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RESEARCH ARTICLE

Remediation of polycyclic aromatic hydrocarbon and metal-contaminated soil by successive methyl-β-cyclodextrin-enhanced soil washing–microbial augmentation: a laboratory evaluation

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Abstract Polycyclic aromatic hydrocarbon (PAH) and metal-polluted sites caused by abandoned coking plants are receiving wide attention. To address the associated environmental concerns, innovative remediation technologies are urgently needed. This study was initiated to investigate the feasibility of a cleanup strategy that employed an initial phase, using methyl-β-cyclodextrin (MCD) solution to enhance *ex situ* soil washing for extracting PAHs and metals simultaneously, followed by the addition of PAH-degrading bacteria (*Paracoccus* sp. strain HPD-2) and supplemental nutrients to treat the residual soil-bound PAHs. Elevated temperature (50 °C) in combination with ultrasonication (35 kHz, 30 min) at 100 g MCD L^{-1} was effective in extracting PAHs and metals to assist soil washing; 93 % of total PAHs, 72 % of Cd, 78 % of Ni, 93 % of Zn, 84 % of Cr, and 68 % of Pb were removed from soil after three successive washing cycles. Treating the residual soil-bound PAHs for 20 weeks led to maximum biodegradation rates of 34, 45, 36, and 32 % of the remaining total PAHs, 3-ring PAHs, 4-ring PAHs, and 5(+6)-ring PAHs after washing procedure, respectively. Based on BIOLOG Ecoplate assay, the combined treatment at least partially restored microbiological functions in the contaminated soil. The *ex situ* cleanup strategy through MCD-enhanced soil washing followed by microbial augmentation can be effective in remediating PAH and metal-contaminated soil.

Keywords Polycyclic aromatic hydrocarbon · Metal · Methyl-β-cyclodextrin · Soil washing · Microbial augmentation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. Excessive inputs from anthropogenic activities have caused serious environmental contamination and threaten to adversely affect human health (Wilcke 2007; Sun et al. 2011). With the signing of the Stockholm Convention and the development of global monitoring programs, many PAH-contaminated sites are left by numerous abandoned coking plants, thermal power plants, and steel plants in major metropolitan areas in China (Ni et al. 2010) and in many industrialized countries worldwide (Mouton et al. 2009; Dalgren et al. 2009). Soils from these sites are often contaminated by a mixture of PAHs and metals, such as Cd, Cr, Ni, Zn, and Pb (Ehsan et al. 2007; Khodadoust et al. 2005; Maturi and Reddy 2008). Currently, most of these contaminated sites face issues of land use conversion for commercial development (Ni et al. 2010) and may pose a threat to residents and the environment. Therefore, it is urgent to develop new soil remediation techniques that are suitable to meet the specific needs (Guo et al. 2009; Gong et al. 2010; Yu et al. 2007).

Proper remediation action to simultaneously remove PAHs and metals from contaminated sites is extremely essential (Ding et al. 2008). Ex situ soil washing in combination with environmentally friendly additives may be a promising technology for achieving the goal. Dalgren et al. (2009) investigated the effectiveness of both single and mixtures of different surfactants in removing PAHs and As from an old wood preservation site. They found that the combination of chelating agent methylglycine diacetic acid and biodegradable, nonionic surfactant alkyl polyglucoside showed very good

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removal capacity for both PAHs and As after 10 min of washing at 50 °C. Huguet and Marshall (2011) observed the highest level of mobilization in three soil washes using a mixture of 0.1 M [*S*,*S*]-ethyelnediaminedisuccinate and 2 % 2-[(*Z*)-octadec-9-enoxy]ethanol (Brij 98) at pH 9 with 20 min of ultrasonication treatment at room temperature. This strategy resulted in the removal of 70 % of As, 75 % of Cr, 80 % of Cu, 90 % of pentachlorophenol, and 79 % of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans that were initially detected in the soil. Sun et al. (2009) also demonstrated that simultaneous removal of PAHs and Cu from heavily contaminated soils was feasible by soil washing using an aqueous ethyelnediaminedisuccinate solution enhanced with ethyl lactate.

The ultimate goal of any remediation process must be not only to remove the contaminant from the polluted soil but also, most importantly, to restore soil health, i.e., its capacity to function (Epelde et al. 2009). Due to their high hydrophobicity and solid–water distribution ratio, often high initial concentrations, and aging processes of soil contamination, the extent and rate of PAH bioremediation are often limited (Talley et al. 2002; Yang et al. 2011). Thus, biodegradation of residual soil-bound PAHs through microbial catabolism may be needed as a follow-up procedure to fulfill the ultimate “cleanup” goal.

Cyclodextrins are nonhazardous and environmentally acceptable organic substances that have been shown to be biodegradable in soil and water after their introduction into an environmental system (Khodadoust et al. 2008). Because of their capacity to promote the release and microbial accessibility of contaminants in soils and solutions, cyclodextrins, such as methyl-β-cyclodextrin (MCD), have been the focus of several recent studies (Semple et al. 2007; Zhou and Zhu 2007; Stroud et al. 2009). MCD is a cyclic, bucket-shaped macromolecule with a hydrophilic exterior and a hydrophobic cavity, thereby increasing their aqueous solubility. MCD is also known to have low toxicity to bacteria and a relatively high water solubility (>500 g in 1,000 mL at 25 °C) (Petitgirard et al. 2009). Although it is known that MCD has the ability to enhance solubility and, thus, the removal of PAHs from soil, few studies have taken into account the coexistence of PAHs and metals in soil as well as the subsequent biodegradation of residual soil-bound PAHs facilitated with specific PAH-degrading bacterium.

Therefore, the aim of this study is to evaluate the utilization of MCD solution for the simultaneous soil washing of a soil contaminated with a mixture of PAHs and metals. The influence of certain factors (i.e., MCD concentration, washing temperature, ultrasonication, and cycles of successive washing) on the removal efficiency was examined. The subsequent biodegradation of residual soil-bound PAHs by inoculation with *Paracoccus* sp. strain HPD-2 was also determined. The effectiveness of the cleanup strategy was assessed using biological tests.

Materials and methods

Chemicals and media

PAH standards (purity >99.5 %, 1,000 ng μL^{-1} Nap, 1,000 ng μL^{-1} Ace, 1,000 ng μL^{-1} Acy, 100 ng μL^{-1} Flu, 50 ng μL^{-1} Phe, 50 ng μL^{-1} AnT, 50 ng μL^{-1} FluA, 50 ng μL^{-1} Pyr, 1 ng μL^{-1} BaA, 50 ng μL^{-1} Chry, 1 ng μL^{-1} B[b]F, 1 ng μL^{-1} B[k]F, 5 ng μL^{-1} B[a]P, 10 ng μL^{-1} DBA, 5 ng μL^{-1} B[ghi]P, and 10 ng μL^{-1} IP), aqueous metal standard solutions of Cd, Ni, Zn, Cr, and Pb (1,000 mg L^{-1}), and MCD (purity >98 %) were purchased from Sigma-Aldrich Co. LLC, USA. Hexane and acetonitrile (HPLC grade) were from Tedia Company, USA. Acetone, cyclohexane, and dichloromethane (analytical grade, Nanjing Chemical Reagent Co.) were distilled prior to use. Anhydrous sodium sulfate (Na_2SO_4) was heated at 200 °C for 4 h. Silica gel (100 mesh) was first activated at 130 °C for 2 h and, then, deactivated by adding deionized water (3 % of the silica gel weight), further homogenized, and equilibrated for 6 h before use. Distilled deionized water was obtained using a Millipore-Q purification system (Millipore, Bedford, MA, USA). Other chemical reagents were all of analytical grade. Minimal medium (MM) contained (per liter) 0.2 g $MgSO_4 \cdot 2H_2O$, 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 0.6 g Na_2HPO_4, 20 mg $MnSO_4 \cdot H_2O$, and 1 g NH_4NO_3. Nutrient agar medium contained 0.5 % peptone, 0.3 % yeast extract, 1.5 % agar, and 0.5 % NaCl (pH 7.0).

Soil preparation

All soil samples were taken from an abandoned coking plant located in the old city district of Beijing, China. This plant was in operation between the 1960s and the 2000s. Soil samples were collected from the surface to a depth of 2 m, where high concentration with PAHs and metals was detected. All samples were homogenized and ground to pass a 2-mm sieve. A portion of the sample was air-dried for 7 days at room temperature, and the remaining sample was kept moist and stored in sealed glass bottles at 4 °C prior to analysis. Physical–chemical analysis shows that the soil has a pH (in water) of 6.2 and contains 9.8 % sand, 53.3 % silt, 36.9 % clay, 2.2 % organic matter, 1.2 gkg^{-1} total N (C/N = 11:1), 63.6 mg kg^{-1} hydrolysable N, 0.43 gkg^{-1} total P, 78.7 mg kg^{-1} available P, and 11.3 gkg^{-1} total K on a dry weight basis. The sum of 16 PAHs was 135.2±7.3 mg kg^{-1} dry soil, with individual 3-, 4-, 5(+6)-ring PAH concentrations of 16.2±1.4, 54.4±2.0, and 64.6±1.4 mg kg^{-1}, respectively (Table 1). The total concentrations of Cd, Ni, Zn, Cr, and Pb were 11.7±0.8, 135.2±8.7,

Table 1 Soil PAHs concentrations obtained by Soxhlet extraction (in milligrams PAH per kilogram dry soil)

PAHs		Concentrations (mg PAH kg^{-1} dry soil)
Compounds	Acronym	
Acenaphthylene	APY	2.8±0.1
Acenaphthene	APE	0.6±0.1
Fluorene	FLE	3.6±0.2
Phenanthrene	PHE	7.8±0.3
Anthracene	ANT	1.3±0.5
Sum of 3-ring PAHs		16.2±1.4
Fluoranthene	FLT	20.4±0.3
Pyrene	PYR	13.6±0.9
Benzo[a]anthracene	BaA	6.8±0.3
Chrysene	CRY	13.6±1.3
Sum of 4-ring PAHs		54.4±2.0
Benzo[b]fluoranthene	BbF	7.1±0.2
Benzo[k]fluoranthene	BkF	16.2±1.7
Benzo[a]pyrene	BaP	10.8±0.7
Dibenzo[a,h]anthracene	DaA	17.3±0.3
Indeno[1,2,3]pyrene	I123P	11.4±1.2
Benzo[g,h,i]perylene	BghiP	2.0±0.1
Sum of 5(+6)-ring PAHs		64.6±1.4
Total PAHs		135.2±7.3

Values are means ± standard deviation of triplicate measurements

236.0±13.8, 310.3±13.4, and 236.0±9.8 mg kg^{-1} dry soil, respectively.

Soil washing

In a 2-L flask with a Teflon-lined cap, 100 g of contaminated soil was placed, to which 1 L of different washing solvents was added. Four treatments were tested, including (1) MCD + 25 °C, (2) MCD + 50 °C, (3) MCD + 25 °C + ultrasonication, and (4) MCD + 50 °C + ultrasonication. Each treatment was replicated four times. The operating concentrations of MCD solution (*w*/*v*) were 0, 50, 100, 150, 200, or 250 gL^{-1}. The flask was shaken at 25±2 or 50±2 °C at 100 rpm for 60 min. Ultrasonication was done after shaking by an ultrasonic homogenizer tuned to 35 kHz for 30 min (XL 2020 Sonic dismembrator, extended horn of 25×1.2 cm, Misonix, Farmingdale, NY). Soil was separated from supernatant by centrifugation at 3,000 rpm for 30 min before further extractions and analyses.

Since preliminary tests indicated that a single washing cycle would not be sufficient to extract most of the PAHs and metals from the test soil under the conditions, successive washings of five cycles were tested to evaluate removal efficiency. At the end of each washing process, the soil was analyzed as described in Section 1.5. Soils after successive washings were combined and air-dried for subsequent microcosm studies.

Biodegradation

Bacterial strain and culture conditions

Paracoccus sp. strain HPD-2 was isolated from a historically PAH-contaminated soil collected from Wuxi, Jiangsu Province, Eastern China (Teng et al. 2010b). This strain was screened for its ability to degrade high-molecular-weight (HMW) PAHs, including fluoranthene, pyrene, and benzo[a]pyrene (B[a]P). After inoculation into the MM medium containing B[a]P at 3.0 mg L^{-1} for 5 days, 89.7 % of the B[a]P was degraded by the bacterium. When this strain was grown with pyrene and fluoranthene at 50 mg L^{-1} for 7 days, 47 and 85 % of these were degraded, respectively. This strain may therefore have the potential to improve HMW PAH biodegradation. Strain HPD-2 was cultured on a slant of nutrient agar medium. After 3 days of incubation at 28 °C, the slant was used to inoculate two 500-mL Erlenmeyer flasks each containing 100 mL of liquid medium containing 3 g beef extract and 5 g peptone per liter. The flasks were incubated for 48 h at 28 °C on a rotary shaker at 200 rpm and produced cell suspensions of approximately 2×10^8 CFU L^{-1}. Cells were harvested by centrifugation at 5,000×g for 20 min, washed twice with autoclaved saline solution, and then resuspended in saline solution.

A nutrient solution (N, P) containing NH_4NO_3 and K_2HPO_4/KH_2PO_4 was prepared. This solution contained

47.5 g N L^{-1} and 2.80 g P L^{-1}; 500 mL of nutrient solution was combined with 50 mL of the above-prepared cell suspension to establish an inoculum population of 1.7 × 10^7 CFU L^{-1} to be used for the soil microcosm experiment.

Soil microcosms

Soil microcosms were set up using a series of beakers (upper diameter 11.5 cm, lower diameter 9 cm, height 18.5 cm) covered with tin foil, each containing 1,000 g of washed air-dried soil. Soil microcosms were inoculated with 80 mL strain inoculum (NI) prepared as above for bioaugumentation. Control microcosms were prepared with the addition of nutrient solution (CK). The final soil N/P ratio is 10:1. Soils in the microcosms were adjusted to 60 % water holding capacity (WHC) with sterilized DI water and maintained at this WHC during incubation by adjusting the weight of the microcosm every 3 days. Each treatment was replicated four times. The microcosms were incubated in a controlled climate chamber for 20 weeks at 28±0.5 °C. Every 2 weeks, approximately 20 g of soil samples was collected from each microcosm using a mini stainless steel soil drill. Each sample was divided into two parts, one was placed in a small plastic bag and stored at 4 °C for subsequent analysis of microbial activity while the other was freeze-dried and passed through a 60-mesh sieve prior to analysis for PAHs.

Extraction and analysis of PAH and metal

PAHs in soil samples were extracted using the Soxhlet extraction procedure (EPA method 3540c). Briefly, 5 g of freeze-dried sample was placed over a filter paper in a porous cellulose thimble (25×70 mm) which is inside a Soxhlet extractor. The extractor was then fitted to a 100-mL round bottom flask containing 60 mL dichloromethane, and the extraction was performed for 24 h. All extracts in the round bottom flasks were dried by rotary evaporation. The residues were dissolved in 2 mL cyclohexane, and 0.5 mL of the solute was transferred, purified with a silica gel column (8×220 mm) and washed with a mixture of hexane and dichloromethane (1:1). The first 1 mL of eluent was discarded because it contained nonpolar saturated hydrocarbons and was less retained than PAHs by silica gel. The second 2-mL aliquot of eluent was collected, dried by sparging with N_2 and then redissolved in 1 mL of acetonitrile for subsequent HPLC determination. Determination of 16 PAHs was carried out according to the method of Ni et al. (2008). Briefly, analysis was conducted on a Shimadzu Class-VP HPLC system (Shimadzu, Japan), with a fluorescence detector (RF-10AXL). A reversed phase column C18 (VPODS 150 4.6 mm I. D., particle size 5 mm), using a mobile phase of water and acetonitrile mixture (1:9, *v*/*v*) at a constant solvent flow rate of 0.5 mL min^{-1}, was used to separate the 16 PAHs. The excitation and emission wavelengths were set specifically for each individual PAH.

An external standard was used for the quantification of the 16 PAHs. The detection limit of the HPLC method for PAHs was in the range of 0.12–1.57 μg kg^{-1}. Method blanks (solvent) and spiked blanks (standards of EPA610 PAH mixture, LA 96245, Supelco, USA used to spike the soil) were extracted and analyzed by the methods described above. Recoveries and relative standard deviations for the 16 PAHs were in the ranges of 74–110 and 0.53–3.57 %, respectively. When blanks extracted under the same conditions were below detection limits, sample results are presented without recovery ratio correction.

The total concentrations of Cd, Ni, Zn, Cr, and Pb were determined by digesting 2 g of soil samples using 50 mL concentrated $HCL–HNO_3–HClO_4$ (4:2:1, *v*/*v*/*v*) and were quantified with a thermo flame atomic absorption spectrophotometer (Liu et al. 2011). For quality assurance, replicate samples, blanks, and a certified reference material were included in all analyses.

The percentage removal of PAH and metal was calculated as follows:

$$\text{Removal\%} = 100 \times (1 - \text{Ms}/\text{Mi}) \tag{1}$$

where Ms is the concentration of PAH or metal in each treatment and Mi is the initial PAH or metal concentration in the soil.

Physiological profiles of the soil microbial community

Physiological profiles of the soil microbial community were obtained as described by Yao et al. (2003). Briefly, 10 g of fresh soil was added to 100 mL of distilled water in a 250-mL flask and shaken for 10 min. Following tenfold serial dilutions, the 10^{-3} dilution was used to inoculate BIOLOG® ECOplates (BIOLOG, Hayward, CA). The plates were incubated at 25 °C, and microbial growth in each well was recorded as optical density (OD) at 590 nm with a plate reader at 12-h intervals. Microbial activity was expressed as average well-color development (AWCD) determined as follows:

$$\text{AWCD} = \sum OD_i/31 \tag{2}$$

where OD_i is the optical density of each well. The Shannon–Weaver index (*H*) was calculated using an OD of 0.25 as the threshold for a positive response (Garland 1996). The Shannon–Weaver index (*H*) and Simpson's diversity index (λ)

(Simpson 1949) were calculated as follows:

$$H = -\sum p_i Lnp_i \tag{3}$$

$$\lambda = \sum {p_i}^2 \tag{4}$$

where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates ($\sum OD_i$).

Statistical analysis

Statistical analysis was carried out using the SPSS 14.0 for Windows software package. Data were analyzed by two-way analysis of variance. Mean values were compared by least significant difference (LSD) at probability level ≤5 %.

Results and discussion

Removal of PAH from soil

Ex situ soil washing has been proposed as a strategy to enhance remediation of PAH-contaminated sites (Ahn et al. 2008; Pannu et al. 2004; Subramaniam et al. 2004). In the present study, all treatments showed significant removal of total PAHs, 3-ring PAHs, 4-ring PAHs, and 5(+6)-ring PAHs using the tested washing parameters ($p<0.05$) (Fig. 1). PAH removals were 57–79 % in treatments containing MCD but only 2–10 % in treatments without MCD. It has long been recognized that high hydrophobicity and initial concentration as well as aging processes promote adsorption of PAH to soil particles and therefore hinder their solubility for effective removal from soil. The obtained data are consistent with previous findings that MCD can facilitate solubilization of a nonpolar solute in water (Badr et al. 2004; Fava et al. 2003; Hanna et al. 2004).

Although PAH removal efficiency increased with increasing MCD concentrations from 25 to 100 gL^{-1}, a slight decrease of PAH removal efficiency was observed at 150 gL^{-1} and a significant reduction was detected from 200 to 250 gL^{-1} ($p<0.05$) (Fig. 1). This phenomenon may be caused by the chemical nature of MCD in aqueous phase. Viscosity of MCD solution increased with the increasing concentration (Allan et al. 2006; Allan et al. 2007). Among the processes controlling the removal, of particular importance in *ex situ* soil washing is desorption because it is often a rate-limiting factor for extraction (Kraaij et al. 2002; Leppanen et al. 2003). High viscosity of MCD solution would hamper the transfer of PAH from soil particle to aqueous phase. Therefore, 100 gL^{-1} was chosen as the MCD concentration to be used in the developed cleanup procedure.

Data in Fig.1 revealed that the PAH removal performance was affected by temperature and ultrasonication. The extent of PAH removal was greatly enhanced when relatively high temperatures were employed ($p<0.01$) (Table 2). When employed, the use of 100 gL^{-1} MCD at 50 °C, approximately 73.3±2.1 % of PAH was desorbed compared with 57.1±3.2 % PAH at 25 °C. This could be attributed to the fact that higher temperature led to a decrease in partition coefficients between contaminants and soil particles, thereby increased desorption rates of PAH from the solid to the aqueous phase. On the other hand, ultrasonication also played a positive role in desorption of PAH. The highest extraction of 66.5±2.4 % PAH was achieved when ultrasonication was introduced (Fig. 1). It was suggested that ultrasonication induced high fluid–solid shear stresses, which promoted mechanical detachment and removal of contaminants (Shrestha et al. 2009; Yin et al. 2004). Abrasion of surface cleaning and leaching of more deeply entrenched material were suggested as two basic mechanisms for acoustically enhanced soil washing (Mao et al. 2004; Mason 2006). Therefore, the combination of elevated temperature and ultrasonication in treatment of this study showed the highest PAH removal rate of 79.6±2.2 %. PAH removal was further significantly ($p<0.01$) enhanced by the addition of MCD at 100 gL^{-1}. Temperature and ultrasonication showed significant interactions (Ftemperature = 7,136.0, Fultrasonication = 1,973.2, Ftemperature × Fultrasonication = 109.0, $p<0.01$). The obtained results support previous findings that when two types of external energy were applied simultaneously, the decreased viscosity of fluid phase would lead to increased flow rate. As a result, the adsorbed contaminants were mobilized, cavitation was developed, and porosity and permeability were increased, which ultimately resulted in increased removal efficiency (Chung and Kamon 2005; Kim and Wang 2003).

Removal of metal from soil

In addition to PAH removal, the above-tested procedure also led to the removal of metals (Cd, Ni, Zn, Cr, and Pb) (Fig. 2). Similar to the removal efficacy of PAH, the total removal of metals was less than 5 % in the treatments without MCD addition and increased with increasing the concentration of MCD solution from 25 to 100 gL^{-1}, but it was followed by significant reductions ($p<0.05$) from 150 to 250 gL^{-1}. MCD possesses high aqueous solubility due to hydroxyl functional groups on their exterior but has a hydrophobic organic cavity in the interior which allows complexation of metals (Fava et al. 2003; Hanna et al. 2004). The presence of MCD may facilitate metal transfer from soil particles to aqueous phase. However, a high concentration of MCD could also hinder the desorption process. In addition, elevated temperature and ultrasonication further enhanced the desorption of contaminants. Therefore, the combination of these two types of external energy in

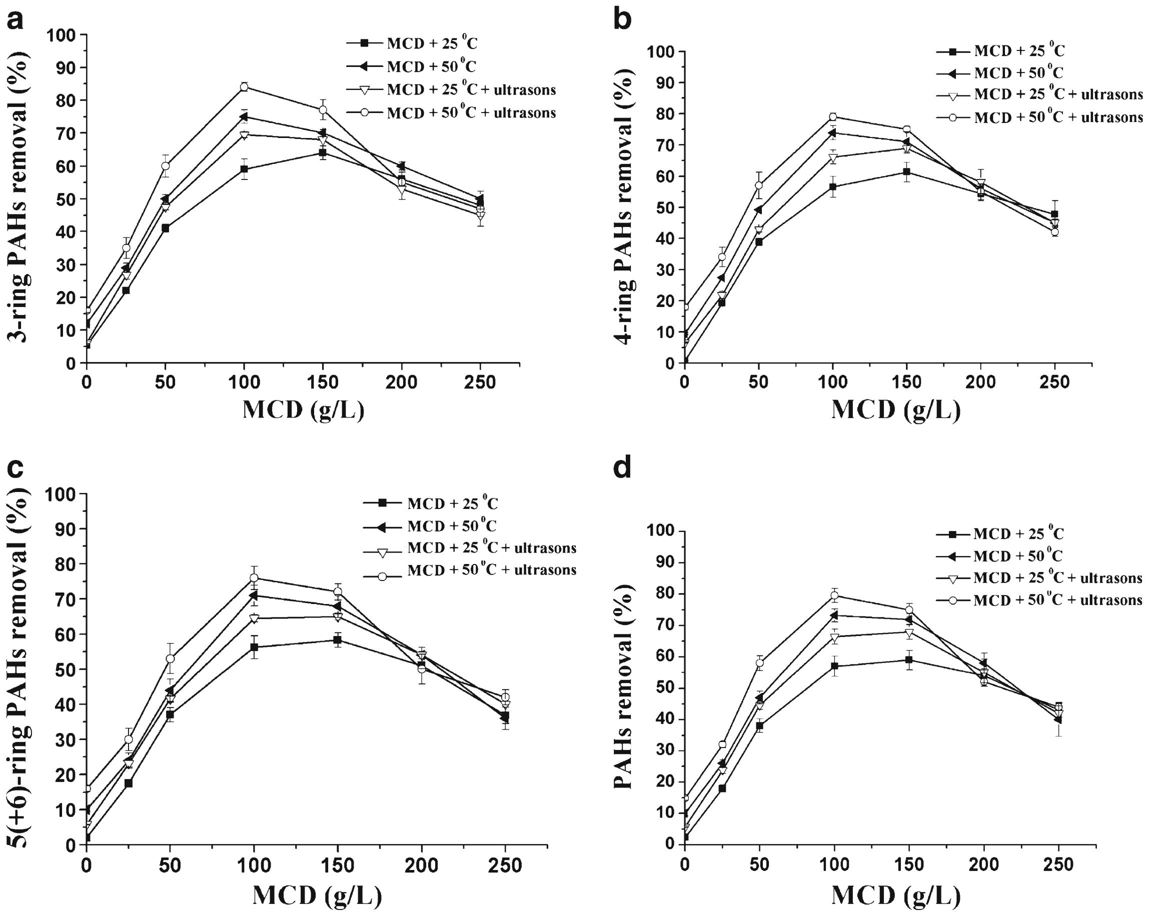

Fig. 1 Removal of **a** 3-, **b** 4-, **c** 5(+6)-ring, and **d** total PAHs in soil after successive washing under different conditions. Values are means ± SD of triplicate measurements

addition to 100 gL^{-1} MCD had significant effects on metal removal, (Table 3) which resulted in the greatest extraction capacity for Cd, Ni, Zn, Cr, and Pb which was 51.4±1.3, 63.6 ±2.6, 72.3±1.3, 68.5±3.3, and 45.5±2.4 %, respectively. Of the four treatments evaluated, the extraction capacity was in the following order: MCD + 50 °C + ultrasonication > MCD + 50 °C > MCD + 25 °C + ultrasonication > MCD + 25 °C.

Successive washing of PAH and metal from soil

By comparing the potential extraction efficiency of PAH and metals simultaneously, the treatment which involves elevated temperature and ultrasonication at 100 gL^{-1} MCD was selected as the better washing parameters for further application. Successive five washing cycles were tested (Table 4). The cumulative removal of PAH and metal increased with increasing washing cycles as expected. Approximately 94.3±4.0 % of PAH, 76.0± 6.3 % of Cd, 82.6±5.9 % of Ni, 95.9±6.5 % of Zn, 87.8±0.8 % of Cr, and 73.5±1.9 % of Pb were extracted after five extraction cycles. Findings reported in literatures suggested high percentages of contaminants being desorbed in soils with relatively low organic matter contents (Khodadoust et al. 2008; Ehsan et al. 2007). The relatively high extraction efficiency achieved in this study could be due to the relatively low organic

Table 2 Two-way ANOVA analysis and the effect of temperature and ultrasonication on the removal of PAHs

Factor	Total PAH		3-ring PAH		4-ring PAH		5(+6)-ring PAH	
	F	p	F	p	F	p	F	p
Temperature	7,136.0	0.000	8,283.4	0.000	5,586.8	0.000	3,365.2	0.000
Ultrasonication	1,973.2	0.000	3,191.8	0.000	1,340.9	0.000	829.9	0.000
Temperature × ultrasonication	109.0	0.000	5.4	0.048	154.4	0.000	35.1	0.000

matter content in the tested soil (2.2 %). In fact, PAH and metals desorbed relatively quickly during the first three washing cycles (Table 4). Soil washing of more than three cycles made limited contributions to desorption of contaminants. The impact of washing cycles on desorption of contaminants varied among metals tested. The obtained results are consistent with our hypothesis that *ex situ* soil washing is a balanced redistribution of contaminants between solid and liquid phases. Because the high initial concentrations of contaminants occurred in the soil over 40 years ago, it is possible that long period of interactions led to increased adsorption of the contaminants. Once the transfer of contaminants achieved a steady state during successive washing cycles, the redistribution of contaminants reached a relative equilibrium. To be cost effective, successive washing of three cycles was chosen in the developed procedure.

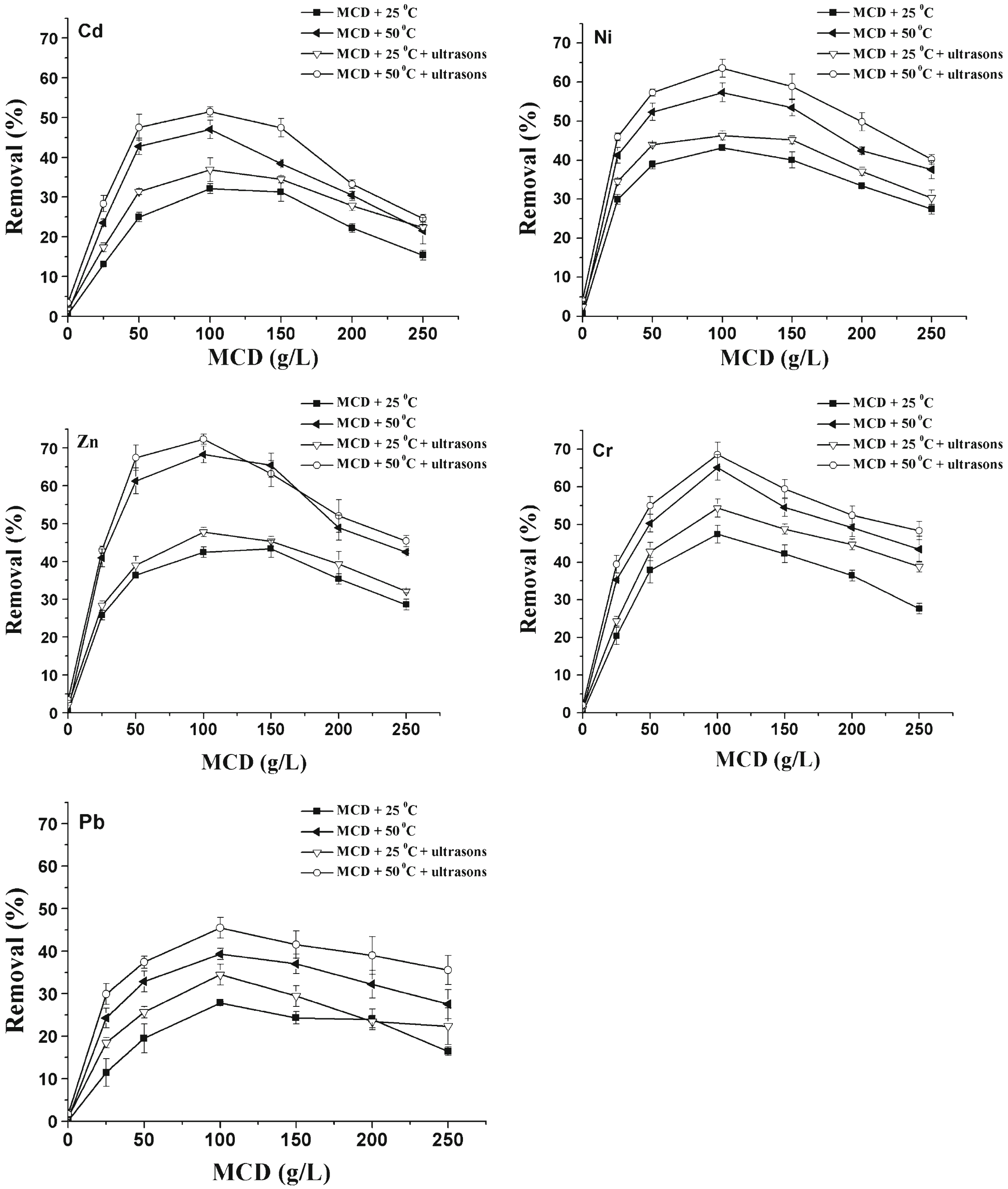

Fig. 2 Removal of Cd, Ni, Zn, Cr, and Pb in soil after successive washing under different conditions. Values are means ± SD of triplicate measurements

Table 3 Two-way ANOVA analysis and the effect of temperature and ultrasonication on the removal of metals

Factor	Cd		Ni		Zn		Cr		Pb	
	F	p	F	p	F	p	F	p	F	p
Temperature	4,359.4	0.000	668.0	0.000	2,078.0	0.000	1,657.7	0.000	9,990.9	0.000
Ultrasonication	4,143.0	0.000	86.3	0.000	711.8	0.000	169.2	0.000	3,244.0	0.000
Temperature × ultrasonication	62.9	0.000	23.9	0.001	5.4	0.049	22.4	0.000	5.6	0.045

Besides, though successive washings efficiently removed PAH and metals from the soil in the laboratory scale, further studies involving different soil types and contaminants under field conditions will be required to elucidate the potential applicability of this strategy. For instance, washing solution will not be eliminated so strongly in situ, and the availability, mobility, and biodegradability of the pollutants may be higher and give different results. Also, systematic research on the influencing factors (i.e., soil/solution ratio, washing cycles, mixing speed, and so on) in relation to removal efficiency of contaminants should be paid further attention.

Biodegradation of residual soil-bound PAHs

Based on microcosm evaluations, inoculation of PAH-degrading bacterium (*Paracoccus* sp. strain HPD-2) with nutrient addition promoted biodegradation of residual soil-bound PAHs (Fig. 3). Effect of bacterial and nutrient addition on metals concentration was not detected because most metals are extremely difficult to be biodegraded but may change metal speciation and bioavailability. It is generally accepted that a low level of bioavailability is one of the most important factors leading to the slow biodegradation of hydrophobic organic compounds in soils. In particular, after three cycles of soil washing, the residual PAHs are bound tightly with soil organic and inorganic components, resulting in an extremely low level of bioavailability (Kraaij et al. 2002). The PAH-degrading bacteria need to secrete various enzymes and regulate physiological metabolism process to adapt to the microcosm (Ding et al. 2008; Epelde et al. 2009). Therefore, the biodegradation process displayed a lag phase in the first six weeks (Fig. 3a), with no more than 8.0±1.3 % of the PAH being degraded. However, the PAH biodegradation rate increased significantly in the following 14 weeks ($p<0.05$). The maximum biodegradation percentage for total PAH, 3-ring PAHs, 4-ring PAHs, and 5(+6)-ring PAHs were 33.9±2.1, 45.3±2.3, 36.4±1.0, and 31.8±2.4 %, respectively. Twenty weeks of biodegradation resulted in a significant decrease in the concentration of total PAHs, 4-ring PAHs, and 5(+6)-ring PAHs in the soil microcosms (Fig. 3b).

Assessment of soil microbial community

To assess the influence of the developed cleanup strategy on soil microbial community, BIOLOG Ecoplate assay was conducted to reveal soil culturable microbial functional diversity (Fig. 4 and Table 5).

The AWCD reflects the sole-carbon-source utilization ability of the soil bacterial community and a measure of

Table 4 Residual concentrations of PAH and metal after successive soil washing cycles (in milligrams per kilogram)

PAH/metal	Background con.	Washing cycles				
		1st Residual con.	2nd Residual con.	3rd Residual con.	4th Residual con.	5th Residual con.
Total PAHs	135.2±7.3 a	28.1±2.8 b	16.3±5.5 c	9.9±0.7 d	9.2±4.2 c, d	7.7±5.4 c, d
3-ring PAHs	16.2±1.4 a	2.6±0.2 b	1.1±0.9 c	0.5±0.5 c	0.2±0.4 c	0.2±0.7 c
4-ring PAHs	54.4±2.0 a	11.4±0.7 b	7.7±2.2 c	3.7±2.2 c	3.3±1.2 c	2.7±0.5 c
5(+6)-ring PAHs	64.6±1.4 a	15.5±2.1 b	7.5±3.7 c	5.7±2.1 c	5.6±0.8 c	4.8±1.1 c
Cd	11.7±0.8 a	5.7±0.2 b	4.3±0.5 c	3.3±0.4 d	3.0±1.0 d	2.8±0.7 d
Ni	131.5±4.9 a	47.9±3.1 b	36.1±6.3 c	28.5±4.9 c	26.5±5.4 c	22.9±7.8 c
Zn	236.0±9.8 a	65.3±3.1 b	39.0±10.0 c	17.8±3.1 d	13.2±5.7 d	9.7±15.3 d
Cr	310.3±16.5 a	97.7±10.2 b	68.4±14.7 c	48.4±15.8 c, d	38.6±21.0 c, d	37.9±2.4 d
Pb	265.7±12.3 a	144.5±6.5 b	115.3±10.3 c	85.4±6.5 d	75.6±18.8d e	70.3±5.0 e

Values are means ± standard deviation of triplicate measurements. Mean values with the same letter are not significantly different among treatments based on LSD tests at $p\leq0.05$

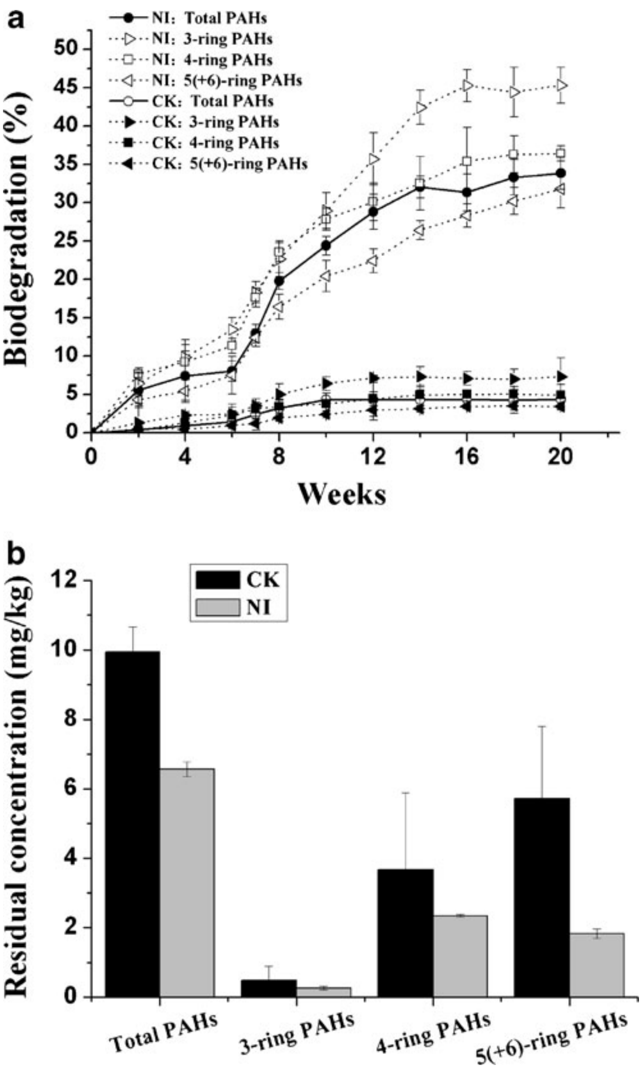

Fig. 3 Biodegradation rate **a** and residual concentrations of 3-, 4-, 5 (+6)-ring, and total PAHs **b** in soil after 20 weeks of biodegradation. CK, control without inoculation of *Paracoccus* sp. strain HPD-2; *NI*, amended with nutrients and inoculated with *Paracoccus* sp. strain HPD-2. Values are means ± SD of triplicate measurements

soil bacteria activity (Garland 1996). Shannon–Weaver index is a measure of actual richness and evenness of the bacterial population, while Simpson index is used to quantify the number of species as well as the relative abundance of each species (Garland 1996; Simpson 1949). Washing followed by 20 weeks of biodegradation led to increased AWCD as well as Shannon–Weaver and Simpson indices. These results confirmed the findings by Teng et al. (2010a, b) in that 28 days of bioremediation with *Paracoccus* sp. strain HPD-2 inoculation and nutrient addition led to marked enhancement of microbial functional diversity. It can be concluded that in spite of the addition of degrading bacteria and nutrient additional reduction of a few percents, the combination of PAH-degrading bacterial inoculation and nutrient addition resulted in at least partial restoration of the microbiological functioning in the contaminated soil.

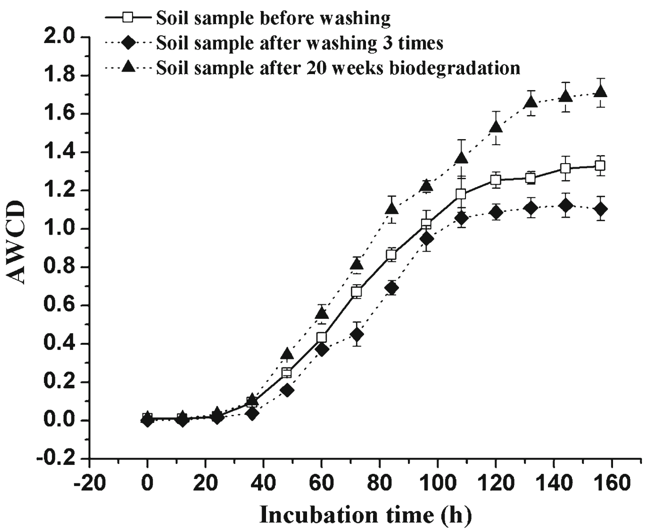

Fig. 4 Average well color development (AWCD) for soil samples from different treatments. Values are means ± SD of triplicate measurements

Conclusions

Successive washing of three cycles using the combined treatment of elevated temperature and ultrasonication at 100 gL^{-1} MCD was shown to be effective in removing PAH and metal contaminants in soil. The subsequent bioremediation with *Paracoccus* sp. strain HPD-2 inoculation and nutrient addition strengthened the biodegradation of residual soil-bound PAH. The combined cleanup strategy is an environmentally friendly technology, evidenced by the increased microbial functional diversity in soil after completing the treatment.

Table 5 Sole-carbon-source utilization activity and functional diversity of soil microbial community after different treatments

Treatment	AWCD	Shannon–Weaver index	Simpson index
CK	1.3±0.1 b	3.3±0.0 b	0.9±0.0 b
TW	1.1±0.1 c	2.9±0.1 c	0.9±0.0 b
BE	1.7±0.1 a	3.5±0.1 a	1.0±0.0 a

Values are means ± standard deviation of triplicate measurements. Mean values with the same letter are not significantly different among treatments based on LSD tests at $p \leq 5$ %

TW after three successive washings, BE after 20 weeks of biodegradation

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