Marine Pollution Bulletin 57 (2008) 707–715

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

PAHs contamination and bacterial communities in mangrove surface sediments of the Jiulong River Estuary, China

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ARTICLE INFO

Keywords:
Polycyclic aromatic hydrocarbons (PAHs)
Mangrove sediments
Bacterial community
Denaturing gradient gel electrophoresis (DGGE)

ABSTRACT

Sixteen sediment samples collected from eight transects in a mangrove swamp of the Jiulong River Estuary, Fujian, China were investigated for their content of polycyclic aromatic hydrocarbons (PAHs) and the biodegradation potential of the indigenous microorganisms. The bacterial community structures in the mangrove sediments and in enrichment cultures were also investigated. The results showed that the total PAHs concentration of mangrove sediments ranged from 280 to 1074 ng g^{-1} dry weight, that the PAHs composition pattern in the mangrove sediments was dominated by high molecular weight PAH components (4–6 rings), and that Benzo[ghi]perylene and Indeno[1,2,3-cd]pyrene were the most dominant at different stations. Abundant PAH-degrading bacteria were found in all the stations, the values of phenanthrene-degrading bacteria ranged from 5.85×10^4 to 7.80×10^5 CFU g^{-1} dry weight, fluoranthene-degrading bacteria ranged from 5.25×10^4 to 5.79×10^5 CFU g^{-1} dry weight, pyrene-degrading bacteria ranged from 3.10×10^4 to 6.97×10^5 CFU g^{-1} dry weight and the benzo(a)pyrene-degrading bacteria ranged from 5.25×10^4 to 7.26×10^5 CFU g^{-1} dry weight. DGGE analysis of PCR-amplified 16S rDNA gene fragments confirmed that there was a remarkable shift in the composition of the bacterial community due to the addition of the different model PAH compound phenanthrene (three ring PAH), fluoranthene(four ring PAH), pyrene(four ring PAH) and benzo(a)pyrene(five ring PAH) during enrichment batch culture. Eleven strains were obtained with different morphology and different degradation ability. The presence of common bands for microbial species in the cultures and in the native mangrove sediment DNA indicated that these strains could be potential in situ PAH-degraders.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread in nature (i.e. soil, water and sediments) because of several polluting anthropogenic activities (Samanta et al., 2002). They have been recognized as a potential health risk due to their intrinsic chemical stability, high recalcitrance to different types of degradation and high toxicity to living organisms. The fates of these compounds in the environment and the remediation of PAH-contaminated sites are, therefore, of high public interest. It is essential to remove PAH compounds from the environment quickly and effectively, thus minimizing their adverse effects. The principal processes for their successful removal are currently believed to be microbial transformation and degradation (Gibson et al., 1975).

Mangrove ecosystems, which are important intertidal estuarine wetlands along the coastlines of tropical and subtropical regions, are exposed to anthropogenic contamination by PAHs from tidal water, river water and land-based sources. Elevated concentrations of PAHs (>10,000 ng g^{-1} dry weight) are recorded in mangrove sediments (Klekowski et al., 1994; Tam et al., 2001; Ke et al., 2002; Zhang et al., 2004). The high levels of productivity that are characteristic of most mangrove estuaries are strongly coupled to the activity of the rich and diverse microbial communities inhabiting them (Holguin et al., 2001). Unlike the oceanic realm where the bulk of organic matter cycling occurs in the water column, in shallow-water estuaries a substantial amount of organic matter settles to the bottom and is subsequently incorporated into the benthos, where it is degraded and modified by dense microbial communities (Köster et al., 2000). The mangrove's unique features of high primary productivity, abundant detritus, rich organic carbon and anoxic/reduced conditions make them a preferential site for the uptake and preservation of PAHs from anthropogenic inputs (Bernard et al., 1996).

PAHs present in soil may exhibit a toxic activity towards different plants, microorganisms and invertebrates. Microorganisms,

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doi:10.1016/j.marpolbul.2008.03.011

being in intimate contact with the soil environment, are considered to be the best indicators of soil pollution. In general, they are very sensitive to low concentrations of contaminants and rapidly respond to soil perturbation. An alteration of their activity and diversity may result, and in turn it will reflect in a reduced soil quality (Schloter et al., 2003). Further characterization of bacterial diversity, and an evaluation of the degradation capacities of the microorganisms inhabiting mangrove sediments, would provide new insights for improving the management of such environments. Recent advances in microbial ecology make it possible to combine molecular and culture-dependent approaches in order to describe bacterial diversity at environmental sites. Molecular methods based on 16S rRNA gene analyses are useful for describing community structures, while bacterial isolation is needed to characterize the degradation pathways. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA genes represents a powerful tool to study bacterial community structures in complex environments as well as in enrichment cultures (Muyzer and Smalla, 1998). However, the combination of both culture-independent and culture-dependent techniques might provide useful and complementary information on the structure of microbial communities. The application of molecular-biology-based techniques has improved our understanding of the composition, phylogeny, and physiology of metabolically active members of the microbial community in the environment (Al-Thukair et al., 2007; Widada et al., 2002).

The Jiulong River Estuary is one of the largest river/estuary systems in south China, with a length of 285 km and an area of 14741 km^2. It is the major source of freshwater to Xiamen's coastal waters. The estuary is characterized by intense agricultural activities and rapid industrial development, and represents a potential major source of pollutants in the Xiamen Special Economic Zone. Since 1986 it has resulted in significant stress to Xiamen Harbour and its surrounding environments. The organic pollution in Xiamen waters has increased steadily in recent years. Fugong mangrove is located at the south of the Jiulong River Estuary, and it has been named as a mangrove nature conservation area of Fugong, Fujian Province of China. The area of the mangrove community is about 670 km^2. Because of its special geographical location, it has been affected by the deposition of ship wastes, and discharge of industrial, livestock, mariculture and household waste and wastewater.

The present study therefore aimed to (1) determine the concentrations of PAHs and the number of PAH-degrading bacteria in the surface sediments of the mangrove; (2) enrich for PAHs degrading communities via successive subculturing to enhance degradation;(3) analyses the community structure of PAH-degrading bacteria using molecular biological techniques that target the 16S rRNA genes; and (4) examine the capability of a bacterial consortium enriched from the mangrove sediments to degrade PAHs.

2. Materials and methods

A flowchart is shown in Fig. 1 to illustrate the workflow of the present study.

2.1. Study area and sampling

In November 2006, sixteen sediment samples were collected along eight transects laid from the non-vegetated mudflat to the mangrove-vegetated mudflat in the Fugong Mangrove Nature Reservation Area, Fujian, China (117°54'–117°55'E, 24°22'–24°24'N). The sampling locations are shown in Fig. 2. Transect 1 (station 1) was on the non-vegetated mudflats; Transect 2 (station 2) was located at the start of the mangrove, Transect 3 (stations 3, 4 and 5) lay at the end of the fresh water; Transect 4 (stations 6, 7 and 8) were sited at the top of the Jiulong River Estuary mangrove;

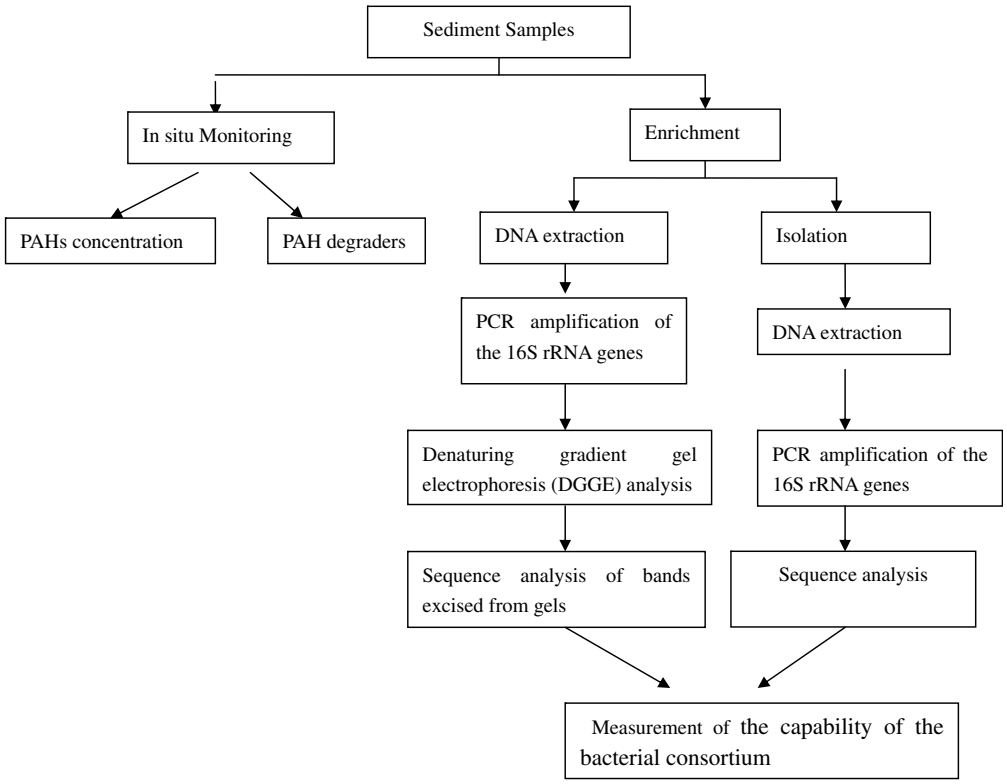

Fig. 1. Flowchart of the present study.

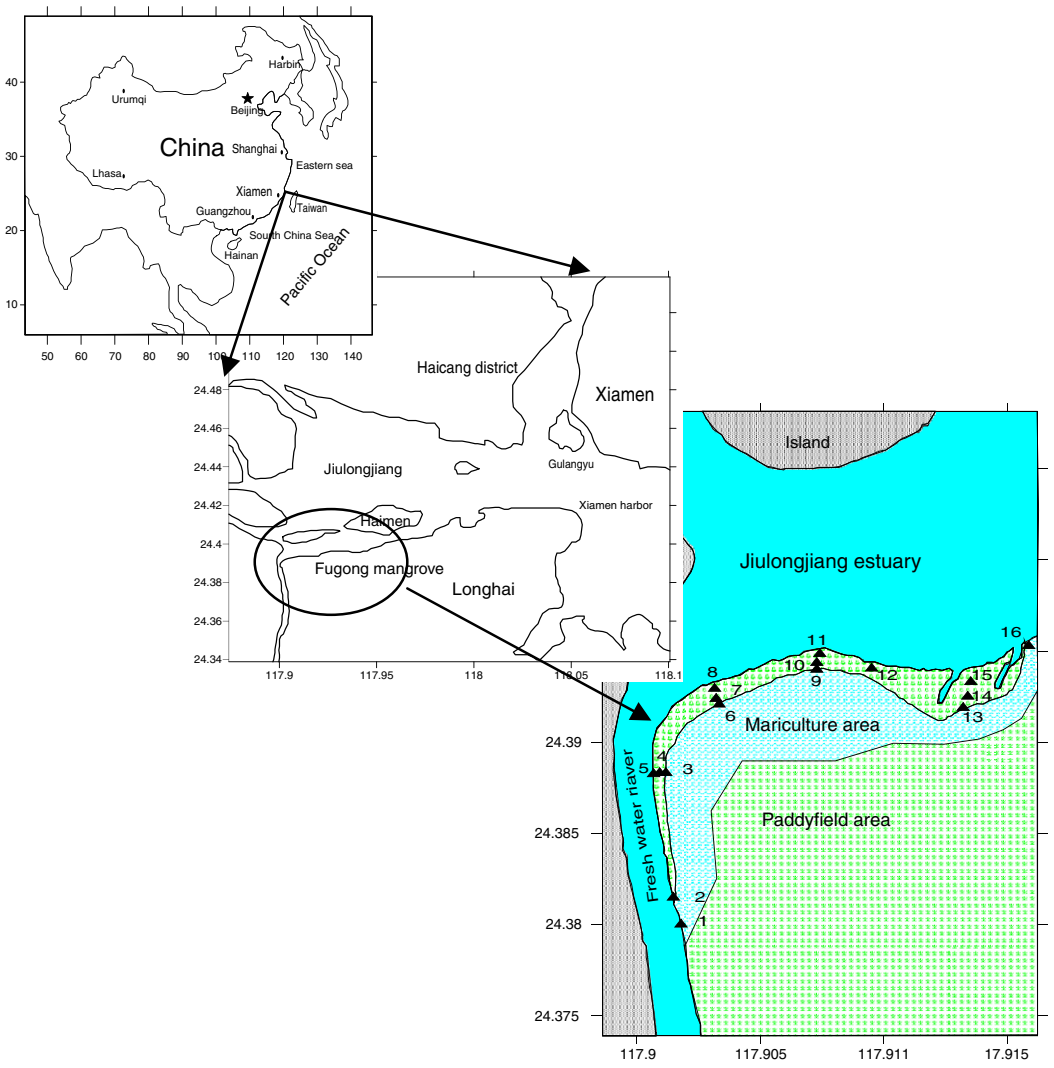

Fig. 2. Sampling location map.

Transect 5 (stations 9, 10 and 11) was at the midstream of the mangrove swamp; Transect 6 (station 12) was also a mudflat in the mangrove forest but not covered with plants; Transect 7 (stations 13, 14 and 15) was a typical mangrove region along the tidal channel where plants grow very well; and Transect 8 (station 16) was at the end of mangrove swamp and was not covered with any plants. The dominant species in this region of mangrove was *Kandelia candel*.

Surface sediment samples (0–5 cm) were randomly collected in triplicate from an area of around 1 m^2 at the centre of each mangrove transect area during low tide. Sediments samples were packed on-site into sealed polythene bags, and transported to the laboratory in an icebox, stored in the dark and kept frozen (−20 °C) prior to PAH analysis. Some sediment samples were transferred to sterilized plastic bottles (100 mL) for microbiological analysis within 24 h of collection. A global positioning system was used to determine the sampling positions.

2.2. Analysis of PAHs

A 10 g freeze-dried sediment sample was transferred to a 50 mL teflon capped bottle and then 2 μg d_{10}-phenanthrene was added to the dehydrated samples as an internal standard. These samples were treated in 20 mL of dichloromethane (DCM) for 15 min in an ultrasonic bath, and then the solvent was filtered through anhydrous sodium sulfate on a GF/F filter to remove the sediment particles. The filter was repeatedly washed with additional fresh DCM. The second and third extraction was conducted with 10 mL and 5 mL DCM, respectively. The three extracts were then combined and concentrated by rotary evaporation to 1 mL under a gentle stream of nitrogen. The extracted samples were cleaned with 3 g activated silica gel and 2 g anhydrous sodium sulfate in glass column (Ø = 11 mm). The aromatic hydrocarbon fraction was eluted with 15 mL of DCM–Hexane (2:3, v/v), then evaporated and the solvent changed to hexane. The fraction and a surrogate standard (2 μg of Naphthalene-d_8) were put into 2 mL autosampler vials. The species and concentrations of PAHs were analyzed using GC/MS (Varian Saturn 2000 GC/MS) installed with a fused silica capillary column (30 m × 0.25 mm i.d., Chrompack CP-Sil 8CB-MS) and Saturn workstation program. High purity He gas was used as a carrier gas. Oven temperature was programmed as follows: 2 min at 80 °C, then an increase of 5 °C/min to 315 °C and then held for 15 min. Aromatic hydrocarbon peaks were identified using the selective ion storage method.

2.3. Microbiological analysis

A spray-plate technique (Kiyohara et al., 1982) with modification was used to enumerate the PAH-degrading bacteria. The composition of the mineral salt medium (MSM) was as follows:

(mg L^{-1}) $(NH4)_2SO_4$ (1000), K_2HPO_4 (800), KH_2PO_4 (200), $MgSO_4$ (1000), $CaCl_2 \cdot H_2O$ (100), $FeCl_3 \cdot 6H_2O$ (5), and $(NH_4)_6Mo_7O_2 \cdot 4H_2O$ (1), 15 g agar. The medium pH was around 7.0–7.2. Each solid medium plate was incubated at 25 °C one night prior to inoculation. Sediment sample (10 g) with 90 mL sterile water were shaken (150 rpm) for 2 h in 250 mL conical flask and then settled for 30 min, the upper 1 mL was removed and decimal dilutions were prepared. Stock PAH solutions were prepared at a concentration of 0.5 mg mL^{-1} acetone for enumeration of the PAH-degrading bacteria. Acetone solutions (0.5 mL) of model PAH compounds (phenanthrene, fluoranthene, pyrene and benzo(a)pyrene) were sprayed onto the plates, and 0.1 mL of the diluted sediment subsample was added to each respective layer of PAH after acetone volatilization. After incubation at 25 °C for three weeks in the dark, PAH-degrading bacteria were determined by the presence of clear-zone-forming colonies on the mineral media plates coated with a crystal layer of the respective PAH (Bogardt and Hemmingsen, 1992; Kästner and Mahro, 1994).

2.4. Enrichment and isolation of the PAH-degrading microbial consortium

Sediment sample (10 g) obtained from the mixture of total 16 stations sediment samples was added to a 250-mL conical flask containing 90 mL MSM with the addition of a model PAH compound (phenanthrene, fluoranthene, pyrene and benzo(a)pyrene) at 50 mg L^{-1}. After 1 week incubation at 25 °C in the dark, 5 mL of the culture was transferred to another 250-ml conical flask containing 45 mL MSM with the same amount of PAHs for the second enrichment. The consortium was enriched in the laboratory for 2 months.

The bacterial colonies were isolated by streaking the enriched consortium on marine broth 2216E agar (peptone 5 g L^{-1}, yeast extract 1 g L^{-1}, $FePO_4$ 0.01 g L^{-1}, agar 15 g L^{-1}, pH 7.6–7.8) plates. Each individual colony was first identified by its color and morphology. The purified bacterium was then identified using 16 S rDNA gene nucleotide sequence analysis according to the method described below.

2.5. Biodegradation of PAHs by the consortium

All enrichment cultures were monitored periodically for the loss of PAHs. The consortium, precultured in marine broth 2216E for 24 h at 25 °C was harvested by centrifugation (6000*g*, 8 min) and inoculated into MSM medium containing 10 mg L^{-1} of the model PAH compound. Flasks containing 20 mL of medium with bacterial inoculum were placed in a rotary shaker (180 rpm) at 25 °C in the dark. MSM medium containing 10 mg L^{-1} PAHs, but no consortium acted as the control. Triplicate samples were taken from the experimental systems at day 0 and day 10 for extraction using the solid-phase extraction (SPE) method followed by analysis of residual PAHs using high-performance liquid chromatography (HPLC) with UV detection at 254 nm, and reversed-phase liquid chromatography with an Agilent Hypersil 4.0 × 250 μM ODS column. Methanol–water (90:10, v/v) was used with a flow rate of 1 mL min^{-1} as a mobile phase.

2.6. Molecular method

2.6.1. Extraction of total DNA

DNA was extracted from sediment samples, enrichment cultures and isolated strains. Total community DNA was extracted from all sediment samples using a PowerSoil DNA Isolation kit (MoBio Laboratories, Inc.). DNA was extracted from enrichment cultures and from the strains isolated according to the method described by Neumann et al. (1992), except that the enrichment cultures were filtrated through a 38 μm filter to remove any remaining PAHs. The DNA concentrations of extracts were measured spectroscopically and were adjusted for PCR amplification.

2.7. 16S rDNA amplification and PCR-DGGE analysis

The V3 region of bacterial 16S rDNA was amplified using primers 341f and 517r (Muyzer et al., 1993). Initial denaturation was at 94 °C for 45 s, amplification was carried out using 30 cycles including denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and DNA extension at 72 °C for 60 s, and final extension at 72 °C for 6 min. An aliquot of 5 μl of the PCR product was run in 1.5% agarose gel at 120 V for 45 min. DGGE was performed using a D-Code 16/16-cm gel system with a 1-mm gel width (Bio-Rad, Hercules, Calif.) maintained at a constant temperature of 60 °C in 7 L of 1 × TAE buffer. The acrylamide concentration in the gel was 8% and gradients were formed between 40% and 60% denaturant (with 100% denaturant defined as 7 M urea plus 40% [vol/vol] formamide). Gels were run at a constant voltage of 110 V for 11 h, at 60 °C. The gels were then stained with 0.5 mg L^{-1} ethidium bromide solution for 30 min, destained in 1 × TAE buffer for 15 min, and photographed.

2.8. Putative identification of bacterial species by sequencing of DGGE bands

Pieces of the DGGE bands of total community DNA to be sequenced were cut out with a sterile scalpel and placed in Eppendorf tubes containing 50 μL of sterilized milli-Q water. The DNA of each band was allowed to diffuse into the water at 4 °C overnight. After centrifugation at 10,000*g* for 6 min, 10 μL of the solution was used as the DNA template in a PCR reaction using the same conditions. PCR products were analyzed using DGGE to confirm that the expected products were isolated.

2.9. Identification of the isolates and the DGGE bands

Bacterial 16 S rRNA genes were amplified using the set of primers 27F (*Escherichia coli* position 8–27, 5V-AGA GTT TGA TCC TGG CTC AG-3V) and 1492R (*E. coli* position 1510–1492, 5V-GGC TAC CTT GTT ACG ACT T-3V) (Ikenaga et al., 2002). The conditions of the PCR were as follows: 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min.

The amplified 16S rDNA was purified from agarose gel and ligated with a pMD18-T vector. The resultant ligation products were transformated into *E. coli* DH5α competent cells and the clones with 1.5 kb insert were sent to be sequenced. The sequences were submitted to the GenBank database to search for similarity with sequences of other bacteria using the Blast alignment tool (Altschul et al., 1997).

3. Results and discussion

3.1. PAHs contamination and PAH-degrading bacteria in mangrove sediments

The total PAHs concentration of sediments ranged from 280.0 ng g^{-1} to 1206.8 ng g^{-1} dry weight, and the PAHs composition pattern in mangrove sediments was dominated by high molecular weight PAH components (4–6 rings), with benzo[ghi]-perylene and indeno[1,2,3-cd]pyrene being dominant at different stations (Table 1). These values were unlikely to cause any adverse biological effects as they were below the effects range-low values (Long et al., 1995) and lower than those in other mangrove sediments around the world such as Puerto Rico (Klekowski et al.,

Table 1
Concentrations of total PAHs and model PAHs, phenanthrene (Phe), fluoranthene (Flu), pyrene (Pyr) and benzo(a)pyrene (BaP), percentages of HMW-PAH to total and ratios of phenanthrene/anthracene (Phe/Ant) and fluoranthene/pyrene (Flu/Pyr) in sediment samples collected from the Fugong mangrove

Sediment	PAH concentrations (ng g^{-1} dw)					Percent of HMW-PAH to total (%)[b]	Specific PHA ratio	
	Total	Phe	Flu	Pyr	BaP		Phe/Ant	Flu/Pyr
Station 1	932.2	6.71	45.9	57.4	41.7	95.72	2.81	0.80
Station 2	741.5	5.72	24.6	59.8	73.8	95.35	2.18	0.41
Station 3	327.3	3.46	23.9	25.5	7.52	85.47	3.71	0.94
Station 4	519.0	ND	ND	39.4	34.4	97.59	NC[d]	NC
Station 5	760.3	ND	55.7	89.0	15.0	91.17	NC	0.63
Station 6	705.1	3.86	ND	91.5	96.5	95.77	0.75	NC
Station 7	1074.5	3.97	49.3	129.7	15.4	95.40	1.11	0.38
Station 8	385.8	4.45	ND	55.7	1.50	95.98	1.06	NC
Station 9	384.9	ND	27.3	35.2	11.3	95.5	NC	0.78
Station 10	719.8	1.50	30.5	38.7	128.1	95.4	0.91	0.79
Station 11	908.3	6.40	30.0	64.3	27.4	98.76	2.0	0.47
Station 12	280.0	3.45	14.6	37.4	8.54	95.44	1.16	0.39
Station 13	413.4	4.18	13.8	27.4	53.7	93.46	6.63	0.56
Station 14	564.5	1.84	ND	59.1	16.6	98.48	0.41	NC
Station 15	714.0	4.20	1.82	63.1	25.8	98.42	1.10	0.03
Station 16	943.2	4.30	34.2	65.3	26.1	98.07	2.79	0.52
ER-L[a]	4022	2404	600	665	430	NA	NA	NA[c]
ER-M[a]	44792	1500	5100	2600	1600	NA	NA	NA[c]

[a] Long et al. (1995), ER-L, effects range-low; ER-M, effects range-median.
[b] HMW-PAHs(high molecular weight-PAHs) including fluoranthene, pyrene, benzo(a)anthracene, benzo(a)pyrene, chrysene, dibenzo(a,h)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-c,d)pyrene and benzo(g,h,i)perylene.
[c] NA, not available.
[d] NC, not calculated due to the concentration of individual PAH compound being below the detection limits.

1994), the Caribbean Islands (Bernard et al., 1996) and Hong Kong (Tam et al., 2001; Yu et al., 2005). However, some high molecular weight PAH compounds such as benzo(b)fluoranthene, diben(a,h)anthracene, indeno[1,2,3-cd]pyrene and benzo(ghi)perylene were detected but their toxicity guidelines were not available, and so this might need a more detailed study. Table 1 also shows that the Phe/Ant ratio in Fugong sediments ranged from NC to 6.63, less than the ratio of 10 for pyrolytic source of contamination proposed by Soclo et al. (2000). On the contrary, their Flu/Pyr ratios (NC to 0.94) were less than 1 suggesting a petrogenic source of contamination. These results indicated that the PAHs in Fugong mangrove sediments might come from a mixture of pyrolytic and petrogenic sources.

The numbers of PAH-degrading bacteria in Fugong mangrove sediments were high at different stations, the values of phenanthrene-degrading bacteria ranged from 5.85×10^4 to 7.80×10^5 CFU g^{-1} dry weight (Table 2); fluoranthene-degrading bacteria ranged from 5.25×10^4 to 5.79×10^5 CFU g^{-1} dry weight; pyrene-degrading bacteria ranged from 3.10×10^4 to 6.97×10^5 CFU g^{-1} dry weight and the benzo(a)pyrene-degrading bacteria ranged from 5.25×10^4 to 7.26×10^5 CFU g^{-1} dry weight. The lowest value was found at station 2.

Table 2
PAH-degrading bacteria (CFU g^{-1} dry weight) in mangrove sediments

Sediments	Phe-degrading bacteria	Pyr-degrading bacteria	Flu-degrading bacteria	BaP-degrading bacteria
Station 1	1.08×10^5	8.25×10^4	1.07×10^5	1.39×10^5
Station 2	5.85×10^4	3.10×10^4	5.25×10^4	5.25×10^4
Station 3	6.45×10^5	6.97×10^5	5.29×10^5	7.26×10^5
Station 4	1.52×10^5	2.15×10^5	1.67×10^5	2.24×10^5
Station 5	4.64×10^5	4.68×10^5	5.79×10^5	4.86×10^5
Station 6	1.24×10^5	2.07×10^5	2.61×10^5	3.94×10^5
Station 7	3.66×10^5	2.47×10^5	3.94×10^5	3.05×10^5
Station 8	3.26×10^5	3.20×10^5	1.89×10^5	1.81×10^5
Station 9	3.61×10^5	3.09×10^5	3.49×10^5	3.59×10^5
Station 10	2.76×10^5	3.58×10^5	2.16×10^5	2.26×10^5
Station 11	3.08×10^5	6.02×10^5	4.57×10^5	4.57×10^5
Station 12	2.72×10^5	1.40×10^5	1.61×10^5	2.40×10^5
Station 13	5.77×10^5	4.09×10^5	3.76×10^5	4.49×10^5
Station 14	2.67×10^5	3.01×10^5	2.54×10^5	3.26×10^5
Station 15	7.80×10^5	4.75×10^5	3.40×10^5	2.03×10^5
Station 16	4.14×10^5	4.36×10^5	2.58×10^5	4.34×10^5

In a comprehensive study of the bacterial diversity of PAH-contaminated sites, Mueller et al. (1997) show that PAH degradation capabilities of bacteria are associated with a few phylogenetically distinct genera and are independent of geographic location. They also discussed the fact that although uncontaminated aquatic sites may naturally harbor bacteria which are capable of PAH degradation, it is unclear if PAH-degrading organisms could be recovered from uncontaminated soil sites. In our case, the concentrations of individual and total PAHs were lower than those in other developed areas, but abundant PAH-degrading bacteria were discovered in mangrove sediments. This suggested that the mangrove sediments might harbor different groups of PAH-degrading bacteria with high potential for PAH degradation.

3.2. DGGE analysis of microbial community structure

Enrichment of microorganisms in MSM supplemented with an individual PAH compound such as phenanthrene, fluoranthene, pyrene, benzo(a)pyrene as the sole carbon and energy source. The DGGE method was used to investigate the bacterial composition and community dynamic changes, and the DGGE profile demonstrated that it was feasible to enrich the PAH-degraders from the original mangrove sediment sample, and that the dynamic changes in the bacterial community were complex under the selective pressure represented by the presence of different PAH compounds from 3 to 5 rings PAH. As Figs. 3–6 show the composition of the bacterial community in enriched cultures were remarkably shifty from first subculture to fourth subculture, but when the enriched time was extended, the composition of the bacterial community tended to become stable. This suggested that the indigenous bacteria are very sensitive to contaminants and respond rapidly to PAH pollution. However, after the sixth subculture the composition of the bacterial community significantly varied under LMW-PAH (phenanthrene and fluoranthene) and HMW-PAH (pyrene and benzo(a)-pyrene) selective pressure. The number of dominant species in the pyrene and benzo(a)pyrene enrichment cultures were much higher

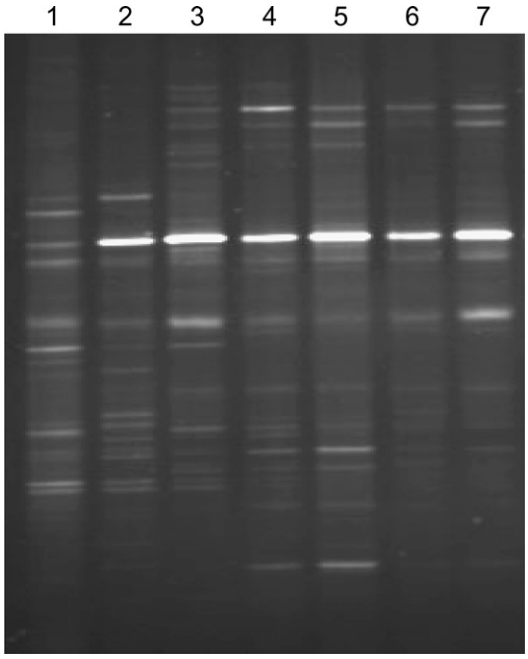

Fig. 3. DGGE analysis of 16s rDNA-V3 fragments obtained from enrichment culture under the selective pressure of phenanthrene. Lanes: 1, 1st subculture; 2, 2nd subculture; 3, 3rd subculture; 4, 4th subculture; 5, 5th subculture; 6, 6th subculture; 7, 7th subculture.

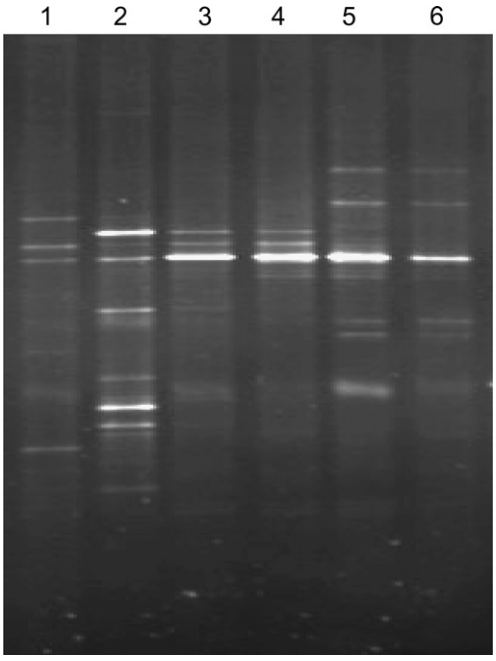

Fig. 4. DGGE analysis of 16s rDNA-V3 fragments obtained from enrichment culture under the selective pressure of fluoranthene. Lanes: 1, 1st subculture; 2, 2nd subculture; 3, 3rd subculture; 4, 4th subculture; 5, 5th subculture; 6, 6th subculture.

than those in the phenanthrene and fluoranthene enrichment cultures. It was therefore postulated in our case that low-molecular-weight hydrocarbons were metabolized by pure strains or simple compositions of species, while the biodegradation of high molecular weight hydrocarbons involved a co-metabolic mechanism, and therefore the whole process of high molecular weight mobilization and biodegradation required the combined efforts of different populations.

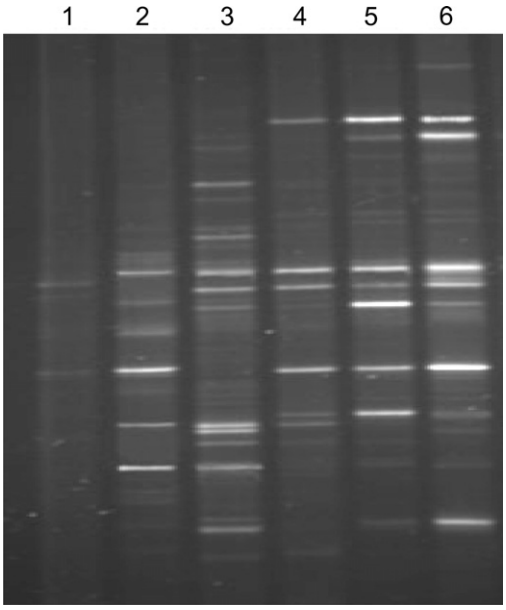

Fig. 5. DGGE analysis of 16s rDNA-V3 fragments obtained from enrichment culture under the selective pressure of pyrene. Lanes: 1, 1st subculture; 2, 2nd subculture; 3, 3rd subculture; 4, 4th subculture; 5, 5th subculture; 6, 6th subculture.

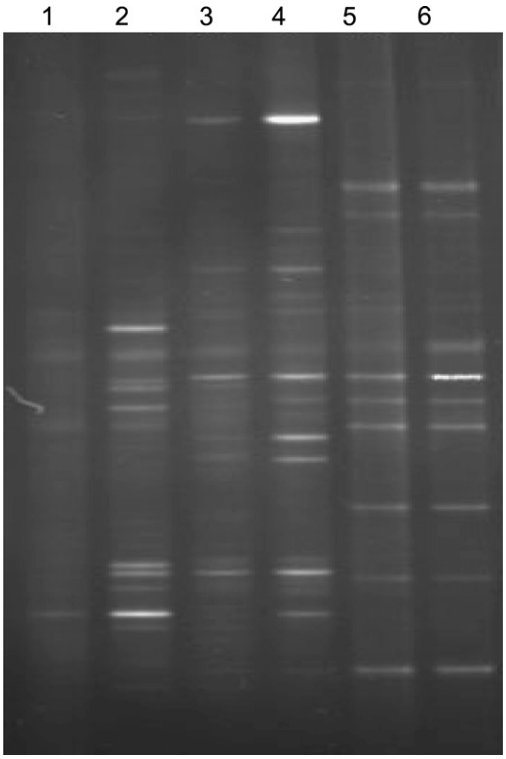

Fig. 6. DGGE analysis of 16s rDNA-V3 fragments obtained from enrichment culture under the selective pressure of benzo(a)pyrene. Lanes: 1, 1st subculture; 2, 2nd subculture; 3, 3rd subculture; 4, 4th subculture; 5, 5th subculture; 6, 6th subculture.

3.3. Position of isolates in the mixed culture analyzed by DGGE

The results of enrichment and DGGE analysis prompted us to isolate the strains from the mixed culture. Five strains, designated as F2, F4, P1, F16 and F19, were isolated from phenanthrene enrichment culture, and the bands of the strains isolated were well

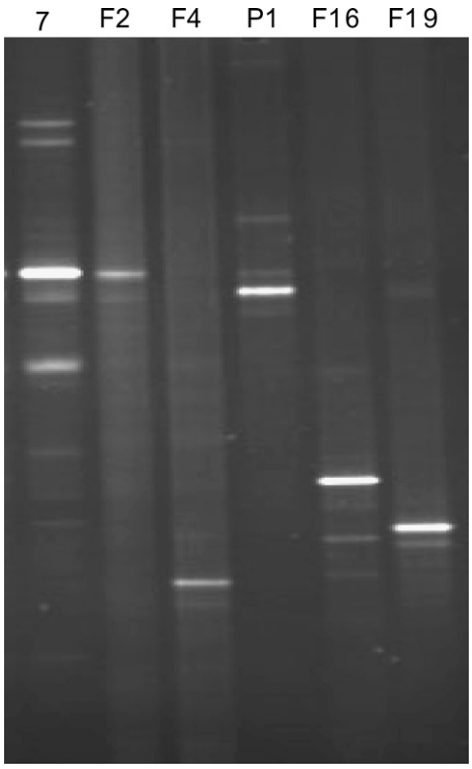

Fig. 7. DGGE analysis and comparison of V3 fragments derived from phenanthrene enrichment culture and bacterial isolates (Lane 1, 7: mixed culture at 7th subculture; lanes 2–6: isolates from the consortium).

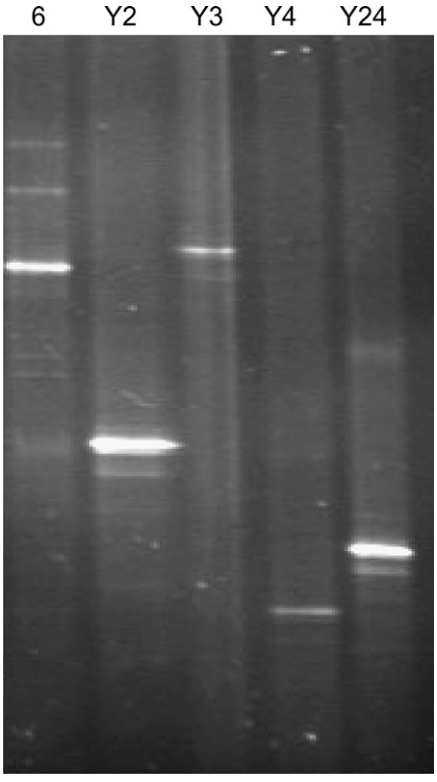

Fig. 8. DGGE analysis and comparison of V3 fragments derived from fluoranthene enrichment culture and bacterial isolates (Lane 1, 6: mixed culture at 6th subculture; lane 2–5: isolates from the consortium).

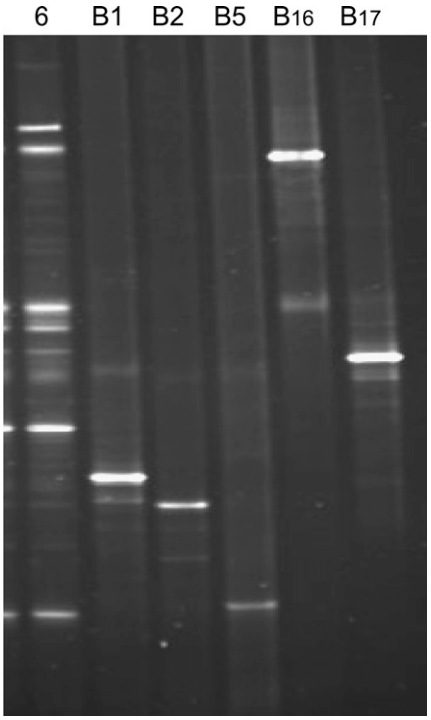

Fig. 9. DGGE analysis and comparison of V3 fragments derived from pyrene enrichment culture and bacterial isolates (Lane 1, 6: mixed culture at 6th subculture; lanes 2–6: isolates from the consortium).

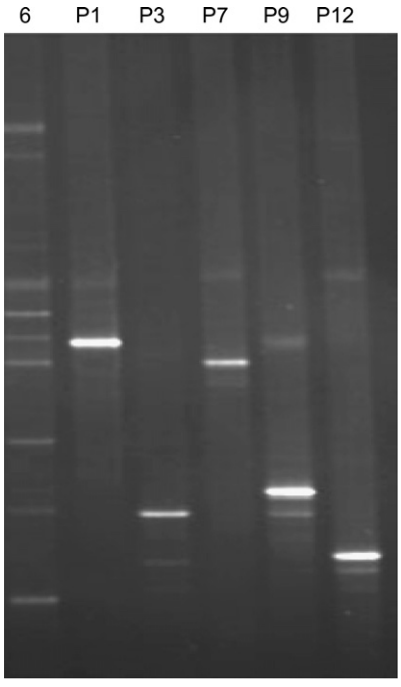

Fig. 10. 0 DGGE analysis and comparison of V3 fragments derived from benzo(a)-pyrene enrichment culture and bacterial isolates (Lane 1, 6: mixed culture at 6th subculture; lanes 2–6: isolates from the consortium).

correlated with those of the mixed culture in the DGGE profile (Fig. 7). Four strains, designated as Y2, Y3, Y4 and Y24, were isolated from fluoranthene culture, but the dominant species could not be isolated from the mixed culture (Fig. 8). Five strains, designated as B1, B2, B5, B16 and B17, were isolated from pyrene enrich-

Table 3
Sequence similarities of isolates

Strain	Closest organism in GenBank database	Accession no.	Similarity (%)	Phylogenetic group
F2/Y2	*Novosphingobium pentaromativorans* strain US6-1	AF502400	99	α-Proteobacteria
F4/Y4/B5	*Stappia* sp. M8	AY307927	99	α-Proteobacteria
B1/Y3	*Kordiimonas gwangyangensis* strain GW14-5	AY682384	99	α-Proteobacteria
B16	*Pseudoalteromonas* sp. A25	AF227237	98	γ-Proteobacteria
B17	*Marinobacter* sp. 61B	DQ629025	97	γ-Proteobacteria
P1	*Vibrio parahaemolyticus*	EF467290	99	γ-Proteobacteria
P7	*Marinobacterium litorale* strain IMCC1877	DQ917760	96	γ-Proteobacteria
F19/Y24	*Marinobacterium litorale* strain IMCC1877	DQ917760	94	γ-Proteobacteria
P9	*Thalassospira* sp. MACL12B	EF198251	99	α-Proteobacteria
P12	*Pseudomonas stanieri*	AB021367	98	γ-Proteobacteria
F16/P3/B2	*Roseobacter* sp. Y2	DQ120728	95	α-Proteobacteria

ment culture, and five strains, designated as P1, P3, P7, P9 and P12, were isolated from benzo(a)pyrene enrichment culture. The bands of the strains isolated were not well correlated with those of the pyrene and benzo(a)pyrene enrichment cultures in the DGGE profile (Figs. 9 and 10).

It was presumed that a five ring PAH such as benzo(a)pyrene was less well decomposed compared to lower molecular weight PAHs, such as phenanthrene, by indigenous microorganisms in the sediment environment. Although benzo(a)pyrene has been detected in a variety of environmental samples (Kanaly and Shigeaki, 2000a), so far, no microorganism is reported that can utilize benzo(a)pyrene as a sole source of carbon and energy. To date, all reported benzo(a)pyrene biotransformations by bacteria therefore occur under cometabolic conditions (Juhasz and Naidu, 2000). DGGE proved to be a valuable first step for obtaining information regarding the dynamics of the consortium and for tentatively identifying the relevant consortium members. The results confirmed the presence of sequence types which were closely related to known HMW-PAH-degrading genera.

3.4. Phylogenetic analysis of isolated strains

All of the isolated strains were identified on the basis of their 16S rDNA homologies with entries in the GenBank-EMBL databases. Accession numbers for isolates sequences and levels of similarity to related organisms are shown in Table 3. Analysis of 16S rRNA encoding sequences showed that the isolates were distributed within 10 distinct genera. Some of them were related to bacterial groups already known for their capacity to degrade hydrocarbons (such as *Pseudomonas*, *Marinobacter*, *Vibrio* and *Roseobacter*) in marine and mangrove sediments (Brito et al., 2006). F2 and Y2 had 99% identity with *Novosphingobium pentaromativorans* sp. US6-1 which degrades high-molecular-mass polycyclic aromatic hydrocarbons of two to five rings, was isolated from muddy sediment of Ulsan Bay, represents a novel species in the genus *Novosphingobium* (Sohn et al., 2004); B1 and Y3 had 99% identity with *Kordiimonas gwangyangensisstrain* GW14-5 which is a marine bacterium capable of degrading high-molecular-mass polycyclic aromatic hydrocarbons was isolated from the sediments of Gwangyang Bay, represents a novel genus and species in the '*Alphaproteobacteria*' (Kwon et al., 2005), other strains (F4/Y4/B5 and P9), were related to isolates *Stappia* sp. M8 and *Thalassospira* sp. MACL12B that have not been thus far detected in hydrocarbon-contaminated sites, nor described for their ability to grow or degrade petroleum hydrocarbons.

3.5. Degradation of PAHs by the bacterial consortium isolated from mangrove sediment

To gain a better understanding of the metabolic capabilities of the consortium, the rates of biodegradation of PAHs by two mixed cultures (M2 and M3) with different species composition were measured. The biodegradation rate of phenanthrene, fluoranthene, pyrene and benzo(a)pyrene are shown in Fig. 11. Phenanthrene (100%) was degraded by both mixed cultures after 10 days incubation. M3 showed higher PAH degradation capability for degrading HMW-PAH compounds, 44.2% of benzo(a)pyrene was degraded by M3 after 10 days incubation.

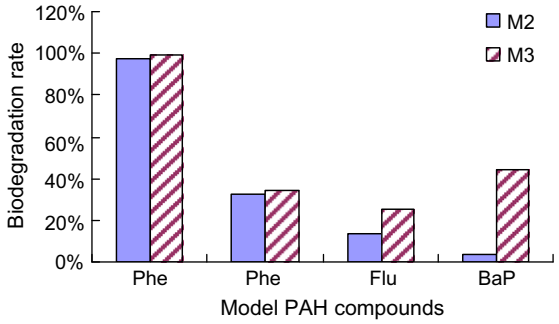

Fig. 11. 1 Biodegradation of PAH by the consortium enriched from mangrove sediments after 10 days incubation

Since the higher molecular weight PAHs, such as benzo(a)anthracene, chrysene or benzo(a)pyrene, are quite resistant to microbial attack, hitherto, the reported degradation rate of benzo(a)pyrene by microorganisms is mostly quite modest. Juhasz et al. (1996, 1997) reported that *Burkholderia cepacia* degrades 20–30% of benzo(a)pyrene in the presence of pyrene as a substrate, after 63 days of incubation. *Mycobacterium* sp. strain RGJII-135 (Schneider et al., 1996), cultivated with yeast extract, peptone and starch as a growth substrate, degraded 40% of benzo(a)pyrene after 32 days incubation. Kanaly et al. (2000b) observed rapid benzo(a)pyrene mineralization kinetics with consortia growing on diesel fuel.

4. Conclusions

PAHs composition pattern in mangrove sediments suggested dominance by high molecular weight PAH components (4–6 rings), and mangrove sediments harbor abundant PAH-degrading bacteria. Comparison of bacterial community structures under different selective pressures using PCR-DGGE analyses showed lower diversity for LMW-PAH enrichment cultures than for HMW-PAH enrichment cultures. The bacterial consortium enriched from mangrove sediments had a good PAH degradation capability. Our results pointed out the rich microbial diversity of the mangroves, whose potential for hydrocarbon degradation is promising for future studies on pollutant bioremediation.

Using the information obtained from DGGE, efforts are being made to determine which individual populations are most impor-

tant in the HMW-PAH biodegradation process and the use of these species in microcosm bioaugmentation trials could help in evaluating their in situ catabolic behaviour in the degradation of HMW PAHs in highly polluted environments.

Acknowledgements

This research project was supported by the National Natural Science Foundation of China (Grants Nos: 30530150, 40476047, 40576054), The NSFC-KOSEF Scientific Cooperation Program and the Program for Innovative Research Team in Science and Technology in Fujian Province University, and the Program for New Century Excellent Talents in Fujian Province University. Professor John Hodgkiss is thanked for his help with the English.

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