\text{FeCl}_3$ , 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 dilution factor (from  $10^{-1}$  to  $10^{-8}$  dilution) was prepared for each retrieved soil sample. An aliquot of 0.1 mL diluted culture was spread over plates, and each dilution had three replicates. Results were expressed as colony forming units (CFU)/g soil.

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

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

## 2.7. Pyrosequencing and data analysis

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

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

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

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

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

## 2.8. Data analysis

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

## 3. Results and discussion

### 3.1. TPH dissipation

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

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

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

status is a key factor for soil bioremediation (Gong, 2012; Maddela et al., 2016; Wu et al., 2017). The deficiency of available nitrogen and/or phosphorus nutrients would lessen biodegradation performance of organic pollutants.

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

### 3.2. Removal of hydrocarbon fractions

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

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

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

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

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

### 3.3. TOC content change

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

The main effect of nutrient addition was to create and maintain, on the entire experimental time period, appropriate levels of nutrients available to both those natural inhabitants and inoculants, thus they are able to multiply and breakdown the petroleum hydrocarbons. It is found that the removal percentages of TOC were lower than those of TPH in respective modes. For example, 33.5%, 28.6% and 37.3% of TOC was removed for BS, RS and BS-RS modes, respectively, with the corresponding TPH removal of 82.1%, 63.7% and 90.2%, respectively. This result indicates that there existed large amounts of hydrocarbon intermediates in the soils after bioremediation. It is known that petroleum hydrocarbons cannot be completely mineralized by microbes to CO<sub>2</sub> and H<sub>2</sub>O, and always leaves more or less complex residues (mainly recalcitrant compounds and

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

### 3.4. Microbial counts

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



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

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

### 3.5. Dehydrogenase activity

Soil DHA is an index for overall microbial activity including total oxidative, which can indicate whether stimulation or inhibition of the microbial communities is present (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 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 increased substantially after incubation in each mode. The maximum DHA was found at week 20 in NA mode [ $84.5 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$ ], week 10 in BS mode [ $238 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$ ], and week 15 in RS mode [ $185 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$ ], when the greatest activity occurred at week 10 in BS-RS mode [ $320 \text{ TPF}/(\text{g soil} \cdot 6 \text{ h})$ ] (Figure 5). From then on, DHA continuously declined with time.

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

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

### 3.6. Microtoxicity analysis

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

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

### 3.7. Evolution of microbial community structure and diversity

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

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

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

#### 4. Conclusions

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

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

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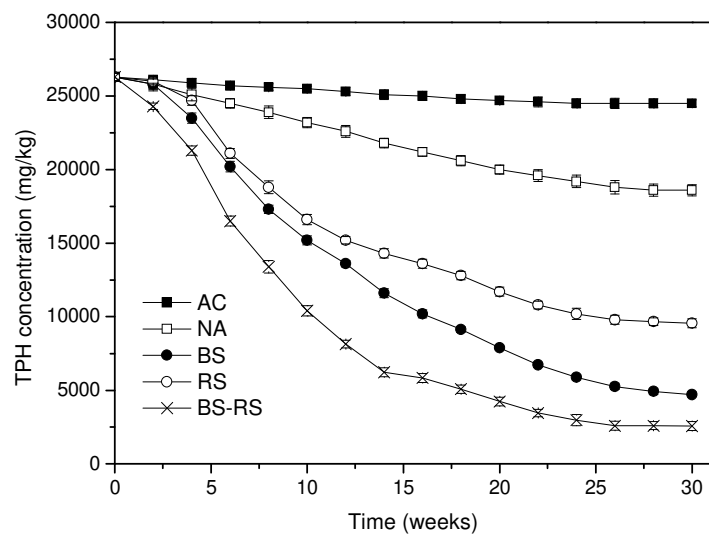


Figure 1. Changes in concentrations of TPH during bioremediation. The TPH concentrations in RS and BS-RS modes have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments ( $n = 3$ ).

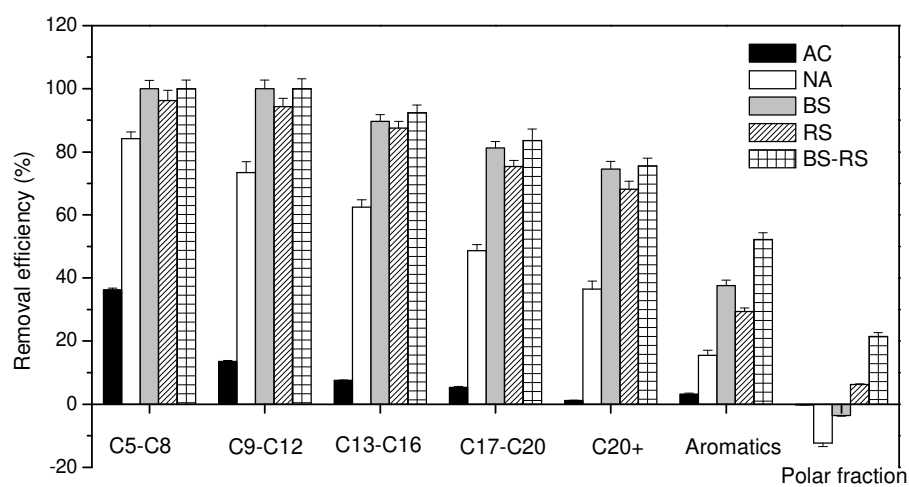


Figure 2. Removal efficiency of individual hydrocarbons at the end of experiments. The hydrocarbon concentrations in RS and BS-RS modes have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments ( $n = 3$ ).

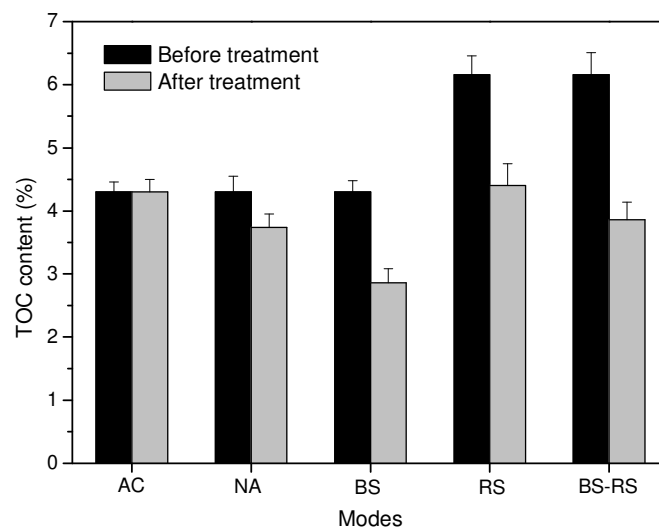


Figure 3. TOC content before and after 30 weeks of bioremediation. The TOC concentrations in the soils have been corrected by considering the dilution effect of aged refuse addition. Error bars represent standard deviations of three independent experiments (n = 3).

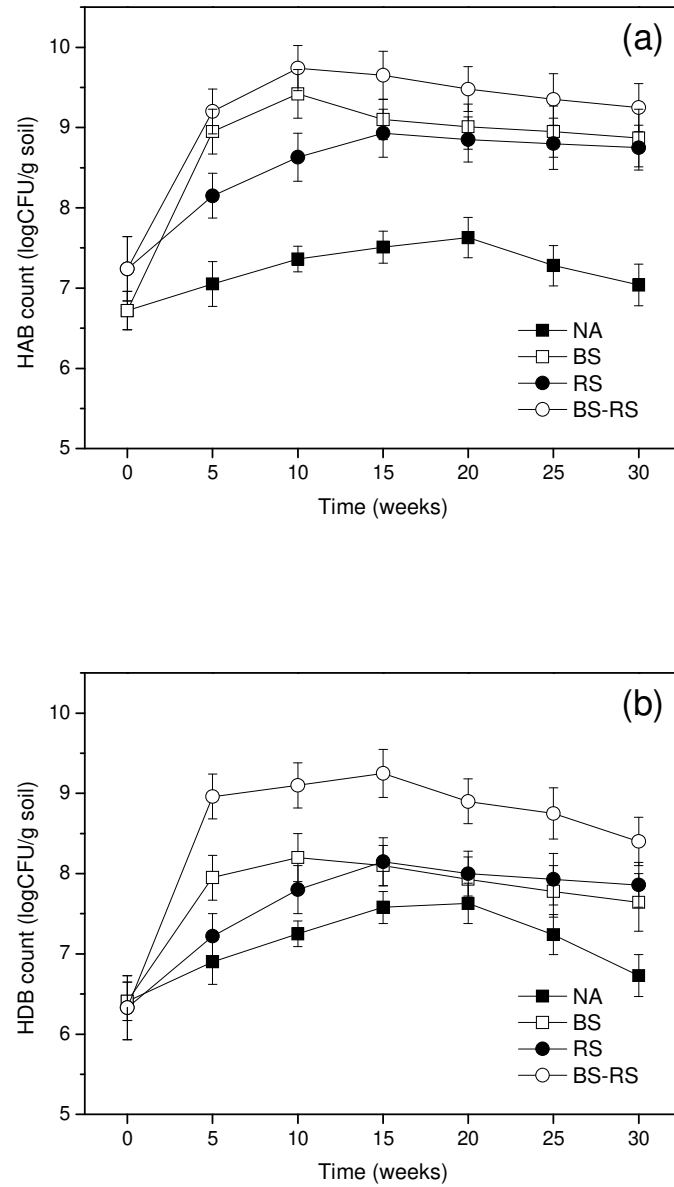


Figure 4. Evolution of (a) HAB and (b) HDB counts in the soil during 30 weeks of incubation.

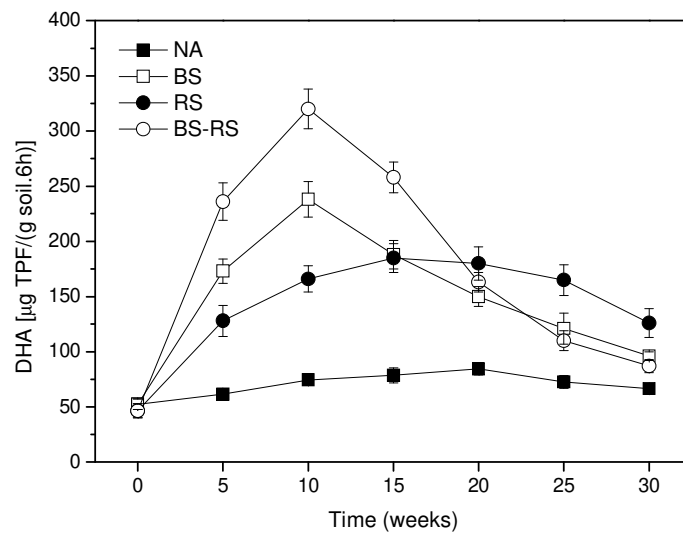


Figure 5. Evolution of soil DHA in the soil during 30 weeks of incubation.



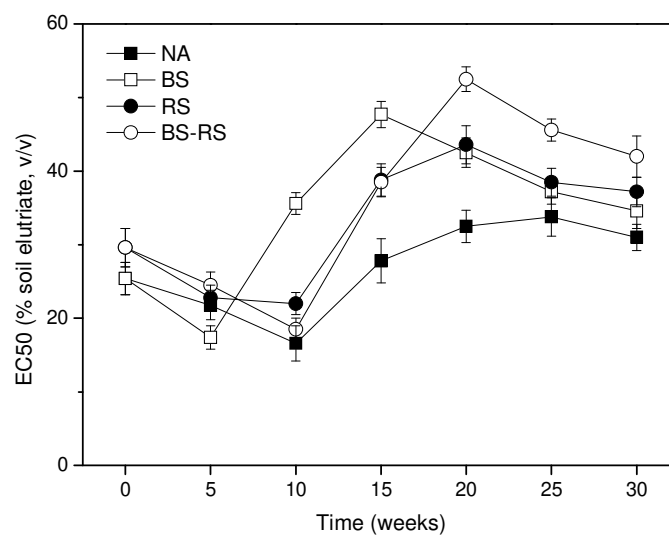


Figure 6. Time course of  $EC_{50}$  value in the soil during 30 weeks of incubation. Data represent  $EC_{50}$  values for single mode with error bars corresponding to 95% confidence intervals calculated using Microtox Data Capture and Reporting software (Ver. 7.8). The higher  $EC_{50}$  value, the lower microtoxicity.

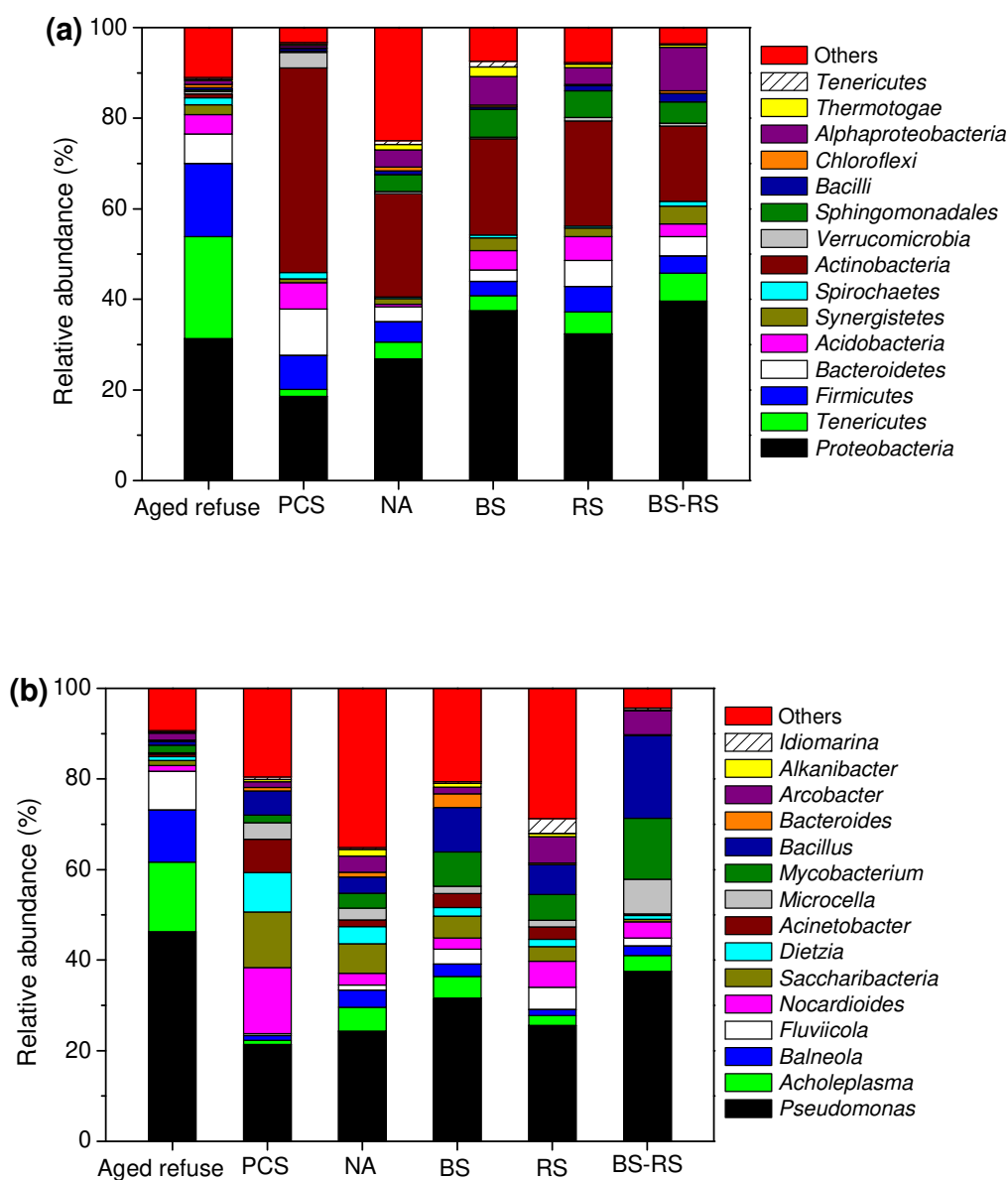


Figure 7. Relative abundance of bacterial phyla (a) and genera (b) in soils before and after 15 weeks. Except for PCS and aged refuse samples, other soils were collected at week 15. The relative abundance was displayed in terms of percentage in total effective sequences.

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 <sup>3</sup> )	1.45
Bulk density (g/cm <sup>3</sup> )	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 <sup>7</sup>