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Remediation potential of immobilized bacterial consortium with biochar as carrier in pyrene-Cr(VI) co-contaminated soil

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) and potentially toxic trace elements (PTEs) soil contamination have become areas of concern. Bioaugmentation is regarded as an effective bioremediation method, however it is difficult to simultaneously degrade organic compounds and remove PTEs with individual microbial strains. Therefore, the objective of this study was to evaluate the feasibility of using immobilized microbial consortia, including two PAH-degrading bacterial strains (W1 and W2) and a Cr(VI)-reducing bacterium (Y2), for the remediation of pyrene-Cr(VI) co-contaminated soil. Three immobilization methods were investigated: (1) bacterial consortium adsorption onto biochar (BC), (2) bacterial consortium entrapment in alginate beads (AC), (3) bacterial consortium adsorption on biochar and sequential entrapment in alginate beads (BAC). In addition, a free bacterial consortium (FC) was also used for comparison. Ten treatments were designed to illustrate the bioremediation efficiency of the free and immobilized consortia. The results show that treatments AC and BAC resulted in more efficient Cr(VI) removal compared with BC and FC. Pyrene levels in AC and BAC microcosms were reduced from 42.33 ± 3.82 to 11.56 ± 1.37 and 7.48 ± 0.39 mg kg⁻¹, respectively. Bioavailable Cr (VI) in AC and BAC was significantly lower than that in other microcosms after 28 days' incubation. Both AC and BAC microcosms exhibited a higher level of dehydrogenase and fluorescein diacetate hydrolysis activity. Furthermore, soil microbial diversity was higher in AC and BAC microcosms compared with the others. Thus, the entrapped consortia may be useful for bioremediation of pyrene and Cr (VI) without compromising soil ecology.

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Immobilized bacterial consortium; pyrene-Cr (VI) co-contaminated soil; Biolog EcoPlate; enzymatic activities; microbial community

Introduction

Soil is a complex system containing organic and inorganic compounds co-existing as a result of human activities. Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants generated from the incomplete combustion of biomass and fossil fuels, vehicle exhaust, and other anthropogenic sources. Hexavalent chromium, Cr (VI), exists widely in the environment and is mainly derived from industrial sources including leather tanning, electroplating, etc. Therefore, PAH-contaminated soil may contain high levels of potentially toxic trace elements (PTEs) such as Cr (VI). It is necessary to note that more than one third of contaminated sites contain at least two types of pollutants [1]. Co-contamination of PAHs and PTEs has become a major environmental problem in soil [2]. The adverse impacts of PAHs and PTEs on soil have received attention due to their persistence in soil, as well as their negative effects on food safety and human health [3]. However, due to the mixed nature of metals and organic compounds, it

is difficult to remove compound pollution using traditional technologies because of the interaction between PTEs and organic matter [4]. PTEs are known to affect biodegradation of organic compounds and vice versa [2,5,6]. Both Cr (VI) and PAHs are often studied separately, and consequently little information exists on effective remediation methods for their removal [7]. Therefore, it is expected that such stable activity of bacteria are present with both metal-resistant and organic matter degrading properties for the successful remediation of the co-contaminated soil [8,9].

Bioremediation through the application of indigenous or introduced microbes is considered a cost-effective technique for restoring PAH-contaminated soils compared to physicochemical remediation technologies [10,11]. However, bioaugmentation strategies can fail if introduced strains are poor survivors or lose their catabolic activities after being inoculated into PAHs and PTEs co-contaminated ecosystems. If inoculated microbes are unable to compete with indigenous

microflora, the bioremediation efficiency of the co-contaminated soil will be limited [12]. It is advantageous to repair contaminated soils with immobilized microbes rather than free cells because immobilized microorganisms may offer more complete and/or more rapid degradation as the immobilization substrates protect the cells from toxicity and natural competition with soil microflora [13].

The choice of immobilization supports is crucial for successful bioremediation of PAHs and PTEs co-contaminated soil. Alginate supports are achieved by cross-linking the carboxyl group of α -L-guluronic acid with a cationic cross linker such as calcium chloride [14]. Compared with other matrices, alginate beads provide nutrients and appropriate conditions such as facilitated oxygen transfer, which is crucial for microbial growth. Several studies have investigated the application of alginate-immobilized microbes for PAHs and PTEs removal [15–17]. Biochar is also a suitable microbial carrier due to its high affinity for PAHs and PTEs [18–20]. The application of biochar into contaminated soils can efficiently reduce the bioavailability of organic contaminants and PTEs [21,22]. Hence, the unique combination of alginate and biochar presents potential for optimizing the localization of inoculants, nutrients, and pollutants, leading to enhanced remediation rates. Such an approach provides a new perspective on sustainable environmental strategies for soil remediation. However, few studies on the co-application of alginate and biochar as immobilization carriers have been reported, especially for the remediation of PAHs and PTEs co-contaminated soil. Further research is still necessary [18].

The addition of exogenous microbes may destroy the microbial ecological balance of the soil if the inoculated microbes influence the indigenous microbial population [23]. Therefore, it is important to note that any bioremediation process should not only remove pollutants from the contaminated soil, but also restore the original soil performance [24]. Any disturbance can rapidly alter soil enzymatic activity and microbial diversity, thus soil microorganisms are very sensitive to ecosystem shifts [25]. Microbiological variables, enzymatic activity, and microbial diversity are considered good indices to evaluate the impacts of pollution on soil [26].

Based on the above consideration, we chose pineapple peels biochar and sodium alginate as the carrier of bacterial consortium and expected to make full use of the advantages in their application in co-contaminated soil remediation. The objective of the current study is to evaluate the feasibility of using immobilized microbial consortia for the remediation of pyrene-Cr(VI) co-contaminated soil. In addition, the effect of the immobilized bacterial consortium on the soil microbial community

was also investigated according to community-level physiological profiles (CLPP) of the soil microbes.

Materials and methods

Preparation of contaminated soil

The topsoil used in this study was collected from Wenzhou University, and no pyrene or Cr(VI) was detected in it. The soil was a Malabon silty clay loam (Pachic Ultic Argixerolls) [27] and its basic physicochemical properties were as follows: organic matter ($3.00\% \pm 0.01\%$), total nitrogen ($0.2\% \pm 0.01\%$), total phosphorus ($6.63\% \pm 0.26\%$), cation exchange capacity (30.01 ± 0.47 cmol/kg), pH (7.02–6.89). Gravel, plant roots, and organic residues in the sampled soil were removed, and the soil was air-dried and passed through a 2-mm mesh. Then, the sampled soil was contaminated with pyrene dissolved in acetone. After solvent evaporation, it was spiked with a $K_2Cr_2O_7$ solution to prepare a co-contaminated soil sample with a certain concentration of pyrene and Cr(VI). The spiked soil was then homogenized and kept in a dark room to equilibrate. After equilibrating for 60 days, the aged soil was analyzed. As a result, the initial concentration of pyrene and Cr(VI) in the form of $CaCl_2$ extraction was 42.33 ± 3.82 mg kg^{-1} and 16.93 ± 1.09 mg kg^{-1} , respectively.

Bacterial consortium and growth conditions

Bacillus sp. W1 (Genbank accession number KT444619) and *Bacillus* sp. W2 (Genbank accession number KT444620) were isolated from PAHs-contaminated sediments collected from Oujiang River wharf of Wenzhou, China. *Microbacterium* sp. Y2 (Genbank accession number KT426691) was isolated from Cr(VI)-contaminated soil near the industrial area of Wenzhou, China.

Previous research has verified this bacterial consortium's capacity to simultaneously remove pyrene and Cr(VI) in solution. Before being immobilized, W1 and W2 were cultured in Erlenmeyer flasks with 100 mL of LB medium and incubated at 30°C under constant agitation at 125 rpm for 24 h, Y2 was cultured and incubated under the same conditions for 72 h. After incubation, equal proportions of each strain were mixed, resulting in a concentration of 2×10^{10} cfu/mL.

Immobilization materials and preparation of immobilized bacterial consortium

Biochar for use as an immobilization carrier was produced from pineapple peels that were oven-dried, crushed, and slowly pyrolyzed at 750°C with a heating

rate 4°C – $5^{\circ}\text{C min}^{-1}$ in a muffle furnace for 2 h. After cooling to room temperature, the charred solids were soaked in 0.1 M HCl solution overnight, washed with distilled water to obtain a neutral filtrate, oven-dried overnight at 50°C – 60°C successively. The final biochar samples were gained [28]. Sodium alginate (chemical pure) was purchased from Sinopharm Chemical Reagent Co., Ltd.

Bacterial consortium W1-W2-Y2 was immobilized on biochar via physical adsorption as follows: 1 g of biochar mixed with a 20-mL cell suspension of bacterial consortium W1-W2-Y2 (2.5×10^8 cfu/mL) was incubated for 24 h at 30°C on a homothermal vibrator. After standing and layering, the biochar and the cell suspension were statically separated after equilibrium. 100 μL of supernatant was plated onto a nutrient agar plate following a 24-h incubation at 30°C . The number of bacterial colony forming units (CFU) on the plate was counted. The cell immobilization efficiency of biochar was calculated with the follow equation [29]:

$$\text{Cell immobilization efficiency} = \frac{\text{CFU}_a - \text{CFU}_b}{\text{CFU}_a} \times 100\%$$

where CFU_a and CFU_b are the CFUs of the initial cell suspension culture and the supernatant, respectively.

The following steps describe the preparation of embedded-bacteria beads: the bacterial consortium culture was fully mixed with a 5% (w/v) sodium alginate sterile solution, and the suspension was added dropwise to a 5% (w/v) CaCl_2 sterile solution using a syringe. The beads solidified in the CaCl_2 solution for 12 h before being harvested with gauze. After that, the beads were rinsed with sterilized water and stored at 4°C before use. The number of CFUs for each bead was 1×10^9 cfu/g. Beads without embedded bacteria were also prepared as a control.

The bacterial consortium was entrapped in alginate beads with biochar carrier by combining the above two methods. First, the biochar and cell suspension were mixed with a 5:100 (w/v) ratio for 2 h, and then 5% (w/v) sodium alginate was fully mixed into the suspension. The mixed suspensions were added dropwise to a well-stirred and sterilized 5% (w/v) CaCl_2 solution. The subsequent steps were the same as those described above.

Preparation of soil microcosms

The experimental design included three replications and the following treatments: (1) inhibiting bacteria control ($\text{CK}(\text{NaN}_3)$), (2) natural dissipation control (CK), (3) free bacterial consortium (FC), (4) biochar adsorbed bacterial consortium (BC), (5) entrapped bacterial consortium

without biochar (AC), (6) entrapped bacterial consortium with biochar carrier (BAC), (7) entrapped beads without bacterial consortium (NA), (8) entrapped beads without bacterial consortium and with NaN_3 ($\text{NA}(\text{NaN}_3)$) addition, (9) entrapped beads without bacterial consortium but with biochar (BA), and (10) entrapped beads without bacterial consortium but with biochar and NaN_3 ($\text{BA}(\text{NaN}_3)$) addition. Each soil microcosm was prepared by placing 100 g of non-sterile soil in a plastic bowl. The above ten treatments were implemented as follows: (1) 2000 mg of $\text{kg}^{-1}\text{NaN}_3$ was added to soil as a microbial inhibitor ($\text{CK}(\text{NaN}_3)$); (2) nothing was added to the natural dissipation control (CK); (3) soil was mixed with 3 mL of LB medium containing 5×10^9 free bacterial consortium W1-W2-Y2 (FC); (4) 1 g of biochar that had adsorbed approximately 5×10^9 bacterial consortium was mixed with soil (BC); (5) 5 g alginate beads entrapped with 5×10^9 bacterial consortium were applied into soil (AC); (6) 5 g alginate beads entrapped with 5×10^9 bacterial consortium with biochar carrier were applied into soil (BAC); (7) soil was mixed with 5 g of alginate beads without bacterial consortium (NA); (8) soil was mixed with 5 g alginate beads without bacterial consortium and with the addition of 2000 mg $\text{kg}^{-1}\text{NaN}_3$ as a microbial inhibitor ($\text{NA}(\text{NaN}_3)$); (9) 5 g of alginate beads entrapped with biochar were put into soil (BA); and (10) soil were blended with 5 g of alginate beads entrapped with biochar and with the addition of 2000 mg $\text{kg}^{-1}\text{NaN}_3$ as a microbial inhibitor ($\text{BA}(\text{NaN}_3)$). All microcosms were incubated for 4 weeks at 30°C in darkness and covered with perforated tinfoil to maintain soil moisture. Soil samples from each microcosm were collected at 14- and 28-day incubation periods and divided into two parts. One part was air-dried and sieved prior to analysis for pyrene and hexavalent chromium in the form of CaCl_2 extraction, and the other was used for soil microbial activity and community analyses.

Analysis of soil samples

The extraction of residual pyrene in soil samples was modified according to the procedure described by Zhang et al. [30]. Briefly, 2.0000-g soil samples were mixed with 2 g of anhydrous sodium sulfate to remove any moisture, and the samples were then extracted with ultrasonication for 30 min using a 1:1 (v/v) solution of acetone and dichloromethane. The solvent was then decanted, collected, and replenished. This process was repeated three times. Samples were rotated to dry and dissolved using 1.2 mL of dichloromethane before being completely transferred to a vial. After natural volatilization of the dichloromethane, pyrene was re-

dissolved using methanol to a final volume of 1 mL for high-performance liquid chromatograph (HPLC) analysis. The (HPLC, Waters e2695, U.S.A) was equipped with a multi λ fluorescence detector (Waters 2475) and a 150 \times 4.6 mm reverse-phase C18 column, with methanol and water (90:10, v/v) as the mobile phase at a flow rate of 1.0 mL min⁻¹. Chromatography was performed at 30°C. Pyrene was detected at 324 nm, and the injection volume was 1 μ L. The biodegradation rate was calculated according to the standard curve.

Hexavalent chromium in CaCl₂ extraction was used to describe the amount of bioavailable Cr(VI). Briefly, 2 g of soil was extracted with 20 mL of 0.01 M CaCl₂ and then shaken at 150 rpm for 24 h. The mixture was filtered, and the concentration of Cr(VI) in the filtrate was measured according to Polti et al. [31].

Soil enzymatic activities

According to the method reported by Zhang et al. [30], we determined the dehydrogenase (DHA) activity as follows: 4 g of soil samples from each treatment were put into a 50-mL Erlenmeyer flask, then 2 mL of 1% TTC-Tris buffer solution and 2 mL of 1% glucose solution were added. After incubation for 24 h at 37°C in the dark, 1,2,5-triphenylformazan (TPF), which is the reduction product of 2,3,5-triphenyltetrazolium chloride (TTC), was extracted with methanol. Next, the absorbance of the solution was measured at 485 nm. The DHA was expressed as micrograms of TPF per gram of dry soil.

Fluorescein diacetate (FDA) hydrolysis was assessed according to a method slightly modified from that described in Tribedi and Sil [23]. Briefly, 30 mL of phosphate buffer (pH 7.6) and 1 mL of FDA (2 g L⁻¹)-acetone solution were added to 1 g of soil in a 50-mL Erlenmeyer flask. After incubation for 2 h at 30°C on a rotary shaker at 200 rpm, the mixture was filtered, and then the

absorbance was measured at 490 nm. FDA was reported as micrograms fluorescein per gram dry soil.

Soil microbial community

Carbon source utilization patterns of the soil microbial community in each treatment were examined as described by Ye et al. [32] with some modifications. The Biolog Eco Plate system contained 96 wells with triplicates of 31 different carbon sources and a blank well with no substrate, as shown in Table 1 [33]. This assay was based on the capacity of microbes to utilize different substrates and generate a metabolic fingerprint providing information on functional diversity in soil [34]. The CLPP were assessed using the method described by Jiang et al. [35]. Briefly, 10 g of fresh soil was suspended in 90 mL of 0.05-M sterilized phosphate buffer (pH 7.0) on a rotary shaker for 60 min. After settling, 150 μ L of 10⁻³ dilution was added to each well of the Biolog Eco-Plate, followed with incubation at 30°C in the dark for 7 days. Then, the optical densities of each well at 590 and 750 nm were determined using a Multimode Reader (CYtation3, USA) every 24 h [36].

After minusing the optical densities at 750 nm, the densities at 590 nm were used to calculate the average well color development (AWCD) after 72 h of incubation, species richness, and the Shannon-Weaver, Simpson, and McIntosh indices, as this is the shortest incubation time that allows the best resolution among treatments [37]. The AWCD was determined using the equation $AWCD = \sum(C-B)/31$, where C is the optical density value from each carbon source well and B is that of the control. The following two equations were used to calculate the Shannon-Weaver index (H) and Simpson index (D), respectively: $H = -\sum p_i (\ln p_i)$ and $D = 1/\sum p_i \times p_i$, where p_i is the ratio of well i absorbance to the total absorbance of all wells. The McIntosh index, which was used to

Table 1. Biolog eco plate carbon source guild groupings.

Well №	Carbon source	Compound group	Well №	Carbon source	Compound group
A1	Water	—	A3	D-Galactonic acid- γ -lactone	Carboxylic and ketonic acids
B1	Pyruvic acid methyl ester	Carbohydrates	B3	D-Galacturonic acid	Carboxylic and ketonic acids
C1	Tween 40	Polymers	C3	2-Hydroxybenzoic acid	Carboxylic and ketonic acids
D1	Tween 80	Polymers	D3	4-Hydroxybenzoic acid	Carboxylic and ketonic acids
E1	α -Cyclodextrin	Polymers	E3	γ -Hydroxybenzoic acid	Carboxylic and ketonic acids
F1	Glycogen	Polymers	F3	Itaconic acid	Carboxylic and ketonic acids
G1	D-Cellulose	Carbohydrates	G3	α -Ketobutyric acid	Carboxylic and ketonic acids
H1	α -D-Lactose	Carbohydrates	H3	D-Malic acid	Carboxylic and ketonic acids
A2	β -Methyl- D-glucoside	Carbohydrates	A4	L-Arginine	Amino acids
B2	D-Xylose	Carbohydrates	B4	L-Asparagine	Amino acids
C2	i-Erythritol	Carbohydrates	C4	L-Phenylalanine	Amino acids
D2	D-Mannitol	Carbohydrates	D4	L-Serine	Amino acids
E2	N-Acetyl- D-Glucosamine	Carbohydrates	E4	L-Threonine	Amino acids
F2	D-Glucosaminic acid	Carboxylic and ketonic acids	F4	Glycyl- L-glutamic acid	Amino acids
G2	Glucose-1-phosphate	Carbohydrates	G4	Phenylethylamine	Amines/amides
H2	D,L- α -Glycerol phosphate	Carbohydrates	H4	Putrescine	Amines/amides

Well №-Well number.

measure species homogeneity in the community, was calculated using the equation $U = \sqrt{\sum (ni)^2}$, where ni is the value of well i absorbance minus that of the blank.

Statistical analysis

All the soil analysis data reported were mean values of three replicates. Differences in soil pyrene removal, CaCl_2 extractable Cr (VI) reducing, enzyme activities, utilization of carbon substrates groups, Shannon-Weaver index, Simpson index, McIntosh index and species richness among the treatments were analyzed with one-way ANOVA followed by Duncan's multiple range test. CLPP data were analyzed by cluster analysis to categorize the treatments with similar characteristics into a group. Statistical analyses were performed with SPSS 20.0 at 0.05 significance levels.

Results and discussion

Residual pyrene and CaCl_2 extracted Cr(VI) in different microcosms

Table 2 shows the pyrene levels in microcosms under different treatments after 14- and 28-day incubations. After 14 days' incubation, treatments BC, AC, and BAC significantly enhanced the dissipation of pyrene in the soil, with resulting pyrene levels of $22.93 \pm 3.52 \text{ mg kg}^{-1}$, $23.71 \pm 8.23 \text{ mg kg}^{-1}$ and $26.38 \pm 7.09 \text{ mg kg}^{-1}$, respectively, whereas treatment FC did not appear to increase pyrene removal ($32.87 \pm 9.27 \text{ mg kg}^{-1}$) compared with CK ($31 \pm 1.89 \text{ mg kg}^{-1}$). After 28 days' incubation, the treatment BAC emerged the strongest pyrene degradation with residual pyrene level $7.48 \pm 0.39 \text{ mg kg}^{-1}$, as well as pyrene in treatment AC had declined significantly ($p < 0.05$). However, treatment BC had no significant difference with FC although both of them represented better pyrene removal than CK ($p < 0.05$). So we can infer that the bacterial consortium are easy to fall off the

biochar because they can't be immobilized on biochar tightly. Besides, the exogenous microorganisms were adapt to the soil environment as time prolonged and played a certain role in pyrene removal, so the pyrene residual decreased significantly from $22.93 \pm 3.52 \text{ mg kg}^{-1}$ (14 d) to $14.99 \pm 6.54 \text{ mg kg}^{-1}$ (28 d) in BC (Table 2). The ranking of the different bioaugmentation treatments with respect to pyrene level was $\text{FC} > \text{BC} > \text{AC} > \text{BAC}$. This indicates that the bacterial consortium immobilized by entrapment (AC and BAC) had a greater ability for pyrene removal compared with the free bacterial consortium (FC) and bacterial consortium immobilized by adsorptive fixation on biochar (BC). This was likely because framework of sodium alginate beads can protect the bacterial consortium from protozoa predation and allow the substrates and products to move freely in and out through the micropores in the bead wall [38]. While, the free bacterial consortium may have experienced an adaptive process after being inoculated into soil, and therefore exhibited a low ability to degrade pyrene in the short term [39]. In addition, pyrene levels in treatments CK(NaN_3), NA(NaN_3), and BA(NaN_3) were significantly higher than those in treatments without NaN_3 ($p < 0.05$), revealing that microorganisms play an important role in pyrene removal.

The concentration of bioavailable Cr(VI) in different microcosms varied from 1.62 ± 0.08 to $6.95 \pm 0.59 \text{ mg kg}^{-1}$ soil (Table 2). The potential bioavailability of Cr(VI) in soil strongly decreased after bioaugmentation with immobilized consortia after 28 days' incubation. However, bioavailable Cr(VI) in soil inoculated with the free bacterial consortium only slightly decreased, with a concentration of $2.58 \pm 0.08 \text{ mg kg}^{-1}$ soil after 28 days' incubation. This is obvious that the matrix increased the exposure of the immobilized cells to Cr (VI) through adsorption resulting Cr(VI) reduction to Cr (III) and provided a barrier against severe environmental conditions [39]. Furthermore, bioavailable Cr(VI) in sterilized soil was higher than that in unsterilized soil,

Table 2. Effects of consortia immobilized with different methods on the removal of pyrene and Cr(VI) in co-contaminated soils after 14- and 28-day incubations.

Treatments	14 d		28 d	
	Pyrene residual (mg.kg^{-1})	CaCl_2 extracted Cr(VI) residual(mg.kg^{-1})	Pyrene residual (mg.kg^{-1})	CaCl_2 extracted Cr(VI) residual(mg.kg^{-1})
CK(NaN_3)	$40.41 \pm 8.45\text{a}$	$6.95 \pm 0.59\text{a}$	$36.46 \pm 2.08\text{b}$	$4.96 \pm 0.43\text{c}$
NA(NaN_3)	$35.89 \pm 6.07\text{b}$	$5.87 \pm 0.87\text{b}$	$35.32 \pm 7.64\text{b}$	$4.65 \pm 0.14\text{d}$
BA(NaN_3)	$36.57 \pm 9.58\text{b}$	$5.69 \pm 0.31\text{b}$	$32.42 \pm 3.98\text{cd}$	$4.02 \pm 0.44\text{e}$
CK	$31.00 \pm 1.89\text{d}$	$3.52 \pm 0.29\text{f}$	$19.72 \pm 1.64\text{h}$	$3.34 \pm 0.08\text{fg}$
NA	$29.33 \pm 5.75\text{e}$	$3.44 \pm 0.23\text{f}$	$14.34 \pm 0.70\text{i}$	$3.07 \pm 0.23\text{ghi}$
BA	$31.84 \pm 6.09\text{cd}$	$3.30 \pm 0.08\text{fgh}$	$13.95 \pm 0.37\text{i}$	$3.05 \pm 0.24\text{ghi}$
FC	$32.87 \pm 9.27\text{c}$	$2.84 \pm 0.36\text{ij}$	$15.21 \pm 0.23\text{i}$	$2.58 \pm 0.08\text{j}$
BC	$22.93 \pm 3.52\text{g}$	$2.53 \pm 0.23\text{j}$	$14.99 \pm 6.54\text{i}$	$1.62 \pm 0.08\text{k}$
AC	$23.71 \pm 8.23\text{g}$	$2.98 \pm 0.16\text{hi}$	$11.56 \pm 1.37\text{j}$	$1.66 \pm 0.00\text{k}$
BAC	$26.38 \pm 7.09\text{f}$	$2.81 \pm 0.16\text{ij}$	$7.48 \pm 0.39\text{k}$	$1.93 \pm 0.16\text{k}$

Values are means \pm standard deviation of triplicate measurements. Mean values with the same letter are not significantly different among treatments by LSD at the 5% level.

which also proved that the bacteria were important for reduction of hexavalent chromium.

Soil biological activities

Microbial activity in unsterilized microcosms was determined after 14- and 28-day incubation periods (Table 3). Compared with the other treatments, DHA activity was significantly enhanced in the AC and BAC treatments (82.25 ± 9.35 and $92.21 \pm 13.59 \mu\text{g TPF g}^{-1}$ dry soil, respectively) after 28 days' incubation. The free bacterial consortium induced microbial activity after 14 days, but reduced activity after 28 days. DHA can be used to determine the presence of viable microorganisms and their oxidative capability. Table 3 shows that soil DHA increased significantly in BAC bioaugmented microcosms. Pyrene or its metabolites were likely used as substrates, thus increasing the enzymes activity. It can also confirm that BAC bioaugmented microcosms showed the highest microbiological activity [40]. Soil FDA hydrolase activity values in all treatments significantly increased from 14 to 28 days, especially in the AC and BAC treatments (51.72 ± 12.09 and $52.08 \pm 9.61 \mu\text{g fluorescein g}^{-1}$ dry soil, respectively). Compared

with the other treatments, DHA and FDA activity was higher in AC and BAC. These results (Table 3) indicated that bioaugmentation of immobilized bacterial consortium enhanced activities of DHA, FDA hydrolase, that is, the soil biological activity, thus raising the degradation rate of pyrene in the soil [41].

Soil bacterial community analysis

To investigate the effects of immobilized consortia on the soil bacterial community, the entire bacterial communities in soil samples from treatments CK, FC, BC, AC, and BAC were extracted and inoculated onto Biolog EcoPlates, which have been successfully used to monitor changes in microbial communities in soil [42]. The variation in AWCD after 28-day bioremediation periods is shown in Figure 1(a). The AWCD reflects the sole-carbon-source utilization ability of the soil bacterial community and soil bacteria activity [40,43]. The Higher AWCD values were found in samples from AC and BAC, whereas the values for samples from FC and BC were lower and similar to those of CK.

To confirm this observation, we also determined the Shannon-weaver index (H), a measure of the actual

Table 3. Soil microbial activities in soils with different treatments after 14 and 28 days' incubation.

Treatments	14 d		28 d	
	DHA($\mu\text{g TPF g}^{-1}$ dry soil)	FDA ($\mu\text{g fluorescein g}^{-1}$ dry soil)	DHA ($\mu\text{g TPF g}^{-1}$ dry soil)	FDA ($\mu\text{g fluorescein g}^{-1}$ dry soil)
CK	$53.16 \pm 8.07i$	$24.36 \pm 1.63e$	$55.21 \pm 13.17h$	$29.35 \pm 7.22d$
NA	$50.90 \pm 6.67j$	$16.73 \pm 0.20gh$	$62.19 \pm 2.56f$	$41.69 \pm 4.28b$
BA	$61.11 \pm 17.25f$	$15.58 \pm 1.43h$	$55.74 \pm 1.01h$	$36.67 \pm 12.73c$
FC	$80.89 \pm 14.58cd$	$14.06 \pm 0.10i$	$58.44 \pm 2.08g$	$30.74 \pm 7.21d$
BC	$71.52 \pm 12.69e$	$25.37 \pm 0.81e$	$70.48 \pm 11.94e$	$37.32 \pm 3.05c$
AC	$87.10 \pm 17.22b$	$17.38 \pm 1.73g$	$82.25 \pm 9.35c$	$51.72 \pm 12.09a$
BAC	$79.92 \pm 7.16d$	$19.46 \pm 0.61f$	$92.21 \pm 13.59a$	$52.08 \pm 9.61a$

Values are means \pm standard deviation of triplicate measurements. Mean values with the same letter are not significantly different among treatments by LSD at the 5% level.

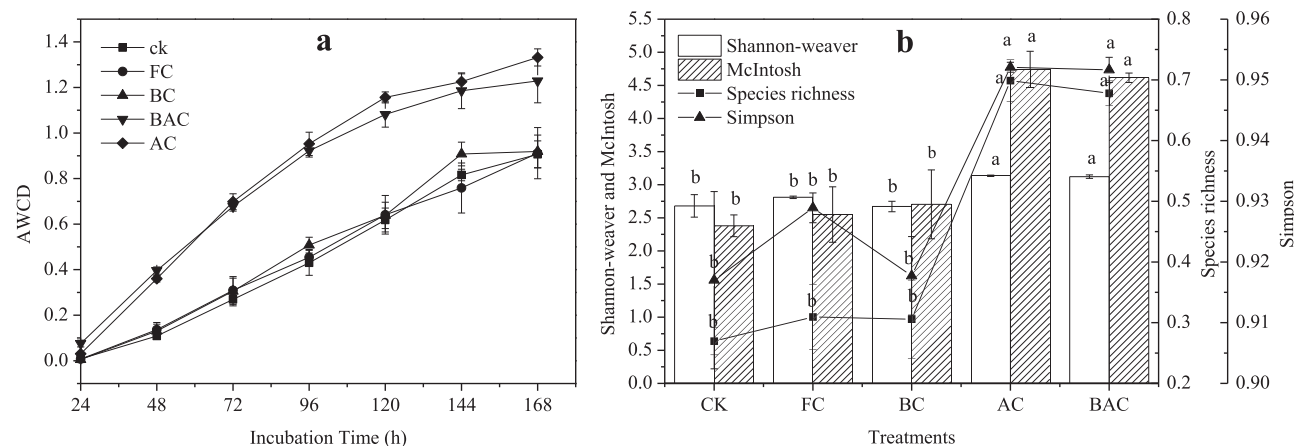


Figure 1. (a) AWCD for different microcosms after 28 days' incubation; (b) Functional diversity of microbial community in different microcosms after 28 days' incubation. Error bars indicate the standard deviation of the means ($n = 3$). Letters indicate significant differences between data ($P < 0.05$).

Table 4. Average utilization of carbon sources from five substrate groups by soil microbes in different microcosms.

Carbon substrates	CK	FC	BC	AC	BAC
CH	0.26 ± 0.09c	0.32 ± 0.04c	0.25 ± 0.05c	0.97 ± 0.05a	0.82 ± 0.02b
CA	0.20 ± 0.03b	0.23 ± 0.06b	0.22 ± 0.01b	0.46 ± 0.06a	0.42 ± 0.02a
AA	0.42 ± 0.11b	0.44 ± 0.12b	0.44 ± 0.14b	0.74 ± 0.07a	0.76 ± 0.06a
PL	0.28 ± 0.09c	0.30 ± 0.04bc	0.42 ± 0.07b	0.55 ± 0.04a	0.62 ± 0.08a
AM	0.12 ± 0.10c	0.22 ± 0.11bc	0.32 ± 0.43bc	0.61 ± 0.20ab	0.94 ± 0.14a

Values are means ± standard deviation of triplicate measurements. Mean values with the same letter are not significantly different among treatments by LSD at the 5% level.

richness and evenness of the bacterial population, the Simpson index (D), often being used to quantify the number of species present as well as the relative abundance of each species, the McIntosh index (U), and Species richness after an incubation period of 72 h for each microcosm (Figure 1(b)). The value of Simpson index ranges between 0 and 1: the greater the value, the greater the sample diversity [6]. Significant differences in H, D, U, and Species richness were found between the AC treatment and CK as well as BAC and CK ($p < 0.05$). However, a similar trend was observed between FC, BC, and CK, indicating that the free bacterial consortium and bacterial consortium adsorbed on biochar did not improve the microbial diversity. These diversity indexes showed significant enhancement of microbial community diversity after 28 d incubation of BAC bioaugmentation. It can be concluded that, as a result of BAC bioaugmentation the microbiological functioning of the pyrene-Cr (VI) co-contaminated soil has been at least partially restored.

The carbon source utilization patterns by various microorganisms in different microcosms were also investigated (Table 4). Treatments AC and BAC resulted in more efficient utilization of all five main carbon sources (carbohydrates, carboxylic and ketonic acids, amino acids, polymers, and amines/amides) compared with CK. However, treatments FC and BC caused a slight increase in polymer and amines/amide utilization, but did not affect the utilization of carbohydrates, carboxylic and ketonic acids, or amino acids compared with CK.

A cluster analysis of heterotrophic bacteria in different microcosms based on consumption of 31 carbon sources was conducted to categorize the treatments into several groups with similar characteristics and to investigate the effects from the different types of bioaugmentation (Figure 2). The cluster analysis revealed that five treatments could be separated into two distinct groups: Group 1 included BC, FC and CK; Group 2 included AC and BAC. The results of the cluster analysis suggested that entrapped microbe addition had distinct impacts on the soil microbial communities comparing with others. FC was quite close to CK, indicating that free bacterial consortium addition present little influence on the soil microbial communities. Meanwhile, entrapped

microbe addition resulted in remarkable changes in substrates utilization. The broadest and highest metabolic activity was found in treatments AC and BAC, in which the microbial communities were able to metabolize 26 out of 31 substrates. In contrast, the communities in treatments BC and FC were only able to utilize 21 and 18 of the total substrates, respectively, which was similar to CK. Thus it can be seen that the exogenous addition of entrapped bacterial consortium to pyrene-Cr(VI) co-contaminated soil improved microbial enzyme activity and diversity. However, the addition of a free bacterial consortium or bacterial consortium adsorbed on biochar had no effect on the soil communities.

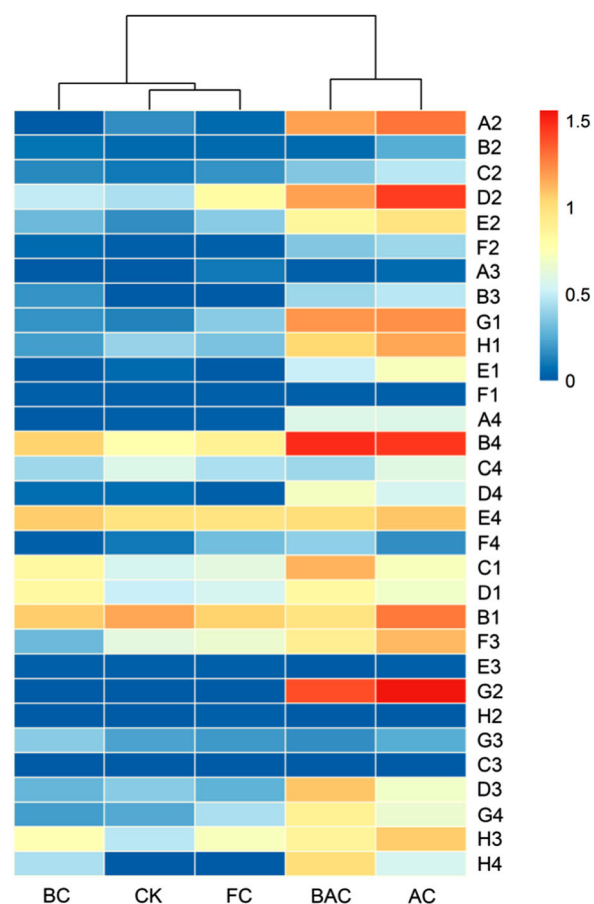


Figure 2. Cluster analysis of microorganisms present in different microcosms based on carbon utilization. Descriptions of A2 to H4 are given in Table 1.

Conclusion

Bioremediation with BAC bioaugmentation used in this soil microcosm study enhanced pyrene dissipation and reduced the content of bioavailable Cr(VI) in the soil compared with the free or biochar-adsorbed bacterial consortium. Further study revealed that the entrapped bacterial consortium significantly improved soil microbial activity and diversity. It suggested that BAC bioaugmentation can increase soil microbiological activity, with some restoration of the microbiological functioning of the pyrene-Cr (VI) co-contaminated soil. Therefore, the entrapped bacterial consortium W1-W2-Y2 can potentially be used for in-situ bioremediation of pyrene-Cr (VI) co-contaminated soil. The BAC bioaugmentation of this strategy is a promising practical bioremediation strategy with further studies involving different soil types and contaminants under field conditions.

Disclosure statement

No potential conflict of interest was reported by the authors.

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