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Contamination and potential biodegradation of polycyclic aromatic hydrocarbons in mangrove sediments of Xiamen, China

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Abstract

Five stations were established in the Fenglin mangrove area of Xiamen, China to determine the concentrations of polycyclic aromatic hydrocarbons (PAHs) and the numbers of PAH-degrading bacteria in surface sediments. Assessing the biodegradation potential of indigenous microorganisms and isolating the high molecule weight (HMW)–PAH degrading bacteria was also one of the aims of this work. The results showed that the total PAH concentration of sediments was 222.59 ng g^{-1} dry weight, whereas the HMW–PAH benzo(*a*)pyrene (BaP) had the highest concentration among 16 individual PAH compounds. The variation in the numbers of PAH-degrading bacteria was 2.62×10^2–5.67×10^4 CFU g^{-1} dry weight. The addition of PAHs showed a great influence in increasing the microbial activity in mangrove sediments. A bacterial consortium, which could utilize BaP as the sole source of carbon and energy, and which was isolated from mangrove sediments and enriched in liquid medium for nearly one year degraded 32.8% of BaP after 63 days incubation.
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Keywords: Benzo(*a*)pyrene (BaP); Biodegradation; Contamination; Mangrove sediments; Polycyclic aromatic hydrocarbons (PAHs)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous persistent environmental contaminants generated by natural combustion processes and human activities. PAHs are considered hazardous because of their cytotoxic, mutagenic, and carcinogenic effects. 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.

Mangrove ecosystems, which are important inter-tidal 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) have been recorded in mangrove sediments (Klekowski et al., 1994; Tam et al., 2001; Ke et al., 2002).

Possible fates for PAHs released into the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, adsorption on soil particles, leaching, and microbial degradation (Cerniglia, 1992). The principle processes for their successful removal are currently believed to be microbial transformation and degradation (Gibson et al., 1975). Biodegradation potentials of microbial strains isolated from hydrocarbon-contaminated environments are as high as, or even higher than those originating from non-contaminated sediments since certain bacteria could have acclimatized and become adapted to

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the contaminated environment (Wild and Jones, 1986; Chaneau et al., 1999; Zhang et al., 2006).

Mangroves are highly productive (Holguin et al., 2001). Bacterial activity plays an important role in forming the high biomass. In tropical mangroves, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2%, respectively (Alongi, 1988). Mangrove's unique features of high primary productivity, abundant detritus, rich organic carbon and anoxic/reduced conditions make them a preferential site for uptake and preservation of PAHs from anthropogenic inputs (Bernard et al., 1996). Ramsay et al. (2000) report that the number of aromatic-degraders in mangrove sediments is very high (10^4–10^6 cells g^{-1} sediments) and such an indigenous community has a considerable potential to degrade oil components. Moreover, the number of oil-degraders could be increased by oil addition. Yu et al. (2005) also report that a bacterial consortium enriched from mangrove sediments has a good PAH degradation capability with 100% degradation of fluorene and phenanthrene after four weeks of growth. These results suggested that the mangrove sediments might harbor different groups of PAH-degrading bacteria with high PAH degradation capability.

In spite of the abundance of information on degradation of PAHs in pure bacterial culture, little work has been done on indigenous PAH-degrading bacterial communities in mangrove sediments and their biodegradation potential (Tam et al., 2002, 2003; Yu et al., 2005). Moreover, there is only limited information regarding the bacterial biodegradation of PAHs with five or more rings in both environmental samples and pure or mixed cultures (Kanaly and Shigeaki, 2000a). The present study therefore aims to (1) determine the concentrations of PAHs in surface sediments of mangrove; (2) enumerate PAH-degrading bacteria using phenanthrene (a 3 ring PAH), pyrene and fluoranthene (4 ring PAHs) as a model PAH mixture; (3) investigate the biodegradation potential of indigenous microorganisms in mangrove sediments; (4) examine the capability of a bacterial consortium enriched from mangrove sediments to degrade benzo(*a*)pyrene(a 5 ring PAH).

2. Materials and methods

2.1. Study area and sampling

A global positioning system (GPS) was used to determine the sampling positions. The sampling stations are shown in Fig. 1.

Surface sediment samples (0–10 cm) were collected with a soil corer during low tides and transferred to pre-cleaned brown glass bottles stored at −20 °C and kept frozen prior to PAH analysis. Some sediment samples were transferred to sterilized plastic bottles (100 mL) for microbiological analysis within 24 h of collection. Three replicates were collected at each station.

2.2. Analysis of PAHs

Freeze–dried sediment samples were passed through an 80-mesh screen-sieve and a 20 g sample was then transferred to a 250 mL pre-washed conical flask, and 1 g activated copper powder was added to remove sulphides. The sediment was spiked with 20 μL internal standard prior to extraction and then shaken with 100 mL trichloromethane and methanol (2:1 v/v), and sonicated in an ultrasonic bath (50 °C) for 20 min. The second extraction was conducted with 100 ml trichloromethane and methanol (1:2 v/v). The two extracts were then combined and concentrated by rotary evaporation under a gentle stream of nitrogen to 1 mL. The solvent extracts were fractionated by using a silica gel/alumina column (1 cm in diameter and 20 cm long), PAHs were eluted with 10 mL dichloromethane and hexane (2:1 v/v). The elute was concentrated by gentle nitrogen flow at 30 °C to about 100 μL.

Sixteen PAH compounds were identified and quantified using HP 6890 Plus GC with HP5973 MSD. The capillary column used for the analyses was an HP-5s (30 m × 0.25 mm × 0.25 μm). The oven temperature was programmed from 60 °C (initial time, 2 min) to 120 °C at a rate of 10 °C min^{-1}, 120 °C to 300 °C at a rate of 4 °C min^{-1} and held at 300 °C for 10 min. GC–MS data were acquired and processed using an HP 3365 Chemstation. All PAH concentrations were reported on a dry weight of sediment basis.

2.3. Microbiological analysis

A 10 g sediment sample with 90 mL sterile water was shaken (150 rpm) for 2 h in a 250 mL conical flask and then allowed to settle for 30 min. The upper 1 mL was removed and decimal dilutions were prepared.

The DAPI direct counting method was used to estimate the number of total bacteria and the spray-plate method was used to numerate the cultivation-dependent bacterial number on marine broth 2216E agar plates (Kästner and Mahro, 1994).

A spray-plate technique with modification was used to numerate PAH-degrading bacteria. The composition of the mineral salt medium (MSM) was as follows: (mg L^{-1}) $(NH_4)_2SO_4$ (1000), K_2HPO_4 (800), KH_2PO_4 (200), $MgSO_4$ (1000), $CaCl_2$ H_2O (100), $FeCl_3$ $6H_2O$ (5), and $(NH_4)6Mo_7O_2$ $4H_2O$ (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. Stock PAH solutions were prepared at a concentration of 0.5 mg mL^{-1} acetone for enumeration of PAH-degrading bacteria. Acetone solutions (0.5 mL) of the model PAH mixture (phenanthrene, pyrene and fluoranthene) were sprayed onto the plates, and 0.1 mL of the sediment dilutions as prepared above were added to each 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 mineral media

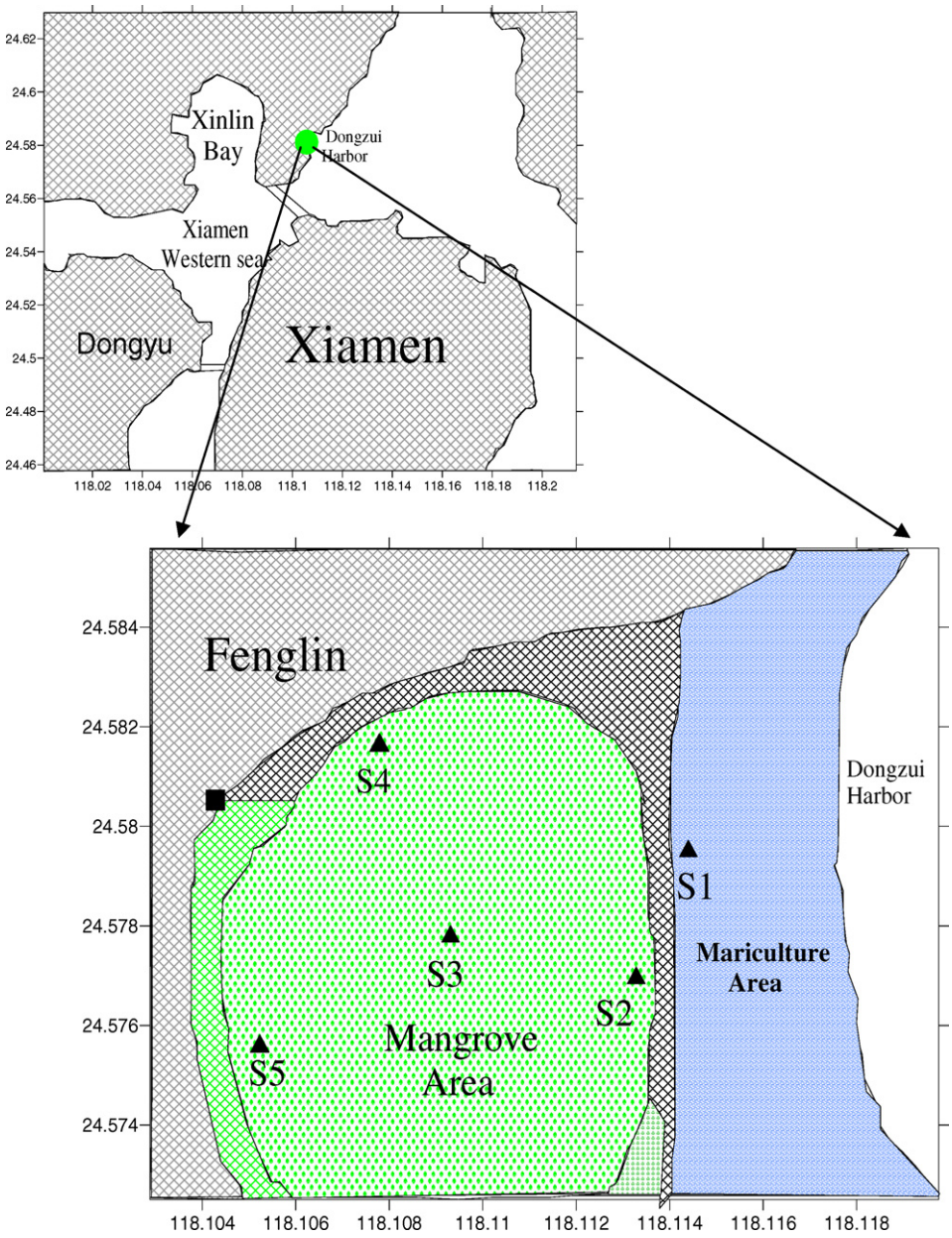

Fig. 1. Map of mangrove area showing sampling stations.

coated with a crystal layer of the respective PAH (Bogardt and Hemmingsen, 1992; Kästner and Mahro, 1994).

2.4. Measurement of microbial activity

Production of CO_2 was used to measure microbial activity in mangrove sediments. Sediment respiration was determined using the method described in Vokou and Liotiri (1999). In brief, sediment samples were put in airtight containers of approximately 100 mL capacity, and a 10 mL vial with 5 mL 0.1 M NaOH was placed on the top of the soil sample in each container. Respiration was estimated under the following conditions: (1) from sediments treated with model PAH mixture, (2) from sediments treated with glucose, (3) from sediments treated with PAHs and glucose, and (4) from untreated sediments as a control. Phenanthrene, pyrene and fluoranthene were used as the model PAH mixture, each at 10 mg L^{-1}, to give a total concentration of 30 mg L^{-1} of PAH mixture. Glucose was added to each treatment container at a concentration of 100 mg L^{-1}. Three replicates were used for each treatment. Experiments were conducted at 25 °C. The amount of CO_2 absorbed by NaOH was estimated by titration with 0.1 M HCl.

2.5. Enrichment, isolation and identification of the PAH-degrading microbial consortium

An aliquot of 10 ml sediment supernatant obtained by shaking 1:5 sediment/seawater (w/v) for 2 h was added to

a 250-ml conical flask containing 90 ml MSM with the addition of BaP at 20 mg L^{-1}. After four weeks 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 BaP for the second enrichment. The consortium was enriched in the laboratory for nearly one year.

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 by 16S rDNA gene sequence after amplification of the gene by PCR 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. PCR products were confirmed using agarose gel (1.0% w/v) electrophoresis, purified and 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).

2.6. Biodegradation of BaP

The consortium, pre-cultured in marine broth 2216E for 24 h at 25 °C, was harvested by centrifugation (6000*g*, 8 min) and inoculated into MSM containing 20 mg L^{-1} BaP. One hundred milliliter conical flasks containing 20 mL of medium with bacterial inoculum were placed in a rotary shaker (150 rpm) at 25 °C in the dark. MSM containing 20 mg L^{-1} BaP, but no consortium was the control. Samples were taken from the experimental systems every week (7, 14, 21, 28, 35, 42, 49, 56, 63 days) and extracted with methylene chloride, adding phenanthrene (20 mg L^{-1}) as the internal standard. The extractions were followed by quantitative analysis of residual BaP using Agilent 1100 series high-performance liquid chromatography (HPLC) with UV detection at 254 nm, using 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. The injection volume was 10 μL.

3. Results and discussion

3.1. PAH concentrations in mangrove sediments

The total sediment concentration of PAHs was 222.59 ng g^{-1} dry weight, whereas benzo(*a*)pyrene had the highest concentration among 16 individual PAH compounds (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., 1994), the Caribbean Islands (Bernard et al., 1996) and Hong Kong (Tam et al., 2001). However, some high molecular weight PAH compounds such as benzo(*b*)fluoranthene, diben(*a*,*h*)anthracene and benzo(*ghi*)perylene were detected but, since their toxicity guidelines were not available, this might need a more detailed study. Table 1 also shows that the Phe/Ant ratio in Fenglin sediments was 4.11, less than the ratio of 10 for the pyrolytic source of contamination proposed by Soclo et al., 2000. On the contrary, their Flu/Pyr ratio was less than 1 suggesting petrogenic source of contamination. These results indicate that the PAHs in Fenglin mangrove sediments might come from a mixture of pyrolytic and petrogenic sources.

3.2. PAH-degrading bacteria and PAH-biodegradation potential of mangrove sediments

The numbers of PAH-degrading bacteria in Fenglin mangrove sediments significantly varied among different stations, and the values ranged from 2.62×10^2 to 5.67×10^4 CFU g^{-1} dry weight (Table 2), and the highest value was found at station 4.

Microbial activity in soil has been measured traditionally using the rates of soil metabolic processes, since organisms make up most of the soil biomass. Some of the metabolic processes used to measure microbial activity in soil are the production of CO_2 and the consumption of O_2 (Regno et al., 1998). In this present study, production

Table 1
Concentrations of total PAHs and mixed PAHs, phenanthrene (Phe), fluoranthene (Flu), pyrene (Pyr) and Benzo(*a*)pyrene (BaP) and ratios of phenanthrene/anthracene (Phe/Ant) and Flu/Pyr in samples collected from the Fenglin mangrove of Xiamen

Soils	PAH concentrations					Specific PHA ratio	
	Total	Phe	Flu	Pyr	BaP	Phe/Ant	Flu/Pyr
	ng g^{-1} dw						
Fenglin mangrove soils	222.6	7.2	23.1	29.6	30.6	4.11	0.78
ER-L[a]	4022	240	600	665	430	NA	NA[b]
ER-M[a]	44792	1500	5100	2600	1600	NA	NA[b]

[a] Long et al. (1995), ER-L, effects range-low; ER-M, effects range-median.
[b] NA, not available.

Table 2
The number of total bacteria and PAH-degrading bacteria in the Fenglin mangrove sediments

Station	Total bacteria[a]	Total bacteria on 2216E	PAH-degrading bacteria
	cells g^{-1}	CFU g^{-1}	CFU g^{-1}
S1	6.26×10^9	1.50×10^6	6.67×10^3
S2	9.19×10^9	9.65×10^6	7.33×10^2
S3	5.17×10^9	4.71×10^6	3.33×10^3
S4	9.13×10^9	5.76×10^6	5.67×10^4
S5	5.40×10^9	2.63×10^6	2.62×10^2

[a] As determined by DAPI direct-count method.

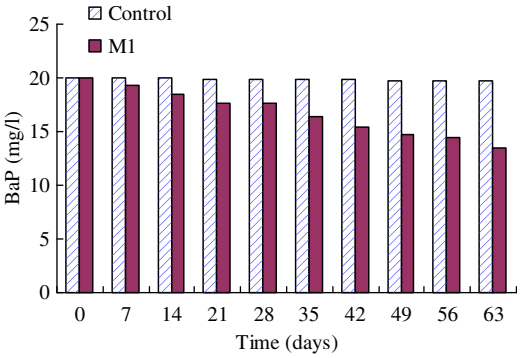

Fig. 3. Biodegradation of BaP by consortium M1.

of CO_2 was used to measure microbial activity in mangrove sediments containing PAHs and other carbon compounds, in order to determine the PAH-biodegradation potential of microorganisms in mangrove sediments. Fig. 2 compares sediment bioactivity (measured as CO_2 production) with added glucose; added glucose and PAHs; added PAHs; and with nothing added (the control) at the five stations.

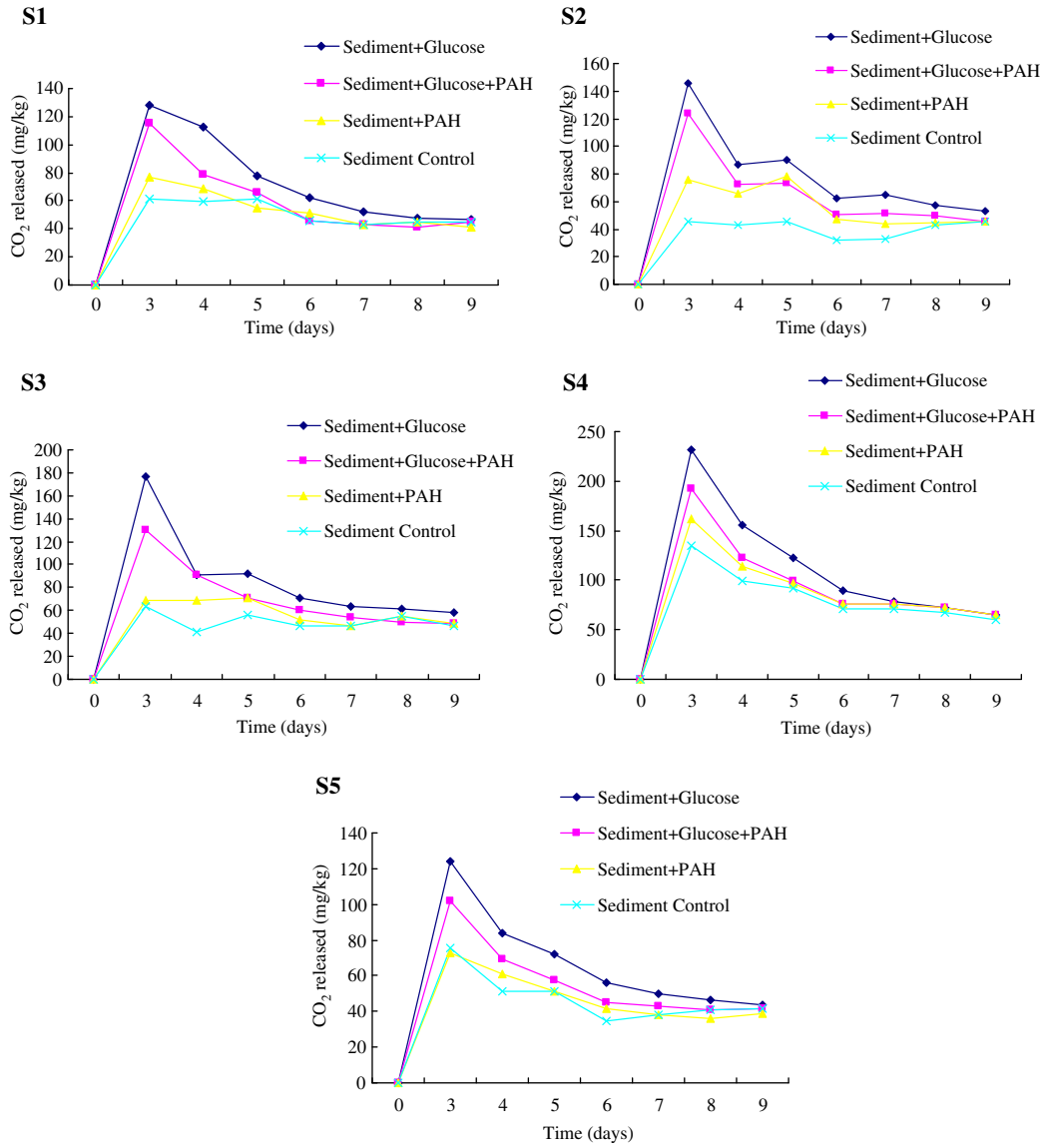

Fig. 2. Effect of the addition of different carbon sources on bioactivity.

The highest value for CO_2 evolution was at station 4, which also had a high number of total bacteria and PAH–degrading bacteria (Table 2). Availability of an easily used carbon source (glucose) enhanced production of CO_2 but did not enhance the degradation of PAHs in the Fenglin mangrove sediments, indicating that carbon is not a limiting factor. This suggested that the microorganisms in this mangrove area have high bioactivity, the capability of adapting to an unfavourable environment, and a high potential to degrade PAHs.

3.3. Biodegradation rate of BaP by the microbial consortium (M1) enriched from mangrove sediments

After 63 days of incubation with BaP as the sole carbon and energy source, 32.84% of the BaP was degraded by the mangrove consortium M1 (Fig. 3). There was degradation from the first week, and after the fourth week, the degradation increased (11.53% was degraded). By the end of the ninth week, 32.84% of BaP (20 mg L^{-1}) had been degraded while the control, which contained BaP without the consortium, only decreased by 1.2%.

Since BaP cannot be utilized both as a carbon and energy source for single microorganisms (Cerniglia, 1992), it is necessary that a growth substrate be supplied to initiate growth of the organism and to induce the production of catabolic enzymes. However, the supply of additional carbon sources may increase the oxygen demand in contaminated sites which could affect aerobic degradation processes and, in addition, the supplied carbon sources may be selective for heterotrophic microorganisms other than BaP degraders (Chen and Aitken, 1999; Kim et al., 2004). Consortium M1 was cultivated in the laboratory, utilizing BaP as the sole carbon and energy source, for nearly a year. Mixed bacteria cometabolize BaP, and thus avoid the disadvantages of supplied carbon sources.

Juhasz et al. (1996, 1997) report that *Burkholderia cepacia* degrades 20–30% of BaP in the presence of pyrene as a substrate, after 63 days incubation. *Mycobacterium* sp. strain RGJII-135 (Schneider et al., 1996), after 32 days incubation, degrades 40% of BaP, cultivated with yeast extract, peptone and starch as a growth substrate. Kanaly et al., (2000b) observe rapid BaP mineralization kinetics with consortia growing on diesel fuel. The mineralization rate in our study was quite modest in comparison to other reported studies. However, consortium M1 can cometabolize BaP as the sole carbon and energy source, whereas other studies were all based on bacteria growing on BaP with other substrates.

3.4. Isolation and identification of the PAH-degrading microbial consortium

Five bacterial isolates were obtained from consortium M1 on 2216E agar plate according to their colony color, shape and diameter, and all of them were gram-positive. The resultant sequences (M1 5-1: 1485 bp, M1 5-2: 1414 bp; M1 5-3: 1489 bp; M1 5-4: 1421 bp; M1 5-5: 1447 bp) were submitted and compared against the 16S rRNA gene sequences held in the Genbank and EMBL (The European Molecular Biology Laboratory). GenBank accession numbers were **AY762054–AY762058**. The phylogenetic trees (Fig. 4) were rooted using *Arthrobacter protophormiae* DNA (APRDNA) as the outgroup. RDP release 8.1 was used for all analyses. Phylogenetic analysis was carried out using 1008 unambiguously aligned nucleotide positions.

Based on the result, the closest relative of M1 5-1 was *Gordona bronchialis* (84.67% 16S rRNA gene sequence identity), indicating that M1 5-1 may represent a new genus. M1 5-2 had 99.504% identity with *Rhodococcus rubber* and *R.* sp.E33, revealing that this strain belonged to the genus *Rhodococcus.* M1 5-3 belonged to the genus *Exiguobacterium*, its identity with *E. marinum* was 97.106% and with *E. gaetbuli* 97.039%. M1 5-4 had 98.236% identity with *Arthrobacter protophormiae*, which indicated M1 5-4 belonged to this genus. The 16S rDNA sequence of M1 5-5 was 98.045%, identical to *Bacillus aquimaris* strain M, which indicated that it belonged to this genus.

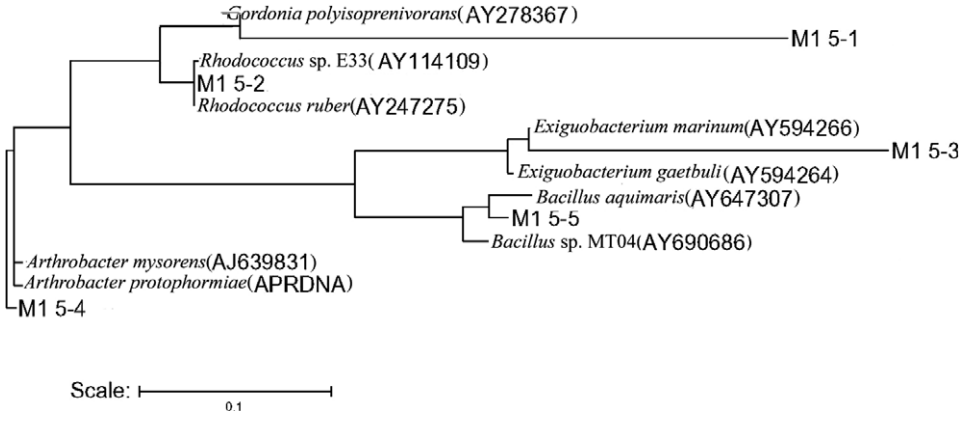

Fig. 4. Neighbor-joining phylogenetic tree with five isolated bacteria and their closest relatives derived from EMBL (The European Molecular Biology Laboratory) based on 16S rRNA gene analysis. Scale bar represents 0.1 substitution per nucleotide position.

Rhodococcus and *Bacillus* have been documented to have the ability of degrading BaP. They are also reported to have the capability of utilizing other PAHs, including naphthalene, anthracene, fluoranthene, chrysene and pyrene etc., as sole carbon and energy sources (Cerniglia, 1992). Although there is no literature concerning biodegradation of BaP by *Arthrobacter*, degradation of other PAHs such as anthracene is reported by Juhasz and Naidu (2000).

4. Conclusions

The present study showed that the total PAH concentration in the Fenglin mangrove sediments was less than those in other mangrove soils, although PAH-degrading bacteria were abundant in the sediments, and numbered more than 10^4 CFU g^{-1}, and the addition of PAHs increased the microbial activity in the sediment. The microorganisms of the mangrove sediments showed high bioactivity, the capability of adapting to an unfavourable environment and the potential to degrade PAHs. The enriched bacterial consortium from mangrove sediments contained five bacterial isolates, which could utilize BaP as the sole source of carbon and energy, and which degraded 32.8% of BaP after 63 days incubation. These genera can metabolize aromatic compounds and become dominant in polluted sediment environments.

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