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ORIGINAL PAPER

Bioremediation of high molecular weight polyaromatic hydrocarbons co-contaminated with metals in liquid and soil slurries by metal tolerant PAHs degrading bacterial consortium

Palanisami Thavamani · Mallavarapu Megharaj · Ravi Naidu

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Abstract Bioremediation of polyaromatic hydrocarbons (PAH) contaminated soils in the presence of heavy metals have proved to be difficult and often challenging due to the ability of toxic metals to inhibit PAH degradation by bacteria. In this study, a mixed bacterial culture designated as consortium-5 was isolated from a former manufactured gas plant (MGP) site. The ability of this consortium to utilise HMW PAHs such as pyrene and BaP as a sole carbon source in the presence of toxic metal Cd was demonstrated. Furthermore, this consortium has proven to be effective in degradation of HMW PAHs even from the real long term contaminated MGP soil. Thus, the results of this study demonstrate the great potential of this consortium for field scale bioremediation of PAHs in long term mix contaminated soils such as MGP sites. To our knowledge this is the first study to isolate and characterize metal tolerant HMW PAH degrading bacterial consortium which shows great potential in bioremediation of mixed contaminated soils such as MGP.

P. Thavamani · M. Megharaj (✉) · R. Naidu
Centre for Environmental Risk Assessment and Remediation (CERAR), University of South Australia, Mawson Lakes, SA 5095, Australia
e-mail: megharaj.mallavarapu@unisa.edu.au

P. Thavamani · M. Megharaj · R. Naidu
Cooperative Research Centre for Contamination Assessment and Remediation of the Environment (CRC CARE), Mawson Lakes, SA, Australia

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Introduction

The ever increasing population coupled with urbanization and city growth have resulted in enormous economic demand for residential, recreational and commercial areas to be developed in former industrialized zones. Consequently, many abandoned industrial sites, such as manufactured gas plant (MGP) sites that were once located in the periphery of urban land have been pushed to the middle of cities due to rapid population growth and urbanisation (US EPA 2004). Thus, many polyaromatic hydrocarbons (PAH) contaminated sites lie in high value commercial land and remediation of such sites has been challenging due to the lack of appropriate cost effective technologies. Often the best solution for such sites has been excavation and transport to landfill sites which is now proving to be expensive due to the recent increases in the landfill levy (EPA Victoria 2009). Consequently, there is significant pressure on owners of contaminated sites to find cost effective in situ remediation methods.

Bioremediation has proved to be effective in removing organics, but metals have been shown to

inhibit organic pollutant biodegradation (Roane et al. 2001; Riis et al. 2002; Sandrin and Maier 2003; Thavamani et al. 2012a, b, c) by impacting both the physiology and ecology of organic degrading micro-organisms. There are many MGP sites in Australia and elsewhere that contain both PAHs and toxic metals (Thavamani et al. 2011). Recently, a number of approaches have been suggested to mitigate metal toxicity and increase organic biodegradation such as the use of metal tolerant bacteria, reduction of metal bioavailability, treatment additives and clay minerals (Wong et al. 2005; Atagana 2006). The isolation of bacteria from mixed contaminated soils which display versatile catabolic activity to tolerate toxic metals and degrade PAHs could be a potential cost effective remediation strategy (Thavamani et al. 2011).

This study was focused on (a) isolation and characterisation of a bacterial consortium able to tolerate toxic metal, Cd^{2+} and degrade HMW PAHs in liquid medium and (b) further evaluation of the ability of this consortium to degrade PAHs in slurries prepared from long term PAHs and metals mixed contaminated soil.

Materials and methods

Enrichment and isolation

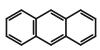
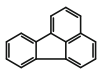
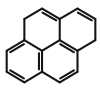
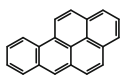
The mixed contaminated soils used in this study were collected from a former manufactured gas plant site in Australia (Thavamani et al. 2011). The site was used for over 90 years but is now no longer used. The site was contaminated with various LMW, HMW PAHs and heavy metals. The physico-chemical properties of five PAHs studied are summarised in Table 1. Stock solutions of individual and PAH mixtures were prepared in dimethylformamide (DMF) and used in all degradation experiments. DMF is water miscible and relatively less toxic to microorganisms hence is a preferred choice of carrier solvent in biodegradation studies using chemicals of extreme low water insolubility (Megharaj et al. 1998). The enrichment and isolation was carried out using a mixture of phenanthrene and pyrene as carbon and energy sources using M9 mineral medium M9 mineral medium contained: g l^{-1}: Na_2HPO_4, 6; KH_2PO_4, 3; NH_4Cl, 1; NaCl, 0.5; $MgSO_4.7\,H_2O$, 0.246; $CaCl_2$, 0.01, pH. 6.9 (Eisenstadt et al. 1994). To enrich the bacterial population from contaminated soils, 20 g of 2 mm sieved soil was shaken in 100 ml of M9 medium for 24 h at 175 rpm, 25 °C. The first enrichment step started by inoculating a 5 ml of this suspension into 45 mL of M9 medium containing a mixture of 100 mg l^{-1} each of phenanthrene and pyrene. When microbial growth was visible, the enrichment broth was subcultured (10 % v/v) into fresh M9 medium containing phenanthrene and pyrene. After several successive subcultures, ten-fold serial dilutions were plated in M9 agar; phenanthrene was supplied as vapour phase in Petri-dish lids (Megharaj et al. 1997). The well grown colonies were further developed in M9 broth with a mixture of phenanthrene and pyrene (each 100 μg ml^{-1}) as carbon sources.

16S rRNA and cloning

The isolated mixed culture was identified based on molecular cloning and 16S rRNA sequencing. The DNA was extracted based on the methods detailed by Bell et al. (1998).The crude DNA extract was diluted 100-fold in sterile water just prior to carrying out PCR under the following conditions. A 25 μl PCR mixture contained 1× concentration of Taq DNA polymerase buffer (Promega, Sydney), 2.5 mM $MgCl_2$, 2 M betaine (Sigma, Sydney), 0.2 mM of each deoxynucleoside trisphosphate, 25 pmol of each forward and reverse primers, 1 U of DNA polymerase (Promega, Syndey), and 1 ul of the diluted DNA extract as template. Almost complete 16S rRNA genes were amplified with the forward primer E8f (50-AGAGT TTGATCCTGGCTCAG-30) and the reverse primer 1541r (50-AAGGAGGTGATCCANCCRCA-30) of Weisburg et al. (1991). The DNA was amplified with an iCycler thermocycler (BioRad, Sydney) using the following program: 5 min of pre-heating at 95 °C, 30 cycles of 30 s of denaturation at 95 °C, 30 s of primer annealing at 55 °C, and 2 min of elongation at 72 °C. A final extension step of 10 min at 72 °C was included.

Purified PCR products were ligated into pGEM-T-vector system (Promega) and transformed into *Escherichia coli* JM109 competent cells as specified by the manufacturer. About four transformed *E. coli* strains were randomly selected and grown overnight in Luria–Bertani broth at 37 °C for 12 h. Plasmids were extracted with a Qiaprep Spin Miniprep kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions. The extracted plasmids were sequenced at Flinders

Table 1 Structures and properties of the PAHs used in this study (modified from ATSDR 2005)

PAH	Structure	Molecular weight (g l^{-1})	Aqueous solubility (mg l^{-1})	Vapour pressure (Pa)	Log K_{ow}	Boiling point (°C)	Melting point (°C)	TEF[a]
Anthracene	 $C_{14}H_{10}$	178	0.045	1.0×10^{-3}	4.54	340	218	0.01
Phenanthrene	 $C_{14}H_{10}$	178	1.1	2.0×10^{-2}	4.57	340	99.5	0.001
Fluoranthene	 $C_{16}H_{10}$	202	0.26	1.2×10^{-3}	5.22	375	111	0.001
Pyrene	 $C_{16}H_{10}$	202	0.132	6.0×10^{-4}	5.18	404	145	0.001
Benzo(a)pyrene	 $C_{20}H_{12}$	252	0.0038	7.0×10^{-7}	5.91	495	179	1

[a] The toxicity equivalence factors based on carcinogenicity (US EPA 1993)

Medical Centre, Adelaide, South Australia using BigDye Terminator version 3.1 sequencing reaction. The 16S rRNA sequences from the isolates were constructed with DNA Star software (SeqMan, version 4.05 for Windows) and aligned with the NAST alignment tool (DeSantis et al. 2006), and chimera formation was checked with the Bellerophon program. Bootstrapped neighbour-joining relationships were estimated with MEGA version 3.1 (Tamura et al. 2007). All sequences obtained were compared with available databases using Basic Local Alignment Search Tool (BLAST).

PAHs degradation in mineral medium-effect of Cd

Many microbial growth media used for toxicity testing contains high concentrations of metal-binding components such as phosphates that can reduce solution-phase metal concentrations thereby causing underestimation of the real toxicity (Megharaj et al. 2003). In our previous study, we developed a new mineral medium by optimising the concentrations of metal binding medium components to allow bacterial growth and at the same time maintain high bioavailable metal (Cd^{2+}) in the medium. The final optimized mineral medium (OMM) contained (per litre): 100 mg KH_2PO_4, 100 mg HNa_2PO_4, 500 mg NH_4NO_3, 500 mg NH_4SO_4, 200 mg $MgSO_4$, 20 mg $CaCl_2$, 2 mg $FeCl_2$, 2 mg $MnSO_4$, pH was adjusted to 6.5. This medium has more than 60 % Cd as Cd^{2+} at pH 6.5 as measured by an ion selective electrode.

PAHs degradation in liquid cultures was conducted in sterile 40 ml amber glass vials fitted with Teflon lined screw caps using OMM. Considering Cd as one of the common heavy metals present in mix contaminated soils, the effect of Cd (5 mg l^{-1} as Cd^{2+}) on PAH degradation was also investigated. Cd stock solution (1,000 mg l^{-1}) was prepared using Cd $(NO_3)_2$ in sterile deionised water. Our preliminary study on tolerance of the bacterial consortium to Cd (0–50 mg l^{-1}) in the optimised mineral medium showed this consortium can tolerate up to 5 mg Cd l^{-1} medium without losing its PAH degradation ability. Therefore, 5 mg l^{-1} Cd^{2+} was chosen as optimum concentration in this study. To obtain fast growth, inoculum was grown in M9 medium supplemented with phenanthrene as the sole carbon

source until growth reached late log phase (~ 48 h). The inoculum was prepared by centrifugation at 10,000× *g*/ 10 min and washed twice with sterile OMM. The cell pellets were resuspended and adjusted to OD_{600} of 0.4 (cell density around 1.7×10^7 cells ml^{-1}) in optimised mineral medium. Unless otherwise stated, 10 ml of liquid culture was incubated with and without PAHs in 40 ml amber vials placed in a shaker set at 175 rpm and a temperature of 25 °C. PAH degradation was conducted in the presence and absence of Cd^{2+} (5 mg l^{-1}) with individual PAHs (pyrene and BaP at 50 mg l^{-1}) as the sole carbon sources or in combination with phenanthrene (250 mg l^{-1}) as a co substrate (pyrene + phenanthrene or BaP + phenanthrene). Also, the ability of consortium to degrade a mixture of five PAHs containing anthracene, phenanthrene, fluoranthene, pyrene and BaP (Table 1) each supplied at 50 mg l^{-1} in the presence and absence of 5 mg l^{-1} Cd was evaluated. The experiment was conducted in triplicate and at designated time intervals. Triplicate samples (entire vials) were sacrificed for PAH analysis. The growth of the bacterial consortium was followed by measuring total cell protein concentration according to Biuret method.

PAHs degradation in soil slurry

An aerobic slurry microcosm was designed to determine the rate and extent of PAHs degradation in contaminated soils. Two soils were selected, one was uncontaminated soil spiked with five PAHs mixture each at a final concentration in soil slurry of 100 mg l^{-1} and 5 mg l^{-1} of Cd. The second soil collected from former MGP site had varying ranges of PAHs and heavy metals (Table 2). The slurry microcosm consisted of 40 ml sterile glass amber vials containing 5 g soil and 10 ml of OMM (1:2 w/v). The inoculum (bacterial consortium) was prepared as described above and added to each vial. Sterile soils (abiotic) and uninoculated non-sterile soils (natural attenuation) served as controls. The slurry microcosms were maintained in the dark at 25 °C with shaking in an orbital shaker set at 175 rpm to facilitate better mixing and aeration.

PAHs extraction and analysis

The triplicate liquid cultures were extracted using methylene chloride (1:1 v/v) by vigorous shaking (90 s). The extract was held for 2 h at room temperature before separating the solvent layer. In soil slurry microcosm the whole vial was extracted twice by adding an equal volume of dichloromethane using sonication. The extracted organic phase was dried over anhydrous sodium sulphate and concentrated to 1 ml under a gentle stream of nitrogen.

PAHs were analyzed using gas chromatograph with a mass selection detector (Hewlett-Packard 5890 Series II, Agilent Technologies, Delaware, USA) according to the standard US EPA 8270 C method (US EPA 1996). The PAHs were separated using a 30-m high resolution glass capillary column DB-5(i.d. 0.25 mm) coated with a 0.25 μm film (J&W Scientific, Agilent Technologies, Delaware, USA). The

Table 2 Physicochemical characteristics of the soils used for soil slurry study

Characteristics	MGP soil	Spiked soil
pH	7.0	7.2
EC (μS)	890	850
Sand (%)	27	25.2
Clay (%)	1	0.7
Silt (%)	72	74.1
CEC_b (cmol (+) kg^{-1})	2.5	2.2
PAHs (mg kg^{-1})		
Naphthalene	18	
Acenapthylene	14	
Acenapthene	8	
Fluorene	36	
Anthracene	77	100[a]
Phenanthrene	41	100[a]
Fluoranthene	66	100[a]
Pyrene	161	100[a]
Benz[a]anthracene	75	
Chrysene	73	
Benzo[b]fluoranthene	19	
Benzo[k]fluoranthene	89	
Benzo[a]pyrene	70	100[a]
Dibenz[ah]anthracene	49	
Indeno[1, 2, 3-cd]pyrene	50	
Benzo[ghi]perylene	43	
Heavy metals (mg kg^{-1})		
Pb	96	
Cd	27	5[a]
Zn	127	

[a] Concentrations in soil slurry

oven temperature was held initially at 40 °C for 4 min, and then ramped up to 270 °C at 10 °C min^{-1}. The calibration was by external standard method, using a certified PAHs mixture (TCL PAHs Mix-Ref 4-8905, Supelco, USA).

Deuterated surrogate standards (1-methylnaphthalene-d8, fluorene-d10, anthracene-d10, pyrene-d10, *p*-terphenyl-d14, BaP-d12, and benzo(*ghi*)perylene-d12) were used to monitor PAH losses during extraction and cleanup. For quality control, experiments on recovery were carried out by spiking a known concentration of PAH standards (5 and 10 μg g^{-1}) in contaminated soil. The results showed a significant recovery of 92 ± 7.2 %. The accuracy and precision of the whole chromatographic operation was checked every ten samples by injecting known standards and a solvent blank.

Results

The physico-chemical properties of five PAHs studied are summarised in Table 1. PAH molecular stability, hydrophobicity and low water solubility appear to be some of the main factors that contribute to their persistence in the environment. Some of these factors have also been correlated to the size of the molecule or total number of aromatic rings (Cerniglia 1992). As it is widely known, HMW PAHs are sparingly soluble in water, electrochemically stable and may be acutely toxic, genotoxic, immunotoxic or act as agents of hormone disruption (Van de Wiele et al. 2005). Due to their elevated octanol–water partition coefficients (K_{ow}), HMW PAHs may separate into organic phases, soil and sediment organic matter and membranes of living organisms. They are also candidates for bioconcentration, bioaccumulation and sometimes biomagnification through trophic transfer to food webs (Kanaly and Harayama 2010).

Isolation and identification

The metal tolerant PAH degrading bacterial consortium designated as consortium-5 was enriched and isolated from a former MGP site soil. Light brown coloured colonies turned dark brown with clearing zone when grown on phenanthrene coated agar, indicating PAH utilization. The visible colonies occurred in M9 plates 3 days after incubation at 25 °C. All the colonies were able to grow using phenanthrene. The individual bacterium within the consortium was identified by cloning on the basis of 16S rRNA sequences. The consortium-5 consisted of four different bacterial species putatively identified as (99 % similarity): *Alcaligenes* sp. (EU726990), *Pseudomonas* sp. (EU726991), *Pandorea* sp. (EU726992), and *Paenibacillus* sp. (EU726993). The evolutionary history of these four species was inferred using the neighbour-joining method (Saitou and Nei 1987). A phylogenetic tree, of the organisms identified in consortium-5, was constructed together with their closest relatives as shown in Fig. 1.

PAHs degradation in liquid culture

The isolated metal tolerant PAH degrading bacterial consortium was initially characterised using, model HMW PAH, pyrene. Then the study was extended to other carcinogenic HMW PAHs including BaP and PAHs mixture. The PAHs degradation was first tested in liquid culture and then tested in soil slurries of spiked as well as long term contaminated soil.

Degradation and co-degradation of model HMW PAH pyrene in the presence of Cd

Pyrene is a four ringed compound, sparingly soluble and widely used as model HMW PAH. The consortium-5 was tested for its ability to utilise pyrene (100 mg l^{-1}) as a sole carbon source with and without Cd (Fig. 2a). Irrespective of the presence or absence of Cd, there was exponential decline in the concentration of pyrene with 50 % of degradation occurring within the first 48 h and complete degradation by the eighth day. Pyrene degradation was followed by an increase in bacterial growth measured in terms of protein. Pyrene degradation was enhanced when phenanthrene was supplied as a co-substrate with 100 % of pyrene disappearing within 6 days, followed by an increase in the bacterial growth measured as protein (Fig. 2b). Presence of Cd did not influence the pyrene degradation by consortium.

Degradation/co-degradation of benzo(a)pyrene (BaP) in the presence of Cd

BaP is a representative HMW PAH of environmental concern due to its known carcinogenicity and

bioaccumulation potential. Although BaP had been detected in a variety of environmental samples to date, not many microorganisms have been reported that can utilize BaP as a sole source of carbon and energy. The consortium-5 was repeatedly grown in liquid mineral medium containing phe + pyr for 8 weeks. After 8 weeks 10 mg l^{-1} of BaP was supplied along with pyrene in the liquid medium and monitored for visual disappearance of BaP. This procedure was followed by repeated subculturing of the consortium every 2 weeks for 60 days to increase the ability of consortium to better adapt and degrade BaP.

The ability of consortium-5 to degrade BaP (50 mg l^{-1}) supplied as a sole carbon source was tested with and without Cd. As shown in Fig. 3a, BaP degradation was slow in both treatments with 67 % of BaP degraded by 60 days of incubation in the absence of Cd whilst only 50 % of BaP degraded in the presence of Cd during the same period. There was an initial lag phase for the first 10 days with approximately 10 % of BaP degraded in the absence of Cd and only 6 % of BaP degraded in the presence of Cd. A relatively rapid growth occurred between 10 and 40 days of incubation, during which period 40 % of BaP was degraded, accompanied by an increase in protein concentration both in the presence or absence of Cd.

When phenanthrene (250 mg l^{-1}) was supplied as a co-substrate, BaP degradation proceeded at a relatively rapid phase compared to BaP alone (Fig. 3b). Thus, 36 % of BaP degradation was observed in 10 days when Phenanthrene was supplied as a co-substrate in the absence of Cd, whereas the presence of Cd delayed BaP degradation, with only

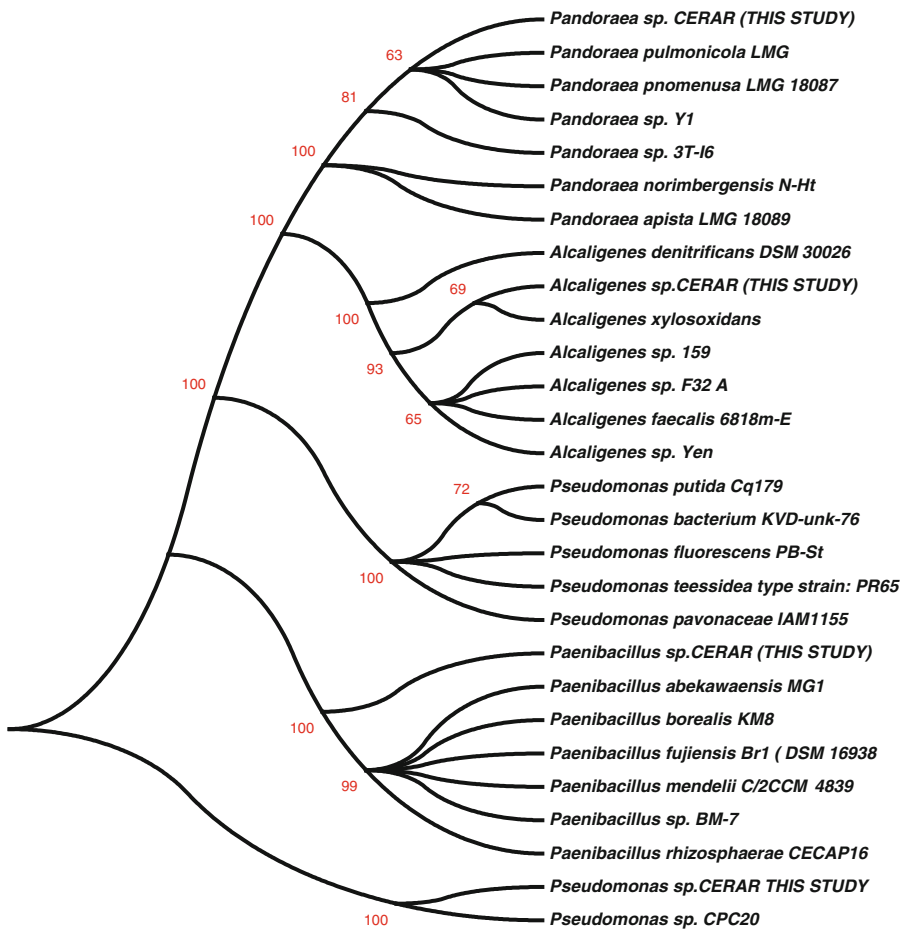


Fig. 1 Phylogenetic analysis of metal tolerant HMW PAHs degrading bacterial species in consortium-5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. All positions containing *gaps* and missing data were eliminated from the dataset

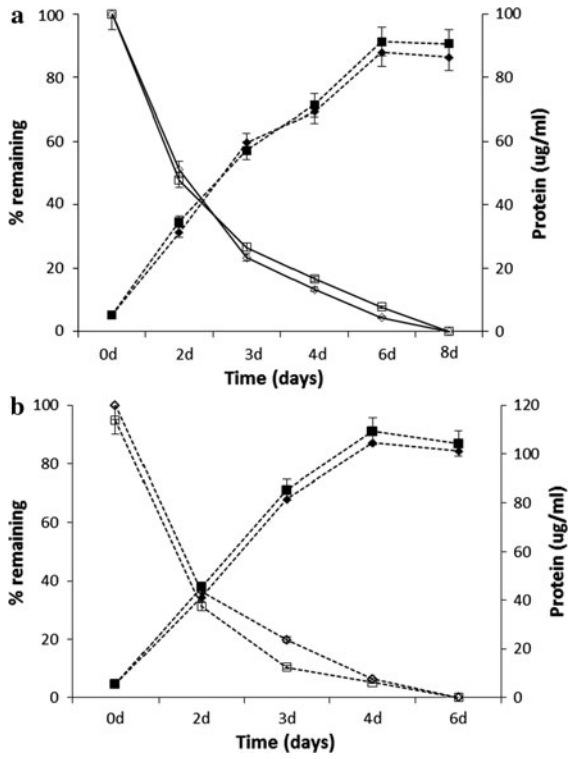


Fig. 2 Degradation of pyrene alone (**a**) and co-degradation with phenanthrene (**b**) by consortium-5 in the absence of Cd^{2+} (*diamond*) and presence of Cd^{2+} (*square*). Corresponding protein concentrations without Cd^{2+} (*filled diamond*) and with Cd^{2+} (*filled square*) presented in the secondary Y axis with *dotted lines*

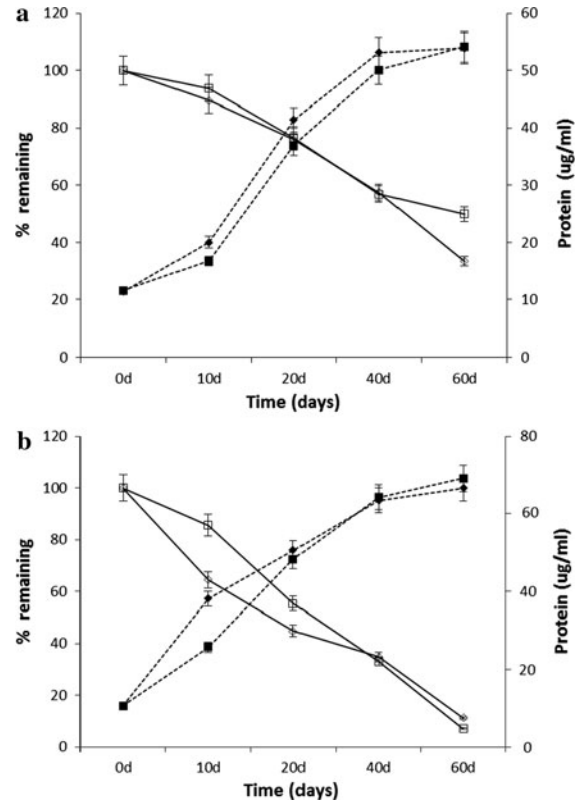


Fig. 3 Degradation of benzo(a)pyrene alone (**a**) and co-degradation with phenanthrene (**b**) by consortium-5 in the absence of Cd^{2+} (*diamond*) and presence of Cd^{2+} (*square*). Corresponding protein concentrations without Cd^{2+} (*filled diamond*) and with Cd^{2+} (*filled square*) presented in the secondary Y axis with dotted lines

14 % BaP degradation occuring during the same period. However, about 90 % of BaP was degraded in the presence of phenanthrene within 60 days.

Degradation of HMW PAH mixtures in the presence of Cd

PAHs occur in the environment as mixtures of various LMW and HMW PAHs. Five PAH congeners (anthracene, phenanthrene, fluoranthene, pyrene and BaP-APPFB) differing in their solubility and ring structures were chosen for this study (Table 1). The ability of the isolated bacterial consortium to grow and utilize PAHs mixtures (50 mg l^{-1}) was tested in liquid cultures with and without Cd, and incubated for 60 days (Fig. 4). The low molecular weight PAHs, phenanthrene and anthracene were completely degraded by the end of a 60 days incubation period in both treatments (presence or absence of Cd). Interestingly, the presence of Cd stimulated the PAH mixture degradation (APPFB + Cd) with the exception of fluoranthene. Thus, among HMW PAHs, 100 and 89 % of degradation was observed for BaP and pyrene, respectively, in the absence of Cd while BaP and pyrene were degraded to 100 and 94 % in the presence of Cd.

Degradation of HMW PAHs in soil slurry

The ability of bacterial consortium-5 to degrade PAHs in soil slurries (1:2 w/v) prepared from PAH spiked uncontaminated soil and real long term contaminated MGP soil was evaluated in laboratory microcosms.

Degradation of PAHs mixture in spiked soil slurry

More than 90 % of anthracene, phenanthrene and fluoranthene were degraded during a 60 day incubation

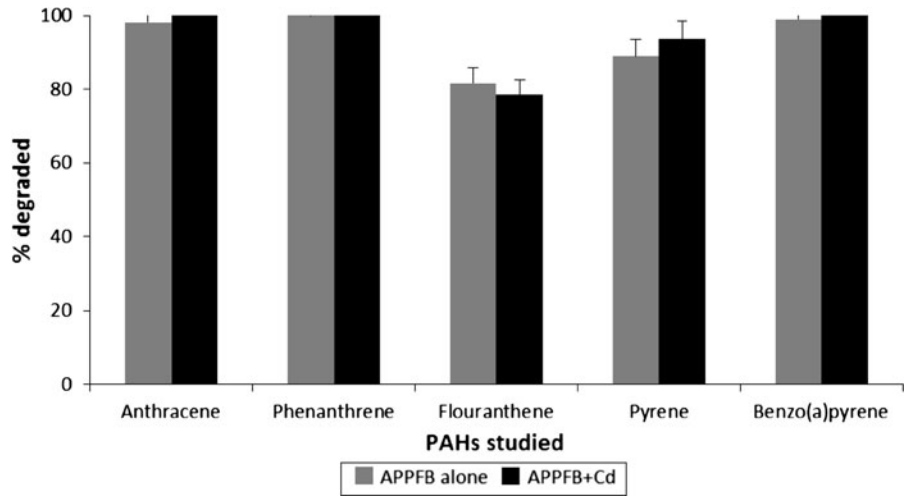


Fig. 4 Degradation of PAHs mixtures by consortia-5 in the presence of Cd^{2+} in liquid culture using optimized mineral medium

period (Fig. 5). In general, the degradation of PAHs decreased with increasing molecular weight. Around 77 % of degradation was observed in pyrene compared to 48 % of degradation in BaP during the 60 day incubation period. Under natural attenuation conditions (without addition of bacterial consortium) LMW PAHs such as anthracene and phenanthrene were degraded up to 25 and 29 %, respectively. However, natural attenuation of HMW PAHs fluoranthene, pyrene and BaP was not significant (<6 %).

Degradation of 16 US EPA PAHs in MGP soil slurry

The MGP soil contained various ranges of all 16 US EPA priority PAHs and heavy metals such as Pb, Cd and Zn (Table 2). Figure 6 shows the ability of consortium to degrade the US EPA priority PAHs, as in long term contaminated MGP soil. Approximately 50 % of all LMW PAHs except phenanthrene (27 %) were degraded. The highest degradation was observed in acenapthene (65 %). More than 40 % of degradation was observed for 4 ring HMW PAHs with pyrene and fluoranthene degraded by approximately 82 and 74 %, respectively. Among five ring HMW PAHs, the degradation of benzo(k)fluoranthene (57 %) and BaP (49 %) appeared to be higher. The degradation of more than five ring HMW PAHs was very low compared to less than five ring PAHs. A slight natural attenuation of LMW PAHs was observed, however natural attenuation of HMW PAHs was insignificant.

Discussion

Polyaromatic hydrocarbons are arguably the most common contaminants encountered on urban land. PAHs typically occur as complex mixtures with inorganic contaminants such as heavy metals. Among various PAHs congeners, those of lower molecular weight are more easily degraded, while in comparison HMW PAHs are more recalcitrant and typically require more time and in some cases different microorganisms to perform such degradation. As more studies are carried out, it is becoming increasingly evident that several microbial species have capacity to use both low and high molecular weight PAHs as sole carbon and energy sources.

Many microbes with the ability to degrade PAHs have been isolated, and PAH degradation mechanisms, enzymes and genes have been widely studied using these isolates (Bastiaens et al. 2000; Uyttebroek et al. 2007; Wong et al. 2005). Only a few reports on the microbial metabolism of PAHs with four or more aromatic rings were published and they too mainly focused on co-metabolic transformations (Kanaly and Harayama 2010). However, PAH degradation by pure cultures may not represent the process under realistic environmental conditions, since the influence of indigenous strains is not considered. Construction of consortia by mixing several known PAH degraders has failed to maximise cooperation among different species using synthetic consortia (Ghazali et al. 2004). A more reasonable way to acquire an efficient degradation system would be to enrich microbes from

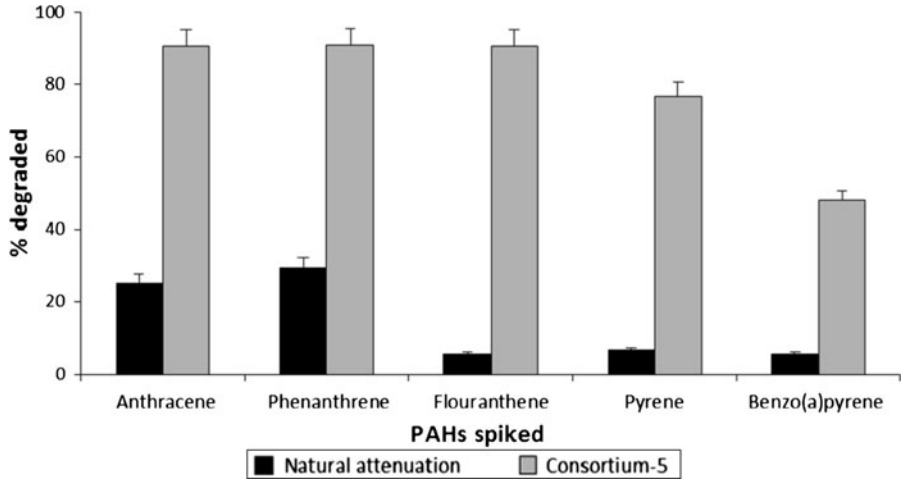


Fig. 5 Degradation of PAHs mixtures by consortia-5 in the presence of Cd^{2+} in spiked soil slurry

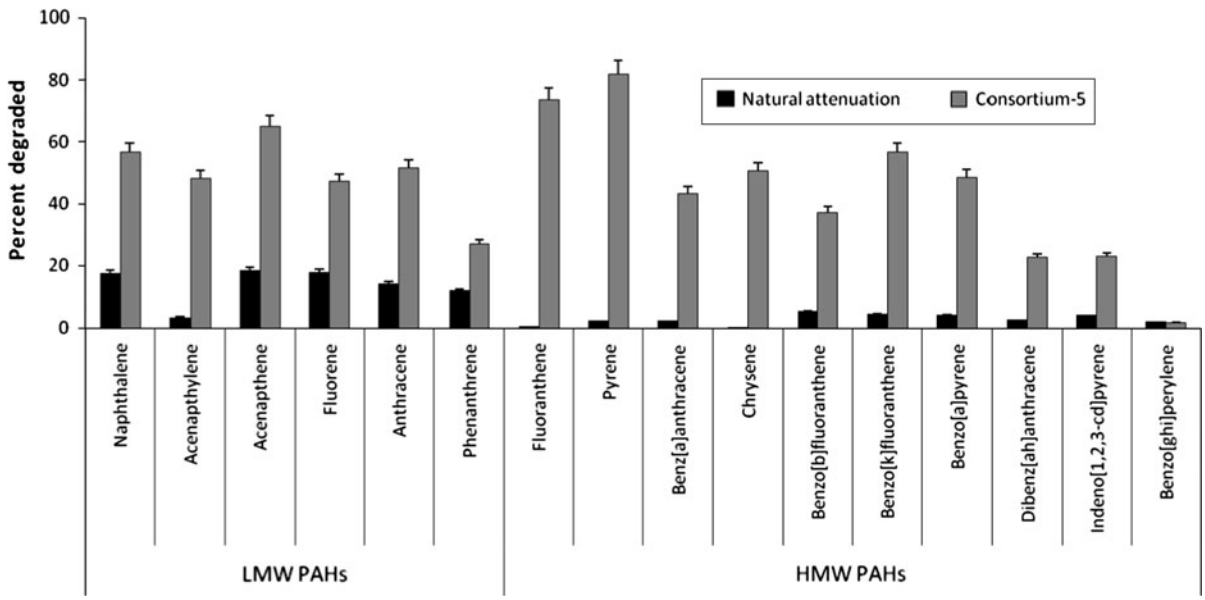


Fig. 6 Degradation of 16 US EPA priority PAHs by consortium-5 in the presence of Cd^{2+} in long-term mixed contaminated (MGP site) soil slurry. Initial concentration of each PAHs was presented in Table 2. Percentage degraded was calculated based on the relative difference to the abiotic control

environments using target compounds as the carbon source.

In this study, a bacterial consortium consisting of four different bacterial species, namely *Alcaligenes* sp.; *Pseudomonas* sp.; *Pandorea* sp. and *Paenibacillus* sp. enriched from long-term mixed contaminated soils was used to study the PAHs degradation in the presence of Cd. This consortium utilized various LMW PAHs such as phenanthrene and anthracene in 3 days. The uniqueness of this consortium which has not been explored so far is its ability to degrade LMW PAHs completely even in the presence of toxic levels of Cd. To our knowledge this is the first study focused on isolation and characterization of metal tolerant HMW PAHs degrading bacterial consortium for remediation of mixed contaminated soils.

In order to evaluate the real effect of toxic metal on degradation of PAHs, all the liquid culture studies

were conducted in OMM to maintain maximum concentration of free ion Cd^{2+} in the system. This was necessary to prevent underestimation of the effect of metal bioavailability and hence toxicity due to the complexation of metal by the ligands (e.g. high phosphate) that are normally present at high concentrations in the growth media. This study clearly demonstrated the ability of consortium-5 to utilize the HMW model PAH compound, pyrene as a sole source of carbon irrespective of the presence of toxic metal Cd and the co-substrate phenanthrene. Co-metabolic degradation of anthracene in the presence of phenanthrene in 6 weeks was reported by Ho et al. (2000). According to McNally et al. (1998), the presence of naphthalene (3 mg l^{-1}) stimulated a two-fold degradation of pyrene (0.13 mg l^{-1}) by *Pseudomonas putida* in 24 h. However, these studies used very low PAH concentrations compared to our study. Apart from Cd resistance, the pyrene degradation ability of bacterial consortium proved to be relatively faster (6 days) than other PAHs degradation studies. Boonchan et al. (2000) reported complete degradation of pyrene (250 mg l^{-1}) in 56 days by fungal-bacterial co cultures whereas Somtrakoon et al. (2008) reported 42 % pyrene (50 mg l^{-1}) degradation over 21 days by *Burkholderia* sp. VUN10013. However, this is the first study to our knowledge that demonstrates such a rapid degradation of pyrene (100 mg l^{-1}) within 6 days that is also in the presence of toxic metal Cd maintained as Cd^{2+}.

One of the main characteristics of PAHs is that they are constituted by mixtures of numerous LMW and HMW compounds. Benzo(*a*)pyrene (BaP), a five ringed PAH, is one of the most potent carcinogenic PAHs. BaP has extremely low water solubility (0.0023 mg l^{-1}) and a high octanol–water partition coefficient (Log K_{ow}, 6.06), which is related to its high recalcitrance to microbial degradation. The consortium-5 was able to degrade 67 % of 50 mg l^{-1} BaP supplied as a sole carbon source irrespective of the presence of Cd in 60 days but with a 10 day lag phase. The lag phase observed during BaP degradation could be due to the time required for adaptation of the culture to the insoluble BaP, including the induction of enzymes and solubilisation of BaP by the production of surfactants. Some bacterial species can solubilise HMW PAHs by producing surfactants and extracellular products. Even though the degradation was slow when BaP was supplied as a sole carbon source, degradation was not significantly affected by Cd. This can be attributed to the metal tolerance mechanisms of consortium.

B*a*P cannot easily be utilized by microorganisms both as a carbon and anenergy source (Cerniglia 1992). Bacterial degradation of HMW PAHs must utilize co-metabolic degradation, which requires a carbon/energy source, an inducer of catabolic enzymes, and oxygen. Hence, we have evaluated the effect of phenanthrene as a co-substrate for degradation of BaP, which clearly showed an enhancement in BaP degradation with almost 100 % of BaP degradation (50 mg l^{-1}) occurring within a 60 day incubation period. Since HMW PAHs, such as BaP, are quite tolerant to microbial attack, until now, the reported degradation rate of BaP by microorganisms was generally quite modest. Juhasz et al. (1997) reported that *Burkholderia cepacia* degrades 20–30 % of B*a*P in the presence of pyrene as a substrate after 63 days of incubation. *Mycobacterium* sp. strain RGJII-135 degraded 40 % of BaP after 32 days of incubation with yeast extract, peptone and starch as growth substrates (Schneider et al. 1996). Kanaly and Harayama (2010) noted rapid BaP mineralization kinetics with consortia growing on diesel fuel. In another study Luo et al. (2009) reported 22.6 % of 10 mg l^{-1} BaP by a consortium in 14 days. Although BaP has been detected in a variety of environmental samples, relatively few organisms have been reported to degrade completely (Zhou et al. 2008; Luo et al. 2009). Boonchan et al. (2000) demonstrated co-mineralisation of BaP by *Sphingomonas maltophilia* VUN10,010 grown on pyrene.

Fluoranthene and pyrene have an identical molecular weight and number of benzene rings, but different ring arrangement and solubility (Table 1). Fluoranthene has a pentane ring in the middle common for many HMW PAHs, which resists complete microbial attack. There may also be competitive inhibition when PAHs are present as mixtures. Such competition is expected when PAHs are metabolized by a common enzyme system (Bouchez et al. 1999). Overall the consortium-5 was able to degrade most of the PAHs mixtures supplied.

Many previous studies indicated that co-metabolism promotes rather than inhibits HMW PAHs degradation. Nuttapun et al. (2001) reported that *Sphingomonas* sp. could degrade PAHs mixtures phenanthrene, pyrene, and fluoranthene in minimal

medium. Yuan et al. (2000) reported that the degradation efficiency improved when acenapthene, fluorene, phenanthrene, anthracene and pyrene are present together compared to PAHs present individually. Cross-acclimation is the key for enhanced degradation of PAHs mixture.

When using consortia the metabolic cooperation of several microorganisms may result in enhanced PAH utilization, since metabolic intermediates produced by some organisms may serve as substrates for the growth of others (Boonchan et al. 2000). The major function of growth substrate during co-metabolism is to induce the enzymes needed to initiate the transformation of HMW PAHs. The induction of enzymes may be particularly important for the degradation of HMW PAHs, which do not induce their own degradation (Somtrakoon et al. 2008). The high degradative capacity of PAHs mixtures by bacterial consortium demonstrated in this study is superior to those reported in previous studies on PAHs mixtures (Mueller et al. 1989; Wiesel et al. 1993). McLellan et al. (2002) reported that phenanthrene and pyrene were potent inducers of BaP degradative activity. This is similar to our results in that LMW PAHs were more effective in improving degradation of HMW PAHs.

The bacterial consortium showed greater PAH degradation ability in soil slurries almost similar to the liquid culture experiments. The fluoranthene degradation was better increased in soil slurry than in liquid culture. This can be attributed to the continuous supply of other carbon sources from the soil, which could accelerate the rate of co-degradation. The natural attenuation (due to indigenous microbes) of PAHs in spiked soil slurries was not significant, which is not surprising given the soil had no previous history of PAH contamination. However, the MGP soil slurry showed varying ranges of degradation in all 16 US EPA PAHs. Weathered long-term MGP soil had less LMW PAHs compared to HMW PAHs, which could be due to loss of volatilization and biodegradation. Most of the remaining LMW PAHs were degraded by consortium-5. As expected, the PAH degradation decreased with an increase in its molecular weight and hydrophobicity. This consortium showed unique ability to degrade even the PAHs containing more than 5 rings. There is only limited information regarding the bacterial degradation of PAHs with five or more rings (Zhou et al. 2008). The consortium-5 could have the special ability to solubilize the high molecular weight PAHs by producing surfactants or any other extracellular polysaccharides. The fact that this consortium was able to successfully degrade the PAHs from real long term contaminated MGP soil which had several heavy metals (Cd, Pb, Zn) in addition to PAHs shows the great potential of this consortium for field scale bioremediation of MGP soils.

Conclusion

A bacterial consortium able to tolerate toxic metal Cd and effectively degrade PAHs has been isolated for the first time from a long term contaminated former MGP site soil. The ability of this consortium to utilise HMW PAHs such as pyrene and BaP as a sole carbon source in the presence of toxic metal Cd was demonstrated. Furthermore, this consortium has proven to be effective in degradation of HMW PAHs even from the real long term contaminated MGP soil. Thus, the results of this study demonstrate the great potential of this consortium for field-scale bioremediation of PAHs in long term mix contaminated soils such as MGP sites.

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