

PAHs contamination and PAH-degrading bacteria in Xiamen Western Sea

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ABSTRACT

Six stations were established in Xiamen Western Sea (24°29' N, 118°04' E) on summer and autumn cruises in July and October 2001 to investigate the level of polyaromatic hydrocarbons (PAHs) and PAHs-degrading bacteria in surface water and sediments. PAHs in the surface water were mainly dominated by low molecular weight PAH compounds (2–3 rings PAH) in July and by high molecular weight PAH compounds (4–6 rings PAH) in October. PAHs in sediments were mainly dominated by high molecular weight PAH compounds (4–6). The percentage of 4–6 rings PAH to total PAHs ranged from 80.4 to 94.9% in July and 78.3 to 88.7% in October. No correlation was found between Fluoranthene-degrading bacteria numbers and Fluoranthene concentration among different stations in the surface water on the two cruises, and the same situation occurred between Pyrene-degrading bacteria numbers and pyrene concentration. But the numbers of fluorene- and phenanthrene-degrading bacteria were positively related to the fluorene and phenanthrene concentrations respectively. In the sediments, a significant positive relationship was found between PAH-degrading bacteria and PAHs concentration, except at station 6 on the two cruises.

Keywords: PAHs, concentration, PAH-degrading bacteria

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous persistent environmental contaminants generated by natural combustion processes and human activities. The PAHs are considered hazardous because of cytotoxic, mutagenic, and carcinogenic effects. Sixteen individual PAH compounds have been identified as priority pollutants by the United States Environmental Protection Agency (USEPA) (Varanasi, 1989). The fates of these compounds in the environment and the remediation of PAH-contaminated sites are, therefore, of high public interest.

In the marine environment, the fate of pollutants is largely determined by biogeochemical process. Some

of these chemical changes enhance the toxicity of the pollutants. Other chemical changes cause the degradation or immobilization of pollutants and as a result, act to purify the waters (self-purification of the marine environment). Possible fates for PAHs, released into the environment, include volatilization, photo-oxidation, chemical oxidation, bioaccumulation and adsorption on soil particles, leaching and microbial degradation (Cerniglia, 1992). The principal processes for their successful removal are currently believed to be microbial transformation and degradation (Gibson *et al.*, 1975). Many studies reported that PAHs-degrading microorganisms were related to the concentration of PAHs in the environment. Bogardt *et al.* (1992) used the over-layer technique to enumerate the numbers of phenanthrene-degrading bacteria to evaluate petroleum-contaminated sites. They reported that diesel fuel-or creosote-contaminated soil and water that were undergoing bioremediation contained 6×10^6 to 100×10^6

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phenanthrene-degrading bacteria per g and $ca 5 \times 10^5$ phenanthrene-degrading bacteria per mL, respectively, whereas samples from untreated polluted sites contained substantially lower numbers. Unpolluted soil and water contained no detectable phenanthrene degraders (desert soil) or only very moderate numbers of these organisms (garden soil, municipal reservoir water). McNally *et al.* (1998) also reported that the species of PAH-degrading bacteria in Keweenaw Bay were less than that in Trenton Bay, and attributed this to greater contamination at the latter Geiselbrecht *et al.* (1996) employed PAH-MPN method to enumerate the naphthalene- and phenanthrene-degrading bacteria in Puget Sound sediments. They found that sediments from a creosote-contaminated contained from 10^4 to 10^7 PAH-degrading bacteria per g of sediment (dry weight), whereas the concentration at an uncontaminated site ranged from 10^3 to 10^4 per g. These studies demonstrated the possibility that PAH-degrading microorganisms was a kind of biomarker of PAH contamination in the environment.

In a given area, the PAHs contents were often affected by several processes dependent on hydrodynamic sources, solubility, adsorption ability, grain size of particles and seasonal change, etc. while the chemical stability, hydrophobicity, bioavailability, concentration of PAHs and pro-exposure time were important factors that affect PAHs-degrading microorganisms. The marine environment is also one in which measurements are difficult because humans introduce materials into the ocean through great variety of mechanisms. Thus, the evaluation of the coastal zone PAHs pollutant by investigating PAH-degrading microorganisms is extremely complex. In this present study, six stations were established in Xiamen Western Sea ($24^{\circ}29' N$, $118^{\circ}04' E$) on Summer and Autumn cruises in July and October 2001 to investigate the level of PAHs in surface water and sediments and used phenanthrene and fluorene (three rings PAH), pyrene and fluoranthene (four rings PAH) as model PAH compound respectively to enumerate PAHs degrading bacteria. The aim was to determine the distribution and source of PAHs and possibility of using PAHs-degrading bacteria as a monitoring index for the evaluation of the level of pollution by PAHs in the coastal marine environment.

MATERIALS AND METHODS

Study area

Xiamen harbour ($24^{\circ}29' N$, $118^{\circ}04' E$) is a semi-enclosed bay due to the presence of a dike connecting Xiamen Island and Mainland China. The water depth ranges from 6 to 25 m with a deep-water coastline of about 30 km, thus providing excellent conditions for harbor activities. The rapid development of Xiamen Economic

Special Zone has resulted in great environmental stress to Xiamen Harbor waters.

Global Positioning System (GPS) was used throughout to determine the sampling positions. The sampling stations in this area are shown in Figure 1. Station 1 is an offshore site; station 2 is located in the channel between Xiamen and Songyu island; station 3 is near Yuandang lake, a municipal sewage-accepting lagoon; station 4 is near Dongdu, a port receiving waste discharge; station 5 is located oil port; station 6 is in the inner bay, close to a mariculture site.

Sampling

Sub-surface (0.5 m) water was sampled from the Xiamen Western Sea on 10 July and 8 October 2001 respectively. Samples were taken using a special water sampler and transferred to pre-cleaned brown glass bottles (5L) for PAHs analysis and sterilized plastic bottles (100mL) for microbiological analysis respectively. Samples were analyzed at laboratory within 24h after collection.

Surface sediment samples were collected with a grab sampler and transferred to pre-cleaned brown glass bottles stored at $-20^{\circ}C$ and kept frozen prior to PAHs analysis, some sediment samples were transferred to sterilized plastic bottles (100mL) for microbiological analysis within 24h after collection.

PAHs analysis

Surface water samples were extracted using a liquid-liquid extraction (G. Witt, 1995; R.J.law *et al.*, 1997).

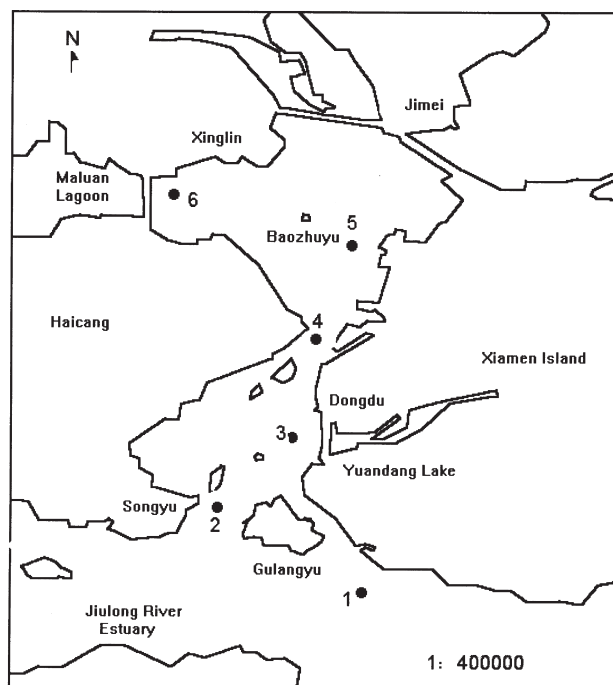


Figure 1 Map of Xiamen Western sea showing sampling stations.

1000 mL surface water samples with 10 μ L internal standard and 100 mL dichloromethane were extracted at one time, and then with 50 mL dichloromethane were extracted twice. The extracts were then combined and concentrated by gentle nitrogen flow.

Sediment samples were filtered through an 80-mesh screen-sieve after freeze-drying. 20 g freeze-dried sediment sample were transferred to 250-mL pre-washed conical flask and 1 g activated copper powder was added to remove sulphides. The sediment was spiked with 20 μ L internal standard prior to extraction. The sediment samples were 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 and under a gentle stream of nitrogen to 1 mL. The solvent extracts were fractionated by 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 μ L.

16 PAH compounds were identified and quantified by HP 6890 Plus GC with HP5973 MSD. The capillary column used for the analyses was a HP-5 (30 m \times 0.25 mm i.d. \times 0.25 μ m). The oven temperature was programmed from 60°C (initial time, 2 min) to 120°C at a rate of 10°C/min, 120°C to 300°C at a rate of 4°C/min and held at 300°C for 10 min. GC-MS data were acquired and processed using HP 3365 HP Chemstation. All PAHs concentrations were reported on a dry weight sediment basis.

PAH-degrading bacteria analysis

Surface water samples were shaken by hand for 1 min, 1 mL water sample was removed and decimal dilutions were prepared. 10 g sediment sample with 90 mL sterile water were shaken (150 rpm) for 2 hours in 250 mL conical flask and then settled for 30 min, the upper 1 mL was removed and diluted as described above.

A spray-plate technique (Kiyohara *et al.*, 1982) with modification was used to enumerate PAH-degrading bacteria. The composition of the mineral salt medium (Mueller *et al.*, 1989) was as follows: (mg/L) $(\text{NH}_4)_2\text{SO}_4$ (1000), K_2HPO_4 (800), KH_2PO_4 (200), MgSO_4 (1000), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (100), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5) (Weissenfels *et al.*, 1990), and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (1), 15 g agar at pH 7.2. Each solid media plate was evenly divided into nine panes by lining on the culture dish bottom and was incubated at 25°C one night prior to inoculation. 10 μ L of an acetone solution of model PAHs compounds (1 mg/mL) were added to the center of each pane of agar plate by Micro Pipette (Nichiryo, NPX-100). 10 μ L dilutions were added to layer of respective PAH after acetone volatilization. After incubation at 25°C for

three weeks in the dark, PAH-degrading bacteria were determined by clear-zone-forming colonies on mineral media coated with a crystal layer of the respective PAH (Kastner *et al.*, 1994).

Environment factors analysis

Salinity, temperature and pH of surface water were measured at sampling site by ocean current meter (Andelar, RCM 9) and Orion 828 pH meter respectively.

The sediment samples were immediately ultracentrifuged (3000 rpm) at 4°C to obtain sediment porewater after collection for salinity, pH analysis.

RESULTS AND DISCUSSION

PAHs concentration and profiles

Total PAH concentration in surface water varied among the different stations as well as between two cruises (Table 1). The values ranged from 99.0 ng L⁻¹ to 551.2 ng L⁻¹ on July cruise. The highest level was found at station 2 located in the channel between Xiamen and Songyu Island. The values ranged from 241.7 ng L⁻¹ to 478.8 ng L⁻¹ on October cruise, and the highest concentration was found at station 3 where received sewage discharges.

The PAH composition in surface water varied significantly between two cruises. PAH in surface water were mainly dominated by low molecular weight PAH compounds (2–3 rings PAH) except station 1 in July, but in October the dominant PAH compounds were high molecular weight PAH compounds (4–6 rings PAH). In a given area, the PAHs contents were often affected by several processes dependent on hydrodynamic sources, *e.g.* solubility, adsorption ability, grain size of particles and seasonal change. Surface water conditions can change very rapidly, especially those affected by tidal coastal waters. These suggest that the PAHs contents were very unstable in the surface seawater.

Total PAH concentration in sediments were relatively homogenous among the different stations and between two cruises, except for station 6, which is located in a densely maricultured area, varying from 683.6 ng g⁻¹ to 2954.2 ng g⁻¹ on July cruise, 564.1 ng g⁻¹ to 2656.0 ng g⁻¹ on October cruise. The highest concentrations of total PAH were both found at station 6 on the two cruises (Table 2).

In contrast to PAHs in the seawater, the PAH composition in the sediments showed remarkable similarity among different stations and between two cruises. PAHs in the sediments were mainly dominated by high molecular weight PAH compounds (4–6). The percentage of 4 to 6 rings PAH to total PAH ranged from 80.4% to 94.9% in July and 78.3% to 88.7% in October. PAHs are hydrophobic compounds and their persistence within ecosystems is due chiefly to their low water solubility. PAHs rapidly become associated with sedi-

Table 1 Concentration of PAHs in surface water of Xiamen Western Sea

Stations	Total PAHs (ng L ⁻¹)		% of 4–6 rings PAH to total		Phenanthrene concentration (ng L ⁻¹)		Fluorene concentration (ng.L ⁻¹)		Fluoranthene concentration (ng L ⁻¹)		Pyrenn concentration (ng L ⁻¹)	
	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.
1	144.8	241.7	64.8	72.9	4.2	21.1	10.1	5.1	2.6	0.0	2.8	29.9
2	551.2	333.7	10.6	69.7	25.1	37.2	50.2	7.0	4.2	0.0	4.7	14.2
3	243.0	478.8	32.1	78.3	24.0	3.0	25.4	17.0	5.0	0.0	6.2	26.7
4	228.7	259.1	25.8	78.1	7.6	21.7	24.8	4.9	1.8	0.0	2.0	7.6
5	186.6	312.5	26.8	73.2	3.2	24.3	23.3	4.8	2.3	0.0	2.6	10.6
6	99.0	284.9	32.1	72.3	13.6	24.4	12.3	5.0	2.8	0.0	3.0	10.5

Table 2 Concentration of PAHs and ratios of different individual PAH compounds in sediment of Xiamen Western Sea

Stations	Total PAHs (ng L ⁻¹)		% of 4–6 rings PAH to total		Phenanthrene concentration (ng L ⁻¹)		Fluorene concentration (ng.L ⁻¹)		Fluoranthene concentration (ng L ⁻¹)		Pyrenn concentration (ng L ⁻¹)	
	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.
1	1097.4	821.8	87.3	79.8	110.0	99.9	0.0	24.2	260.1	141.7	192.6	127.6
2	795.4	875.3	90.1	86.9	70.5	79.2	3.4	0.0	138.1	147.4	111.0	120.7
3	1028.5	564.1	91.3	78.3	66.9	85.9	3.0	7.2	155.9	151.1	140.5	143.9
4	1010.2	846.7	80.4	86.9	95.4	99.8	5.25	6.0	189.9	179.8	243.5	150.8
5	683.6	857.5	87.4	86.3	71.5	91.1	4.0	0.0	207.9	148.5	183.3	128.6
6	2954.2	2656	94.9	88.7	134.3	217.6	3.4	9.0	1327.5	939.0	1060.1	866.9

ments where they may become buried and persist until degraded, resuspended, bioaccumulated, or removed by dredging (Cerniglia, 1992).

PAH-degrading bacteria

The numbers of fluorene-, phenanthrene-, fluoranthene-, and pyrene-degrading bacteria in the surface water significantly varied among different stations and between two seasons (Table. 3). The four kinds of PAH-degrading bacteria numbers on the summer cruise were higher than that on the autumn. The lowest value of PAH-degrading bacteria numbers were all found at station 6 on the summer cruise, but the same situation was not found on the autumn.

The numbers of fluorene-, phenanthrene-, fluoranthene-, and pyrene-degrading bacteria in the sediments significantly varied among different stations and between two seasons (Table. 4) and tended to be higher

than those in the surface water. Numbers of the four kinds of PAH-degrading bacteria found on the summer cruise were higher than those found in the autumn. The lowest value of PAH-degrading bacteria numbers were almost found at station 6 on the two cruises.

No correlation was found between fluoranthene-degrading bacteria numbers and fluoranthene concentration among different stations in the surface water on the two cruises, and the same situation occurred between pyrene-degrading bacteria numbers and pyrene concentration (Figure 2). But the numbers of fluorene- and phenanthrene-degrading bacteria were positively related to the fluorene and phenanthrene concentration, respectively (Figure 2). Microbial degradation of PAHs in aquatic and terrestrial ecosystems is influenced strongly by a wide variety abiotic and biotic factors which include: temperature, pH, soil type, aeration, nutrients, depth, diffusion, microbial adaptations, water

Table 3 numbers (cells/mL) of PAHs-degrading bacteria in surface water in Xiamen Western Sea

Stations	Phe-degrading bacteria (cells/ml)		Flo-degrading bacteria (cells/ml)		Flu-degrading bacteria (cells/ml)		Pyr-degrading bacteria (cells/ml)	
	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.
1	1.67×10^3	7.33×10^2	1.33×10^3	1.0×10^3	8.27×10^3	9.0×10^2	7.0×10^3	1.33×10^3
2	4.13×10^3	8.67×10^2	5.93×10^3	6.7×10^2	1.23×10^3	6.67×10^2	1.6×10^3	2.33×10^2
3	5.97×10^3	0	2.27×10^3	3.0×10^3	1.23×10^3	6.0×10^2	1.5×10^3	4.67×10^2
4	1.27×10^3	5.33×10^2	1.8×10^3	2.33×10^2	1.63×10^3	5.33×10^2	3.73×10^3	4.47×10^4
5	1.0×10^3	6.67×10^2	2.93×10^3	3.33×10^2	2.3×10^3	1.0×10^2	2.53×10^3	2.33×10^2
6	2.93×10^3	7.67×10^2	0.77×10^3	5.33×10^2	0.2×10^3	1.4×10^2	0.53×10^3	1.67×10^3

Table 4 Numbers (cells/mL) of PAHs-degrading bacteria in sediment of Xiamen Western Sea

Stations	Phe-degrading bacteria (cells/ml)		Flo-degrading bacteria (cells/ml)		Flu-degrading bacteria (cells/ml)		Pyr-degrading bacteria (cells/ml)	
	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.	Jul.	Oct.
1	10×10^4	4.33×10^3	1.5×10^4	5.0×10^3	4.7×10^4	2.33×10^3	4.33×10^4	1.0×10^3
2	1.27×10^4	1.33×10^3	1.1×10^4	1.67×10^3	6.0×10^4	2.67×10^3	1.2×10^4	1.0×10^3
3	2.73×10^4	2.67×10^3	3.23×10^4	2.67×10^3	2.6×10^4	2.33×10^3	3.67×10^4	2.33×10^3
4	5.4×10^4	4.33×10^3	5.7×10^4	1.68×10^3	3.78×10^4	5.0×10^3	4.57×10^4	6.0×10^3
5	3.7×10^4	3.3×10^3	4.43×10^4	1.33×10^3	3.47×10^4	3.0×10^3	3.67×10^4	1.0×10^3
6	1.97×10^4	1.33×10^3	0.90×10^4	0.33×10^3	1.4×10^4	0.67×10^3	1.27×10^4	1.0×10^3

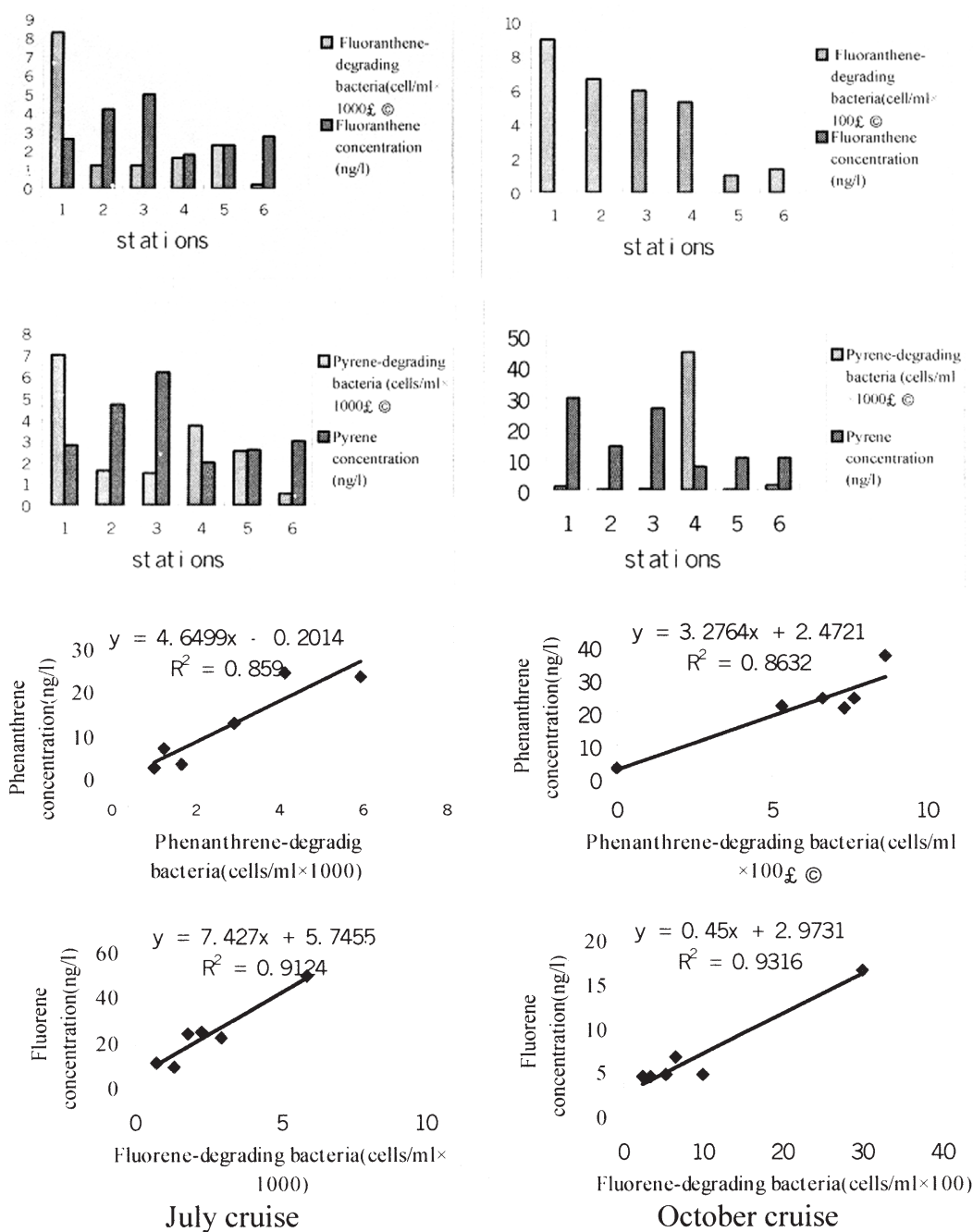


Figure 2 The relationship between PAH-degrading bacteria numbers and PAH concentration in surface water of Xiamen Western Sea.

availability, previous chemical exposure, sediment toxicity, physico-chemical properties of PAH, concentration of the PAH and seasonal factors (Cerniglia, 1992). In the present study, the levels of fluoranthene and pyrene in the surface waters were very low during the two cruises (Table 1). The concentration of fluoranthene ranged from 1.8 ng L⁻¹ to 5.0 ng L⁻¹ on the summer cruise, and fluoranthene were not detected on the autumn. The concentration of pyrene ranged from 2.0 ng L⁻¹ to 6.2 ng L⁻¹ on the summer cruise and 7.6 ng L⁻¹ to 29.9 ng L⁻¹ on the autumn. Fluoranthene and pyrene were high molecular weight (MW 202.3) PAH compounds. Generally, an increase in the number of fused rings increase the chemical stability and hydrophobicity of PAH molecules (Harvey, 1997), thus making them less amenable to biodegradation. In addition, the unstable water condition and tide made very short exposure time of microorganisms to PAH compound. These suggested that the inducement of fluoranthene and pyrene to microorganisms was limited by their low concentration in the surface water, high chemical stability and hydrophobicity and short exposure time.

Compared with fluoranthene and pyrene, fluorene and phenanthrene with low molecular weight (MW 166.2 and 178.2 respectively), have relatively low chemical stability, hydrophobicity and high solubility; the solubility of phenanthrene is 1.300 mg/L while that of fluoranthene and pyrene is 0.260 mg/L and 0.140 mg/L (Cerniglia, 1992). In the present study, the concentration of fluorene and phenanthrene in the surface water were higher than that of fluoranthene and pyrene. Although in the same water condition, the inducement of fluorene and phenanthrene to microorganisms were stronger than that of high molecular weight PAH compounds. These suggested that the numbers of Fluorene- and Phenanthrene-degrading bacteria were positively related to the fluorene and phenanthrene concentration respectively.

The relationship between the numbers of fluorene-, phenanthrene-, fluoranthene-, and pyrene-degrading bacteria and the concentration of the four kinds of PAH compound in the sediments are shown in Figures 3 and 4. A significant positive relationship was found between PAH-degrading bacteria and PAHs concentration, except at station 6 on the two cruises. The concentration of fluoranthene and pyrene in the sediments were high. The value of fluoranthene concentration ranged from 138.1 ng L⁻¹ to 1327.5 ng L⁻¹ on the summer cruise and 141.7 ng L⁻¹ to 939.0 ng L⁻¹ on the autumn. The highest level was both found at station 6. The concentration of pyrene ranged from 111.0 ng L⁻¹ to 1060.1 ng L⁻¹ on the summer cruise and 120.7 ng L⁻¹ to 866.9 ng L⁻¹ on the autumn. The highest level was also found at station 6. Lower molecular weight PAHs are degraded rapidly in sediment, whereas higher molecular weight PAHs

are resistant to microbial attack. The potential biodegradation rates for PAHs are higher in PAH-contaminated sediment than in pristine sediments (Herbes and Schwall, 1978; Foght and Westake, 1988; Wang *et al.*, 1990; Cerniglia, 1992). High concentration of PAHs, long exposure time and relative stable environment, all promote microbial adaptations to PAHs.

Station 6 with highest value of total PAHs in the sediments of Xiamen Western Sea located in the Maluan Lagoon where it has been known for a long time to be densely maricultured. The highest value of phenanthrene, fluoranthene and pyrene concentration were all found at this station, but the numbers of these kinds of PAH compounds degrading bacteria at this station were lowest among different stations. Antibiotics have been used widely in recent years with the development in the aquaculture industry (Zheng, 1993). The serious overuse and abuse of some antibiotics exists in the mariculture area because of the lack of direction. Overuse and abuse of some drugs may not only cause physiological hazards to the aquaculture animal and pollute the aquatic environment, but also lead to decreasing numbers of the total bacteria and of biomass. This suggests that the numbers of PAH-degrading bacteria were not increasing with the concentration of PAHs, mainly due to the overuse and abuse of antibiotics at station 6. That also is the main reason that the level of PAHs was highest in the sediments at station 6, further suggesting that microbiological degradation of PAHs is the major process that results in the decontamination of sediment.

CONCLUSION

The present study shows that the concentration of total PAHs and individual PAHs in the surface water varied among the different stations as well as between two cruises, indicating the contents and composition of PAHs in the surface water were unstable. In the surface water the level of high molecular weight PAH compounds fluoranthene and pyrene were very low during the two cruises. No correlation was found between the concentrations of high molecular weight PAH compounds and the numbers of their degrading bacteria, but the correlation was found between the concentrations of low molecular weight PAH compounds (fluorene and phenanthrene) and the numbers of their degrading bacteria. This implies that the inducement of high molecular weight PAH compounds to bacteria was limited by their low concentration in the surface water, high chemical stability and hydrophobicity and short exposure time. Thus the numbers of high molecular weight PAH degrading-bacteria cannot indicate the extent of the contamination of the surface water by PAHs.

Total PAH concentration and the PAH composition in the sediments were relatively homogenous among the different stations and between two cruises, PAHs in

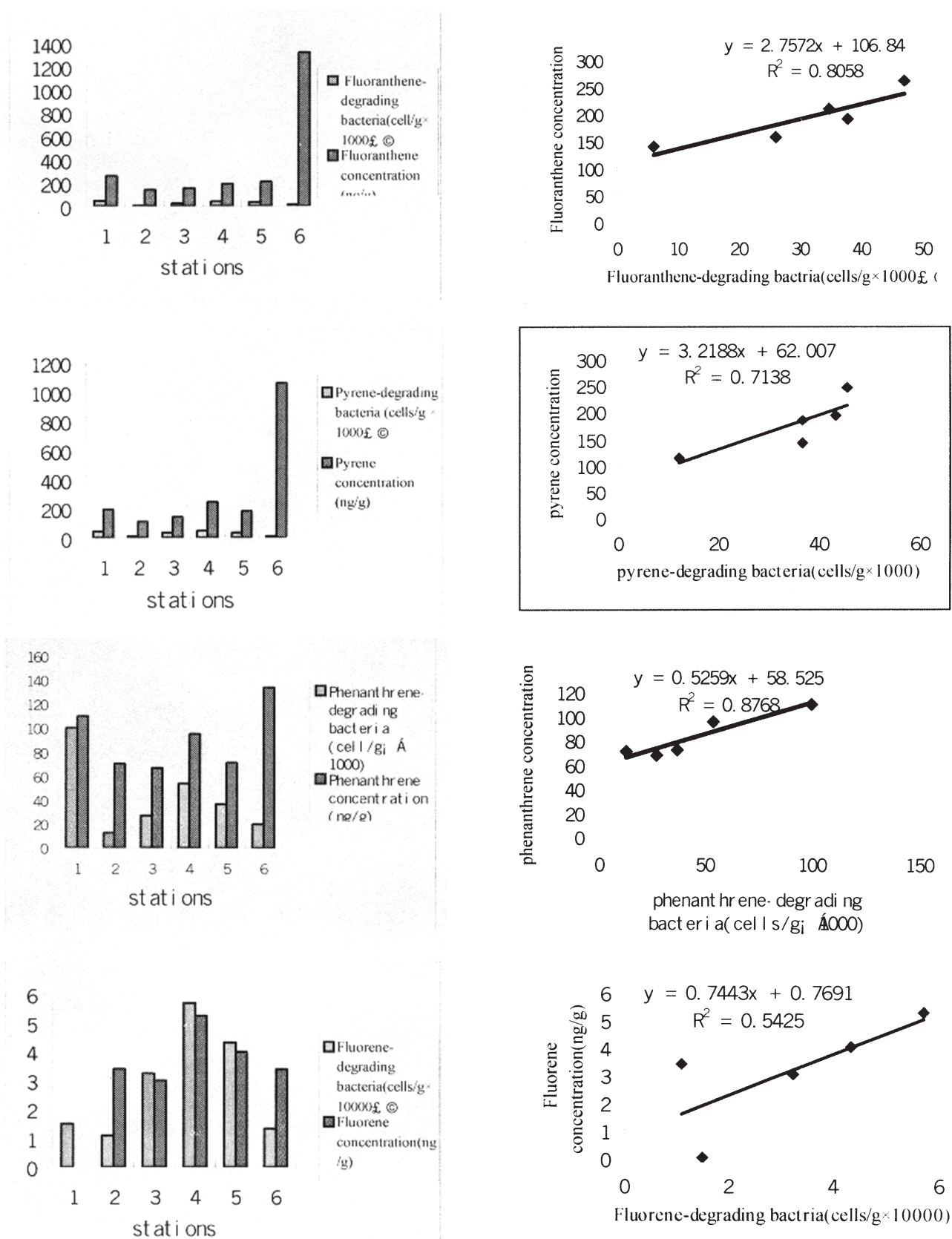


Figure 3 Relationship between PAH-degrading bacteria numbers and PAH concentration in sediment of Xiamen Western Sea on July cruise. Left panels show the relationship between PAH-degrading numbers and PAH concentration among six stations; right panels show the relationship between PAH-degrading number and PAH concentration among five stations except station 6.

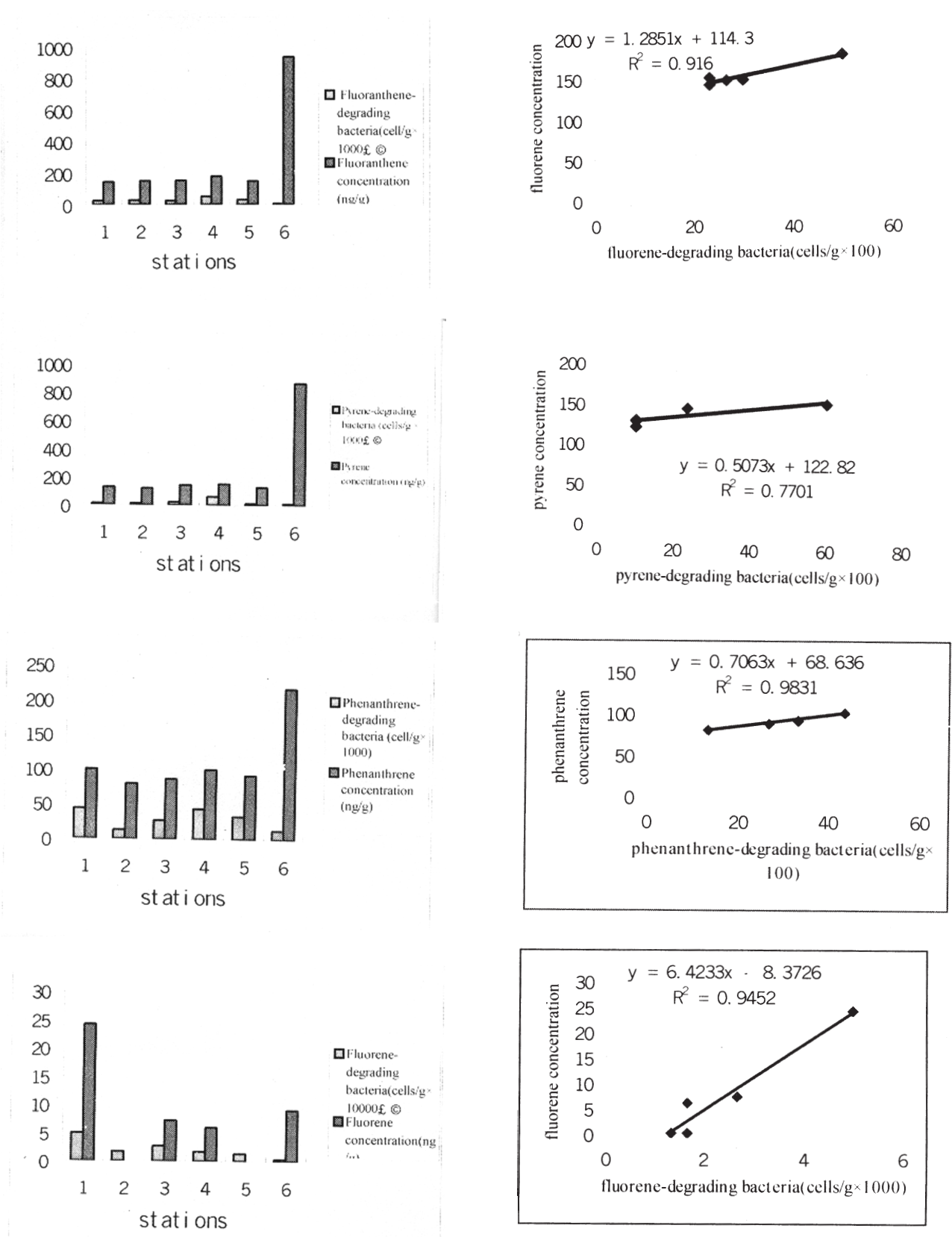


Figure 4 Relationship between PAH-degrading bacteria numbers and PAH concentration in sediment of Xiamen Western Sea on October cruise. Left panels show the relationship between PAH-degrading numbers and PAH concentration among six stations; right panels show the relationship between PAH-degrading number and PAH concentration among five stations except station 6.

the sediments were mainly dominated by high molecular weight PAH compounds (4~6), especially, fluoranthene and pyrene. In the sediments, significant positive correlation was found between the numbers of PAH-degrading bacteria and the concentrations of PAHs, except at station 6 where the overuse and abuse of antibiotics has lead to the decreasing of the total bacteria numbers and biomass. It is suggested that the relationship between the concentrations of high molecular weight PAH compounds and their degrading bacteria numbers is only significant for highly contaminated sites and long-term exposure; the numbers of PAHs-degrading bacteria can indicate the contamination by PAHs to some extent in the relative stable sediment environment, but it is necessary to consider other influential factors.

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