— **Regular Article** —

Interactive Effect of Biosurfactant and Microorganism to Enhance Phytoremediation for Removal of Aged Polycyclic Aromatic Hydrocarbons from Contaminated Soils

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To improve phytoremediation efficiency of polycyclic aromatic hydrocarbons (PAHs), pot experiment was conducted to introduce arbuscular mycorrhizal fungi, aromatic hyrocarbon degrading bacteria (ARDB), and rhamnolipids into phytoremediation system. Alfalfa biomasses, the number of heterotrophs and ARDB, dehydrogenase activity, polyphenol oxidase activity and residual PAHs concentration were determined after 90 days of alfalfa growth. The results indicated that the average removal efficiency of total PAHs by multi-technique phytoremediation system reached to 60.48%, which was 251.83% greater than that of phytoremediation itself (17.19%). Importantly, the multi-process system was capable of removing most of the high molecular weight PAHs (HMW-PAHs) from soil, the highest average removal percentage of HMW-PAHs, such as fluoranthene, pyrene and benzo[*a*]pyrene were 89.39%, 88.36% and 92.31%, respectively. A sharp increase in the size of the heterotrophic and aromatic hyrocarbon degrading microbial populations was observed, which resulted in increase of soil dehydrogenase and polyphenol oxidase activities. The key elements for successful phytoremediation were the use of biosurfactant that increase bioavaliable of PAHs in soil, and inoculation of microorganisms (arbuscular mycorrhizal fungi and ARDB) that accelerate plant growth and increase PAHs removal from heavily contaminated soils. The synergistic use of these approaches resulted in rapid and massive biomass accumulation of plant tissue in contaminated soil, putative providing more active metabolic process, and led to more rapid and more complete removal of PAHs.

Key words —— phytoremediation, polycyclic aromatic hydrocarbon, biosurfactant, arbuscular mycorrhizal fungi, aromatic hyrocarbon degrading bacteria

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of the most widespread organic pollutants, distributed widely in soil, sediment and oily substances. Nowadays, their fates in the environment had attracted considerable concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic.[1)] During the last 20 years, various methods have been introduced to remediate PAHs-contaminated soils, including physical, chemical, and biological methods. Physical removal of contaminated soil and washing of those soils with solvents are relatively fast but energy-cost and highly-expensive. Chemical oxidation have a significant influence on soil physical-chemical properties and microbial community.[2)] Bioremediation, including phytoremediation, microbe remediation and their interactive remediation, provide an alternative way to remove contaminants from the soils.

Phytoremediation, the use of vegetation for *in situ* treatment of contaminated soils and sediments, is a more attractive and cost-effective alternative than those traditional approaches. Some plant

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species, *e.g.* alfalfa, ryegrass and clover, usually showed a good phytoremediation performance due to their extensive root systems as well as their high adaptability and resistance to survive environmental stress.[3,4] Plant roots can enhance the dissipation of organic pollutants in soils, and it was mainly attributed to the co-metabolism, in which the degradation of organic pollutants was improved by root exudates for activating microbial community in the rhizosphere.[5,6] However, the efficiency of phytoremediation in aged PAHs-contaminated soils is always limited, becaused the residual components of PAHs in aged soil are with poorer water solubility and more strongly adsorbed by soil particles, which led to a lower biodegradation compared with fresh PAHs-contaminated soils.[7–9]

To the above-mentioned question, it may be possible to facilitate phytoremediation efficiency by introducing surfactant to improve desorption and bioavailability of the compounds, resulting in enhanced biodegradation of aged PAHs in soils. Previous studies have shown that the application of chemical surfactants (such as nonionic-surfactant Tween 80) could increase both aqueous solubility as well as desorption of PAHs in soil-aqueous systems.[10] Recently, biosurfactants, *e.g.* rhamnolipids, had caused more attention due to the environmental compatibility of these compounds.[11–13] Other than having no phytotoxicity, biosurfactants could even promote plant growth with a certain concentration range.[14] Therefore, the combination of biosurfactants with phytoremediation might supply an effective approach to remediate aged PAHs-contaminated soils.

In most situation, microorganisms in toxic organic pollutant-contaminated soil might be depressed, thus the amount and activity of microbes often be reduced.[15] Since the presence of active metabolic degraders is the key to successful bioremediation,[16] inoculation with microoganisms that have the capability to degrade PAHs is also a recommended practice for remediation of aged PAHs-contaminated soils.[17–19] In addition, the potential role of arbuscular mycorrhiza (AM) fungi in phytoremeidation of contaminated soils is becoming evident although it needs to completely understand the ecological complexities of the plant-microbe-soil interaction.[20] Increased degradation of PAHs in mycorrhizosphere has been observed in pot experiments with inoculation of *Glomus mossease*,[21] Wu *et al.* found arbuscular mycorrhizal alfalfa and a non-ionic surfactant Triton X-100 could consistently promote phenanthrene dissipation in the soil.[22] There are many reports about application of AM fungi, aromatic hydrocarbon degrading bacteria (ARDB), and rhamnolipids alone to remove PAHs from contaminated soils, but very little information is available for their interactive effects on enhancement of phytoremediation in aged PAHs contaminated soil.

In the present study, a pot experiment was conducted in aged PAHs-contaninated arable soil, by comparing the effects of AM fungi, ARDB, and rhamnolipids applied alone or in combination on phytoremediation efficiency of aged PAHs-contaminated soil using alfalfa, the objectives of this paper were to evaluate interactions of plant, AM fungi, ARDB, and rhamnolipids in the specific remediation system and to find a high efficient phytoremediation approach of aged PAHs-contaminated soil.

MATERIALS AND METHODS

Soil —— Soil samples were collected from a farmfield located in Wuxi, Jiangsu Province, eastern China (30°36′14″N, 120°28′33″E). The farmland was 1 km far from a foundry and had severely contaminated with PAHs due to a long history of sewage irrigation. Soil samples were collected in July 2008, the topsoil (0–20 cm) from the site was collected by a stainless steel auger, and placed in airtight bags and transported to the laboratory. Subsequently, the samples were kept at 4°C until the phytoremediation experiments. The soil physical and chemical properties are presented as following; C_{org} of 19.2 $g \cdot kg^{-1}$, total N of 1.0 $g \cdot kg^{-1}$, total P of 0.5 $g \cdot kg^{-1}$, total K of 14.2 $g \cdot kg^{-1}$, CEC of 21.5 $cmol \cdot kg^{-1}$ and pH of 6.4. Organic carbon content was determined by the Walkley-Black method using $FeSO_4$ for titration. Total N content was analyzed with Kjeldahl Digestion Method. Soil pH was determined in a 1 : 5 ratio of soil to water. The initial concentration of 15 PAHs components are shown in Table 1.

Experimental Design —— The soil were carefully collected, homogenized, air-dried and passed through a 2 mm sieve. Eight treatments were designed with four replicates of each treatments as Table 2.

Alfalfa (*Medicago sativa* L.) was selected for the experiment because its extensive, widely branched root system providing a large root surface

Table 1. Treatments of Experimental Design

	Alfalfa	AM fungi	ARDB[a)]	Rhamnolipids
CK	+[b)]			
AM	+	+		
DB	+		+	
RH	+			+
AM + RH	+			+
AM+DB	+	+	+	
DB+RH	+		+	+
AM+DB+RH	+	+	+	

a) ARDB, aromatic hydrocarbon degrading bacteria; *b*) +, represented to corresponding treatments.

Table 2. The Limits of Detection or Quantitation of All PAHs ($ng \cdot ml^{-1}$)

PAHs	limits of detection	limit of quantification
NAP	0.26	0.86
ACN	0.06	0.21
FLE	0.02	0.05
PHE	0.05	0.17
ANT	0.02	0.05
FLA	0.21	0.71
PYR	0.12	0.39
CHR	0.03	0.09
BAA	0.03	0.09
BBF	0.06	0.18
BKF	0.01	0.03
BAP	0.01	0.03
BGP	0.05	0.16
IPY	0.10	0.32
DBP	0.03	0.10

NAP, naphthalene; ACN, acenaphthene; FLE, fluorene; PHE, phenanthrene; ANT, anthracene; FLA, fluoranthene; PYR, pyrene; CHR, chrysene; BAA, Benzo[*a*]anthracene; BBF, benzo[*b*]fluoranthene; BKF, benzo[*k*]fluoranthene; BAP, benzo[*a*]pyrene; BGP, benzo[*g*,*h*,*i*]perylene; IPY, indeno[1,2,3-*cd*]pyrene; DBP, dibenzo-[*a*,*h*]pyrene.

for the growth of microbial population.

Rhamnolipids was produced by fermentation of *Pseudomonas aeruginosa* (provided by Nanjing Agriculatral University). It is the mixture of the monorhamnolipid and dirhamnolipid with a concentration of $4.3\,g \cdot l^{-1}$. The concentrationof rhamnolipids amended to the soil was $150\,mg \cdot kg^{-1}$ on a dry basis.

The mycorrhizal fungi used in this experiment is *Glomus caledonium*, which was propogated by clover growth in sterilized sand : soil (1 : 3 w/w) mixture. The air-dried inoculum, containing AM hyphae, spores, and root pieces, was added to the amount of 4% (dry basis) by placing the inoculum in the middle layer of the pots.

The aromatic hydrocarbon degrading bacterial inoculum, which mainly contained two strains *Flavobacterium* sp. and *Bacillus* sp., were cultivated in tryptic soy broth[23)] at 37°C for about 2 days. About 10^7 cells were inoculated to one gram of soils on a dry basis.

The pots (12 cm in diameter, 18 cm in height) with 3 kg of dry soils were placed into the greenhouse. Alfalfa seeds were sterilized in 10% (v/v) H_2O_2 for 20 min and washed three times with distilled water. Fifteen seeds were sown in four replicates and the seedlings were thinned to 10 seedlings after germination. The growth conditions were: 25°C during a 12-hr day and at 20°C during a 12-hr night. The light intensity was 4500–7300 lux and the soils were watered daily and adjusted to approximately 50% of the water holding capacity during plants growth.

Sampling and Analysis —— The soils were collected by soil auger from the surface (0–20 cm) in vicinity of the root 90 days after germination. The soil and the root were separated by repeated vigorous rubbing and shaking the root system. Soils was manually crushed and homogenized, then passed through the sieve (2 mm). The subsamples were stored at 4°C for assessing soil enzymes and enumeration of soil microorganisms. Shoots and roots were harvested, respectively. Root fragments were collected by sieving the soil and adding them to the root samples. Roots were first carefully washed with tap water to remove any adhering soil particles. Then shoots and roots were freeze-dried and weighed. For evaluating mycorrhizal colonization, about 1 g fresh root was cut into 1 cm segments and stained with trypan blue as described by Koske and Gemma.[24)] Mycorrhizal colonization was determined by the grid line intersect method according to Leyval and Binet.[25)] Soil dehydrogenase acitivity (DHA) was determined by the reduction of triphenyltetrazolium chlorid (TTC) to triphenylformazan (TPF) as described by Tabatabai.[26)] Soil polyphenol oxidase activity was determined by standard colorimetric methods.[27)] Total number of heterotrophs in soils were counted using tryptone soy broth plate. PAHs degraders were enumerated using the most-probable-number (MPN) method with five replicates per dilution.[28)] A mixture phenanthrene ($10\,g \cdot l^{-1}$), anthracene ($1\,g \cdot l^{-1}$), fluorene ($1\,g \cdot l^{-1}$) and fluoranthene ($1\,g \cdot l^{-1}$) was supplied as the sole carbon source to a mineral medium.[28)] Serially diluted soil solution, ranging from 10^{-3} to 10^{-6}, were performed, inoculated into

Table 3. Plant Growth and Mycorrhizal Colonization in Different Treatments

Treatments	Root dry weight g/pot	Shoot dry weight g/pot	Mycorrhizal colonization %
Control	2.17 ± 0.30a[a)]	2.96 ± 0.35a	6.50 ± 3.54a
AM	2.24 ± 0.62ab	3.10 ± 0.65a	17.00 ± 3.00b
DB	2.75 ± 0.39ab	3.65 ± 0.42abc	12.67 ± 3.51a
RH	2.33 ± 0.62ab	3.31 ± 0.79ab	8.00 ± 2.00a
AM+DB	3.23 ± 0.48b	4.77 ± 0.74c	18.00 ± 6.08b
AM+RH	3.20 ± 0.30ab	3.55 ± 0.31ab	16.50 ± 3.19b
DB+RH	2.98 ± 0.69ab	3.74 ± 0.43abc	12.33 ± 2.08ab
AM+DB+RH	3.24 ± 0.53b	4.26 ± 0.27bc	11.67 ± 0.58ab
Significance of			
AM	N.S.[b)]	N.S.	*
DB	N.S.	N.S.	N.S.
RH	N.S.	N.S.	N.S.
AM×RH	N.S.	N.S.	*
AM×DB	*	*	*
RH×DB	N.S.	N.S.	N.S.
AM×DB×RH	*	*	N.S.

a) Data are means ± S.D. ($n = 4$). Values within the same column not followed by the same letter differ significantly ($p < 0.05$). *b*) N.S., not significant.

the medium and incubated at 28°C in the darkness. After 3 weeks, the medium turned yellow or brown were treated as positive.

Another subunit of soil samples were stored at −20°C for PAHs analysis. Five gram frozen dried samples were extracted with 60 ml dichloromethane in a Soxhlet apparatus for 24 hr. Extracts were then concentrated using a rotary evaporator and purified with chromatography column filled with activated silica gel. Purified extracts (10 µl) were analyzed using HPLC (Waters, Milford, Massachusetts, U.S.A.), which was fitted with a PAHs special column (particle size 5 µm, C18 covered, 250 mm × 4.6 mm Inner Diameter (ID), Waters) and a guard column packed with the same material (Waters). A mobile phase acetonitrile/water gradient was used. The gradient started at ratio of acetonitrile/water 6 : 4 (v/v) at 0–12 min and 1 : 0 at 12–25 min, 6 : 4 (v/v) at 25–45 min. Separation was performed at 30°C and the flow rate of 1.0 ml·min^{-1}. A fluorescence detector with changing wavelenths (Waters 2475) was used for PAHs analysis. Excitation/emission wavelenths were 215/330 nm from 0.0–8.5 min, 290/335 from 8.5–11.4 min, 240–375 nm from 11.4–16.7 min, 235–420 nm from 16.7–22.3 min. Individual PAHs were identified by the retention time according to PAHs standards. The limits of detection or quantitation of all PAHs were shown in Table 2.

Statistical Analysis —— Data were subjected to three-way analysis of variance using SPSS version 13.0 software package to determine the significance of AM inoculation PAHs-degrading bacteria inoculation with or without rhamnolipids addition as sources of variation. Comparisons of means was made by calculation of least significant difference (LSD) test at the 5% level.

RESULTS

Plant Biomass and Mycorrhizal Root Colonization

The means of biomasses of plants in different treatments varied from 2.17 to 3.24 g·pot^{-1} in root dry weight and from 2.96 to 4.77 g·pot^{-1} in shoot dry weight, respectively (Table 3). Single application of AM fungi, PAHs-degrading bacteria (DB), and rhamnolipids slightly increased shoot and root dry weight, but without significant difference. However, the treatments "AM+DB" and "AM+DB+RH" (RH: rhamnolipids), significantly improved the plant biomass, with 43.9% and 61.2% greater in shoot dry weight, and 48.9% and 49.3% greater in root dry weight.

The mean proportion of mycorrhizal colonization, which ranged from 6.5% to 18.0% was only significantly ($p < 0.05$) affected by AM fungi inoculation. Irrespective of rhamnolipid addition or ARDB inoculation, root colonization rates were not much higher in inoculated treatment than that in uninoculated treatments. The indirect effect of RH

Table 4. PAHs Concentration of Different Treatments in Soils (mg·kg^{-1} soil)

PAHs[a)]	Intial concentration	Residual concentration			
		CK	AM	DB	RH
NAP	N.D.[b)]	N.D.	N.D.	N.D.	N.D.
ACN	N.D.	N.D.	N.D.	N.D.	N.D.
FLE	0.13 ± 0.02a[c)]	0.03 ± 0b	0.02 ± 0.01b	0.02 ± 0b	0.04 ± 0.01b
PHE	0.82 ± 0.18a	0.43 ± 0.06b	0.32 ± 0.02bc	0.20 ± 0.03cd	0.53 ± 0.14b
ANT	0.03 ± 0a	N.D.	N.D.	N.D.	N.D.
FLA	2.45 ± 0.01a	1.89 ± 0.16a	1.17 ± 0.01ab	1.60 ± 0.03ab	1.64 ± 0.35a
PYR	3.18 ± 0.73a	2.95 ± 0.12a	1.09 ± 0.11bcd	0.70 ± 0.05bc	2.72 ± 0.33b
CHR	0.73 ± 0.19a	0.71 ± 0.02a	0.64 ± 0.09ab	0.55 ± 0.13ab	0.61 ± 0.14ab
BAA	0.98 ± 0.23a	0.87 ± 0.13a	0.79 ± 0.15ab	0.69 ± 0.22abc	0.71 ± 0.22ab
BBF	1.27 ± 0.23a	1.11 ± 0.04a	0.68 ± 0.01abc	0.83 ± 0.22abc	0.92 ± 0.27ab
BKF	0.56 ± 0.01a	0.48 ± 0.02a	0.32 ± 0abc	0.34 ± 0.14abc	0.37 ± 0.15ab
BAP	0.65 ± 0.07a	0.52 ± 0.04b	0.43 ± 0.06bc	0.38 ± 0.07bc	0.45 ± 0.09b
BGP	0.19 ± 0.06a	0.15 ± 0.04ab	0.07 ± 0.01bc	0.10 ± 0.04bc	0.06 ± 0.01bc
IPY	0.61 ± 0.08a	0.46 ± 0.06ab	0.41 ± 0.01bc	0.40 ± 0.03bc	0.33 ± 0.07bc
DBP	1.23 ± 0.31a	1.19 ± 0.21a	0.83 ± 0.06ab	0.93 ± 0.15ab	0.85 ± 0.19bc
Total	12.85 ± 0.21a	10.01 ± 0.45a	6.78 ± 0.29bc	6.41 ± 0.28bc	9.41 ± 0.48ab
PAHs[a)]	Intial concentration	Residual concentration			
		AM+DB	AM+RH	DB+RH	AM+DB+RH
NAP	N.D.[b)]	N.D.	N.D.	N.D.	N.D.
ACN	N.D.	N.D.	N.D.	N.D.	N.D.
FLE	0.13 ± 0.02a[c)]	0.01 ± 0b	0.03 ± 0.01b	0.05 ± 0.02b	0.03 ± 0.01b
PHE	0.82 ± 0.18a	0.11 ± 0.01d	0.44 ± 0.06b	0.39 ± 0.03bc	0.17 ± 0.02d
ANT	0.03 ± 0a	N.D.	N.D.	N.D.	N.D.
FLA	2.45 ± 0.01a	1.30 ± 0.11ab	1.48 ± 0ab	1.34 ± 0.37ab	0.26 ± 0.12b
PYR	3.18 ± 0.73a	0.53 ± 0.10c	1.09 ± 0.12bcd	0.91 ± 0.03cd	0.37 ± 0.03d
CHR	0.73 ± 0.19a	0.39 ± 0.14ab	0.49 ± 0.07ab	0.40 ± 0.04ab	0.14 ± 0.01b
BAA	0.98 ± 0.23a	0.48 ± 0.09bc	0.73 ± 0.03abc	0.63 ± 0.21abc	0.14 ± 0.02c
BBF	1.27 ± 0.23a	0.22 ± 0.08bc	0.79 ± 0.07abc	0.69 ± 0.02abc	0.10 ± 0.02c
BKF	0.56 ± 0.01a	0.24 ± 0.10bc	0.37 ± 0.02abc	0.31 ± 0.03abc	0.05 ± 0.01c
BAP	0.65 ± 0.07a	0.30 ± 0.04bc	0.35 ± 0.02bc	0.28 ± 0.05bc	0.05 ± 0.01c
BGP	0.19 ± 0.06a	0.04 ± 0bc	0.08 ± 0.01bc	0.08 ± 0.01bc	0.06 ± 0.01bc
IPY	0.61 ± 0.08a	0.39 ± 0.02bc	0.40 ± 0.03bc	0.33 ± 0.03bc	0.32 ± 0.02bc
DBP	1.23 ± 0.31a	0.68 ± 0.25ab	0.98 ± 0.08ab	0.80 ± 0.01ab	0.46 ± 0.02b
Total	12.85 ± 0.21a	5.37 ± 0.09c	7.21 ± 0.53bc	6.69 ± 0.19bc	4.46 ± 0.26c

a) NAP, naphthalene; ACN, acenaphthene; FLE, fluorene; PHE, phenanthrene; ANT, anthracene; FLA, fluoranthene; PYR, pyrene; CHR, chrysene; BAA, Benzo[*a*]anthracene; BBF, benzo[*b*]fluoranthene; BKF, benzo[*k*]fluoranthene; BAP, benzo[*a*]pyrene; BGP, benzo[*g*,*h*,*i*]perylene; IPY, indeno[1,2,3-*cd*]pyrene; DBP, dibenzo[*a*,*h*]pyrene. *b*) N.D., not detected. *c*) Data are means ± S.D. ($n = 4$). Values within the same row not followed by the same letter differ significantly ($p < 0.05$).

addition on the PAHs desorption from soil particle, increase concentration of PAHs have a toxic effect on the plant growth. Although without significant difference, inoculation of ARDB could increase the indigenuous mycorrhizal colonization, compared with control and "RH." In particular, mycorrhizal colonization in treatment "AM+DB+RH" was slightly higher than the control, but lower than that of "AM."

Dissipation of PAHs in Soils

15 Environmental Protection Agency (EPA)-PAHs concentration of contaminated soil initially 12.85 mg·kg^{-1} (Table 4), high molecular weight PAHs (HMW-PAHs), refering to the PAHs with more than three rings (4, 5, and 6 rings PAHs), accounted for 92.48% of total PAHs, while the low molecular weight PAHs (LMW-PAHs) refering to the PAHs with 2 and 3 rings PAHs, only accouned 7.52%. After 90 days, the removal percentage of total PAHs in no plant treatment (only soil) was 4.91% (not be shown in this paper). Considering the natural dissipation of PAHs, the effect of phytoremediation by alfalfa only was 17.19% in this

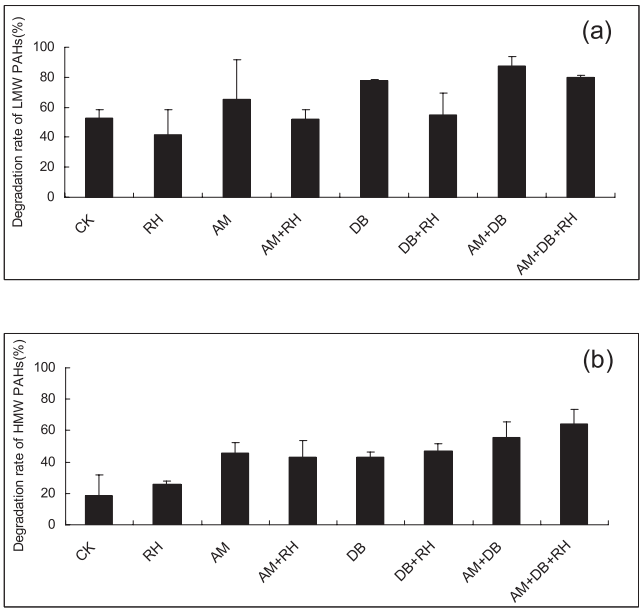

Fig. 1. Percentage of Degradation for PAHs in Different Treatments
(a) LMW-PAHs and (b) HMW-PAHs.

paper. Among all the treatments, "AM+DB+RH" treatment had achieved the highest removal rate of total PAHs (60.48%) which was 251.83% higher than that of the control treatment, 176.67%, 42.74% and 33.78% higher than those of single application of rhamnolipids (RH), AM fungi (AM), and aromatic hydrocarbon bacteria (DB), respectively (Table 4).

The removal rate of LMW-PAHs were above 50% in the phytoremediation using alfalfa (control) (Fig. 1). There were not significant differences in LMW-PAHs degradation rate between the treatments, however, "AM+DB" had the highest removal rate of LMW-PAHs among all the treatments. the removal ratio of LMW-PAHs decreased by RH addition at different degree in the corresponding treatments contrasted to those treatments without RH (Fig. 1). At the same time, phytoremediation alone had little effect on dissipation of HMW-PAHs, the removal rate was only up to 19.55% within 90 day experiments. Compared to control, single and multiple application of ARDB, AM fungi and rhamnolipids had an markable improvement on the dissipation of HMW-PAHs. The highest removal ratio of HMW-PAHs was observed by 64.11% in the treatment of "AM+DB+RH," 2.27 times higher than that of control, and 0.4, 0.5 and 1.51 times higher than those of "AM," "DB," and "RH." The highest degradation of individual PAHs (fluoranthene, pyrene and benzo[*a*]pyrene) disappeared in soil by 89.39%, 88.36% and 92.31%, respectively.

Soil Microorganisms and Soil Enzyme Activities

As shown in Table 5, the number of heterotrophic bacteria (AHB) and ARDB showed marked variation responded to the different treatments, ranging from 3.3 to 37.3×10^7 g^{-1} and from 2.8 to 37.0×10^6 g^{-1}, respectively. Single application of AM fungi and aromatic hydrocarbon bacteria significantly increased the number of soil ARDB, with 4.3 and 10.7 times higher than that of control. In contrast, single application of RH had little effect on increase number of ARDB. And as our expectation, all the treatments inoculated with ARDB ("DB," "AM+DB," "DB+RH" and "AM+DB+RH") resulted in a higher number of ARDB than the uninoculated treatments.

Soil dehydrogenase activity in treatments of aromatic hydrocarbon degrading bacterial inoculation were from 2.4 to 3 times higher than those of uninoculated treatment (Table 5). It indicated ARDB inoculation significantly increased soil dehydrogenase activity, while AM fungi inoculation and RH addition had a negligible effect on the total activity of soil microorganisms. Although no significant differences in soil polyphenol oxidase activity were observed within the different treatments (Table 5), there was a trend that soil polyphenol oxidase activity was higher in multi-application treatments than those of single application treatments, and the highest activity were observed in "AM+DB+RH" treatment.

DISSICUSION

According to Canadian Environmental Quality Guidelines,[29] this soil was not suitable for agricultural land uses as well as residential or parkland uses due to high concentration of PAHs. Especially, a large percentage of HMW-PAHs, such as fluoranthene and pyrene, were the most dominant components widely distributed in soil. Slow release of PAHs from the soil matrix to the aqueous phase represents a long-term contamination source and hinder remediation efforts.[30,31] and it would be very difficult, if not impossible, to completely remove all of these chemicals in a relative short time by a single technique.[32] For example, during the phytoremediation (control), 77% of fluorene and 48% of phenanthrene were able to dissipate, while only 20% of

Table 5. Enumberation of Soil Microbes and Soil Enzyme Activities in Different Treatments

Treatments	AHB[a)] $\times 10^7$ CFU·g^{-1}	ARDB $\times 10^6$ MPN·g^{-1}	Dehydrogenase mg TPF kg^{-1}·d^{-1}	Polyphenol oxidase mg purpurigallin g^{-1}·3 h^{-1}
CK	3.32 ± 0.01a[b)]	2.78 ± 0.04a	78.69 ± 0.66a	0.72 ± 0.04a
AM	8.16 ± 0.16b	14.73 ± 2.13b	71.20 ± 4.96a	0.81 ± 0.01a
DB	18.87 ± 0.05c	32.51 ± 0.78d	194.15 ± 7.34b	0.79 ± 0.04a
RH	3.34 ± 0.14a	2.81 ± 0.01a	68.32 ± 10.25a	0.78 ± 0.14a
AM+DB	29.16 ± 0.58d	33.06 ± 1.45e	215.19 ± 12.81d	0.94 ± 0.07a
AM+RH	11.48 ± 0.51b	15.36 ± 1.54c	79.44 ± 7.48a	0.74 ± 0.07a
DB+RH	23.17 ± 0.32c	32.08 ± 1.34d	257.45 ± 21.82c	0.83 ± 0.21a
AM+DB+RH	37.28 ± 0.26e	36.98 ± 0.98f	185.94 ± 12.71b	0.95 ± 0.23a
Significance of				
RH	N.S.[c)]	N.S.	N.S.	N.S.
AM	*	N.S.	*	N.S.
DB	*	N.S.	**	N.S.
AM × RH	N.S.	N.S.	**	N.S.
AM × DB	*	*	*	N.S.
RH × DB	N.S.	N.S.	N.S.	N.S.
AM × DB × RH	*	*	**	N.S.

a) AHB, aerobic heterotrophic bacteria; ARDB, aromatic hydrocarbons degrading bacteria. *b*) Data are means ± standard deviation (S.D., $n = 4$). Values within the same column not followed by the same letter differ significantly ($p < 0.05$). *c*) N.S., not significant; * and **, significant at 0.05 and 0.01 levels, respectively.

Benzo[*a*]pyrene, 21% of benzo[*g*,*h*,*i*]perylene and 25% of indeno[1,2,3-*cd*]pyrene were removed from the soil.

Surfactants usually were employed to increase bioavailability and biodegradation rate of PAHs, biosurfactants have the ability to solubilize PAHs but have several additional advantages over synthenic surfactants that make them surperior candidates in bioremediation schemes.[33–35] When rhamnolipids was added alone during the phytoremediation process, removal percentage of 15 EPA-PAHs was not significantly promoted (21.86%) and the number of ARDB in treatment "RH" was similar with that in control, and significantly lower than the other treatments (Table 5). This could be simply explained that a lack of aromatic hydrocarbon degrading microorganisms existed in the soil.[16] So it is necessary to increase the number of ARDB and to stimulate the dissipation of PAHs by bioaugmentation or other approaches.

The AM fungi played an important role in bioremediation, and enhanced plant survival and growth by improving pollutant tolerance and nutrient uptake, moreover, it also could enhance PAHs dissipation through increasing the number of aromatic hydrocarbon degrading microorganism in the mycorrhizosphere.[6, 16, 21, 22] In the present experiment, inoculation of AM fungi alone had no significant effect on shoot and root dry weight and mycorrhizal colonizition rate maintained at a relatively low level (below 20%, Table 2), it maybe attribute to AM fungi exposure to high PAHs stress. Verdin *et al.*[36, 37] reported that AM fungi presented a reduce development of extraradical mycelium and a decrease in sporulation, root colonization and spore germination when exposed to PAHs substances, such as anthracene and phenanthrene. However, AM fungi inoculation contributed to an obvious increase of culturable heterotrophic and PAH DB, which resulted in the addition enhancement in dissipation rate of total PAHs and HMW-PAHs (Fig. 1). This was consistent with the results of Corgie *et al.*,[38] who showed that colonization of roots by *Glomus mosseae* (*G. mosseae*, BEG 69) modified the structure and density of bacterial populations in the mycorrhizosphere, compared to the rhizosphere of non-mycorrhizal plants. *G. mosseae* increased the density of culturable heterotrophic and PAH DB beyond the immediate rhizosphere in the presence of phenanthrene.

Of the methods tested, the multi-technique phytoremediation system ("AM+DB+RH") was most effective to remove of total PAHs from contaminated soil, at the same time, it was really important that HMW-PAHs, that are highly hydrophobic and strongly bound to soil particles, were also successfully effective to be removed from soil. There were several possible explanations for this result. Firstly,

the effect of a surfactant on biodegradation was a combination of the solubilizing power of the surfactant and the bioavailability of the substrate within the surfactant micelles. In our study, removal rate of HMW-PAHs was more effectively improved in contrast with the trend that removal percentage of LMW-PAHs were slightly decrease in contaminated soil when rhamnolipids was added. This result was consistent with that of Chun *et al.*,[39] who showed markedly different tedencies with coexistent PAHs with surfactant. In the prensence of less hydrophobic solutes, the solubility of more hydrophobic solute was increased. Especially, phenanthrene was in greatly influenced by co-solutes than naphthalene. The solubility of phenanthrene was greatly enhanced in presence of naphthalene but reduced in presence of pyrene. The explanation for these results could be that less hydrophobic compounds can be solubilized at the interfacial region of a hydrophobic core, which reduces the interfacial tension between the core and water, and then the reduced interfacial tension can support a larger core volume for the same interfacial energy. Secondly, there was also evidence that dissipation of HMW-PAHs by AM fungi inoculation was more pronounced than that of LMW-PAH. Since root exudates are influenced quantitatively and qualitatively by the plant growth and development,[40] the more significant plant growth promotion by AM fungi inoculation was expected to have impacted more root exudation of the mycorrizal plant compared to the nonmycorrhizal plant. On one hand, root exudates are known to influence the genetic structure and diversity of microbial communities in the rhizosphere.[20] On the other hand, root exudates from mycorrizal plant, such as carbohydrates, amino acids and phenolicsas, may drive HWM-PAHs co-metabolism as substrate.[41,42] Finally, inoculation of aromatic hydrocarbon degrading microorganism enriched by HMW-PAHs had a direct influence on the dissipation of HMW-PAHs.[43] Under the improved condition that aqueous solution and bioavailability of PAHs were increase with RH addition, and root exudate of alfalfa was considered as co-metabolism substrate, HMW-PAHs were rapidly degraded by these microorganisms.[44,45] Interactive effect of above three main ways in the phytoremediation system resulted in the phenomena that HMW-PAHs were rapidly and efficiently removal from aged PAHs contaminated soil.

In conclusions, the use of biosurfactant would increase the bioavaliability of PAHs in aged contaminated soils. The inoculated ARDB would then metabolize PAHs as substrates more efficiently, increase degradation rate and in turn reduce the toxic effect to the plants. The inoculated AM fungi would improve plant growth, and provide a more favorable living environment for DB, which were helpful to increase the dissipation of aged PAHs from soils. The present study has demonstrated that the residual concentrations of PAHs in soils decreased significantly by interactive effect of AM fungi and ARDB with RH when planted with alfalfa. The findings of this study provide preliminary evidence for the potential of AM inoculation, ARDB inoculation and biosurfactant application in the decontamination of organic pollutants in agriculatural soil. However, detailed and comprehensive studies will be required to establish effective remediation methods under field condition.

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