Bull. Environ. Contam. Toxicol. (2003) 71:625–632
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DOI: 10.1007/s00128-003-8841-x

Bulletin of Environmental Contamination and Toxicology

Biodegradation of Polycyclic Aromatic Hydrocarbons in Sludge

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Received: 2 December 2002/Accepted: 9 May 2003

Polycyclic aromatic hydrocarbons (PAHs) are introduced into the environment via the combustion of fossil fuels or other organic material, the accidental spilling of processed hydrocarbons and oils, coal liquefaction and gasification, or organic oil seepage and surface run-off from forest/brush fires and natural geological processes (Cerniglia 1984; Freeman and Cattell 1990). The US Environmental Protection Agency has identified sixteen PAHs as priority pollutants for which levels in industrial effluents require monitoring (Heitkamp and Cerniglia 1988). Wastewater analyses have revealed high PAH concentrations resulting from such sources as industrial waste, domestic sewage, atmospheric rainfall, airborne pollutants, and road surface runoff. Due to their low solubility and high hydrophobicity, PAHs are adsorbed onto solid particles during the wastewater treatment process, resulting in sewage sludge containing between 1 and 10 mg/kg of individual PAHs (Wild and Jones 1989).

Biological degradation represents the major route through which PAHs and other organic chemicals are removed from contaminated environments. The aerobic biotransformation of single PAHs present in sludge has been observed (Barr and Aust 1994; Sepic et al. 1997). However, little attention has been paid to situations where multiple PAHs are present simultaneously. In addition, little is known about controlling PAH degradation in sludge, which has been amended with a phenanthrene-adapted bacterial mixture. The successful establishment of a phenanthrene-adapted bacterial mixture, with stable degradation power, from sediments collected at sites of long-term petrochemical pollutant discharge in southern Taiwan has been described previously (Yuan et al. 2000). The bacterial mixture consists of six Gram-negative strains (B1 to B6), each with distinct colony morphology; strains B1 through B5 are rod-shaped, and strain B6 is coccus-shaped. Positive identifications have only been achieved for strains B1 (*Pseudomonas fluorescens*) and B2 (*Haemophilus* sp.). Each isolated strain is capable of degrading phenanthrene, but degradation rates of the phenanthrene-adapted bacterial mixture are significantly faster than those of individual strains.

The use of surfactants has the potential to increase the degradation rates of hydrophobic organic compounds in contaminated environments by increasing the total aqueous solubility of these compounds (Edwards et al. 1991). PAH

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degradation was found to be enhanced in the presence of hydrogen peroxide for environmental purposes (Vazquez-Duhalt 1999). In the present study the effects of the following factors on PAH degradation rates were investigated in sludge treated with the aerobic bacterial mixture: sludge source, the presence of individual or a mixture of PAHs, pH value, temperature, and the addition of either nitrogen source, carbon source, yeast extract, hydrogen peroxide or surfactants.

MATERIALS AND METHODS

Polycyclic aromatic compounds (phenanthrene, acenaphthene, anthracene, fluorene, and pyrene), 99.0% analytical standards, were purchased from Aldrich Chemicals (Milwaukee, WI). The chemical structures of the PAHs used in this study are represented in Figure 1. Surfactants (Brij 30, SN70, and Triton N101) and all other chemicals were purchased from Sigma (St. Louis) or Mallinckrodt, Inc. (Paris, KY). Stock solutions of each PAH dissolved in dimethyl sulfoxide were initially made up at concentrations of 10,000 mg/L, and then diluted to 500 mg/L before being used in experiments.

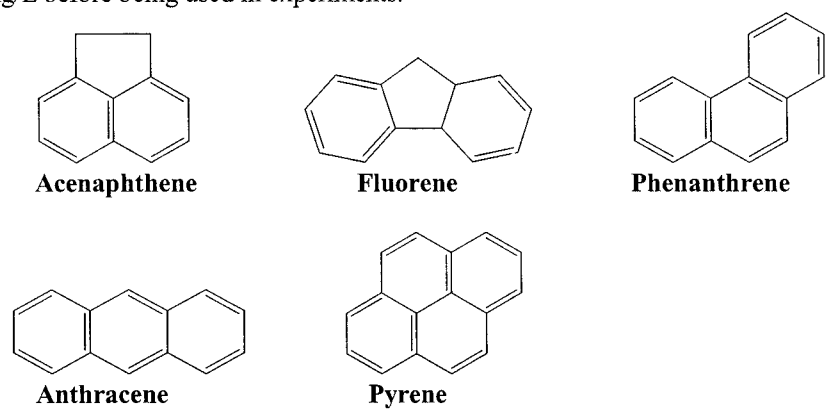

Figure 1. The chemical structures of PAHs used in this study.

Sludge samples were taken from wastewater treatment plants. Sewage sludge samples were collected from Minshi municipal sewage treatment plants in Taipei (total solids 1.26 g/L, pH6.8, microbial numbers 3.63×10^9 CFU/mL). Petrochemical sludge samples were collected from oil refineries in Taoyuan, a heavily industrialized area approximately 100 miles south of Taipei (total solids 0.45 g/L, pH6.5, microbial numbers 4.25×10^7 CFU/mL). Adaptation was performed by adding phenanthrene (1 μ g/g) to 500 g of sediment at 7-day intervals, under static incubation at 30℃ without light, for three years. The resulting mixed culture was then defined as a phenanthrene-adapted bacterial mixture.

The experimental medium consisted of (in g/L distilled water): $K_2HPO_4 \cdot 3H_2O$ (4.25), $NaH_2PO_4 \cdot 3H_2O$ (1.0), NH_4Cl (2.0), $MgSO_4 \cdot 7H_2O$ (0.2), $FeSO_4 \cdot 7H_2O$ (0.012), $MnSO_4 \cdot 7H_2O$ (0.003), $ZnSO_4 \cdot 7H_2O$ (0.003), and $CoSO_4 \cdot 7H_2O$ (0.001).

All experiments were performed using 40 mL vials containing sludge (total solids concentration 5 g/L), 0.5 mL phenanthrene-adapted bacterial mixture, and 1 μg/g of each of the five PAHs (phenanthrene, acenaphthene, anthracene, fluorene, and pyrene). The PAH degradation rates in autoclaved control sludge samples and in untreated sludge with or without addition of the phenanthrene-adapted bacterial mixture were initially compared. Sterile controls were produced via autoclaving at 121℃ for 20 min. Inoculated controls consisted of sludge samples treated with the bacterial mixture.

The following factors were then adjusted to study their effects on PAH degradation after addition of the phenanthrene-adapted bacterial mixture: sludge source (sewage or petrochemical sludge); total solids concentration (5, 10 g/L); pH (5, 6, 7, 8 and 9); temperature (20, 30 and 40℃); presence of five PAHs (phenanthrene, acenaphthene, anthracene, fluorene, or pyrene at concentrations of 1 μg/g) individually or simultaneously; carbon source (sodium acetate 50 mM; sodium pyruvate, 50 mM; sodium lactate, 50 mM); nitrogen source (potassium nitrate or ammonium chloride at 0.2 or 2.0 g/L); yeast extract 50 mg/L; hydrogen peroxide 1.0 % and nonionic surfactants (Brij 30, SN 100 or Triton N101 at concentrations of 0.1%). Sample bottles were incubated for a period of 20 days with shaking at 30℃ in darkness. Samples were periodically collected for the purpose of measuring residual PAH concentrations. All experiments were conducted in duplicate.

Three extractions of residual PAHs were made with hexane, each followed by centrifugation for 10 min at 12,000 g and filtering through 0.45 μm filters (Lida Corp.). Extracts were analyzed with a high performance liquid chromatograph equipped with a fluorescence detector (model FL-1), pump (model 125), autosampler (model 502) and system gold (all from Beckman), plus a polymetric-bound silica column (Phenomenex). Fluorescence detector excitation and emission wavelengths were set at 254 nm and 390 nm, respectively. The mobile phase employed a 40:60 mixture of acetonitrile and water. Detection limits for phenanthrene, acenaphthene, fluorene, anthracene and pyrene were set at 0.1, 0.5, 0.5, 0.01 and 0.1 mg/L, respectively. Remaining PAH percentages were calculated as residual substrate concentration divided by initial concentration. Degradation rates were measured in terms of time taken for substrate reduction, determined as 2 to 4 points along a linear portion of graphed data. Significant differences were calculated using a standard variance F-test.

RESULTS AND DISCUSSION

Degradation rates were first compared for acenaphthene, fluorene, phenanthrene, anthracene, and pyrene in sludge samples taken from the two sources and treated with or without the phenanthrene-adapted bacterial mixture during a 20-day incubation (Table 1). The results show that PAH degradation in both sludge samples occurred as a result of microbial action. PAH degradation was enhanced by treatment with the bacterial mixture in sewage sludge, but was not

Table 1. PAH degradation with or without addition of the bacterial mixture in two sludge samples during a 20-day incubation.

Treatment	PAH remaining percentage (%)									
	Acenaphthene		Fluorene		Phenanthrene		Anthracene		Pyrene	
	T	M	T	M	T	M	T	M	T	M
Sterile control	89.8	95.5	94.9	93.9	98.1	91.9	95.3	92.9	98.1	95.5
Without bacterial mixture	—[a]	79.6	—[a]	60.5	—[a]	45.3	—[a]	35.5	—[a]	43.4
With bacterial mixture	—[b]	66.5	—[b]	36.6	—[b]	—[c]	—[b]	—[c]	—[b]	—[c]

T and M represent petrochemical and sewage sludge, respectively.
The standard deviation is less than 10%.
[a] Acenaphthene, fluorene, phenanthrene, anthracene and pyrene were completely degraded within 1, 1, 1, 4 and 7 days, respectively.
[b] Acenaphthene, fluorene, phenanthrene, anthracene and pyrene were completely degraded within 1, 1, 1, 3 and 6 days, respectively.
[c] Phenanthrene, anthracene and pyrene were completely degraded within 7, 8 and 7 days, respectively.

significantly influenced by treatment with the bacterial mixture in petrochemical sludge.

Table 2 presents data on PAH degradation after addition of the bacterial mixture to the sewage and petrochemical sludge samples. In the sewage sludge when PAHs were present simultaneously, degradation rates for acenaphthene, fluorene, phenanthrene, anthracene and pyrene were measured as 0.017, 0.042, 0.131, 0.099 and 0.117 μg/g/day, respectively. Degradation rates decreased in the following order: phenanthrene → pyrene → anthracene → fluorene → acenaphthene. In the petrochemical sludge samples, degradation rates for the five PAHs were 0.992, 0.905, 0.821, 0.426 and 0.318 μg/g/day, respectively. Degradation rates were found to decrease in the following order: acenaphthene → fluorene → phenanthrene → anthracene → pyrene. The results show that PAH degradation rates were higher for all PAHs in the petrochemical sludge samples, although a different order was noted in the two sludge samples. In petrochemical sludge, autochthonous microorganisms may play a major role in PAH degradation (Yuan et al. 2002). PAH biodegradability is primarily dependent upon the complexity of their chemical structures and corresponding physicochemical properties (Walton and Anderson 1988). With reference to the structure diagrams presented in Figure 1, the molecular weights for acenaphthene, fluorene, phenanthrene, anthracene and pyrene are 154, 166, 178, 178 and 202, respectively. The lower the molecular weight of the PAH, the higher the rate at which it is degraded; higher molecular weight molecules are generally more resistant to biotransformation than those with lower molecular weights (Park et al. 1990). In sewage sludge, the phenanthrene-adapted bacterial mixture may play a major role in PAH degradation. Since we added phenanthrene during the three-year adaptation process, it was not surprising that a higher degradation rate was noted for this particular PAH in the municipal sludge samples.

Table 2. PAH degradation rates when the five PAHs were present either individually or simultaneously in the two sludge samples.

Treatment	PAH degradation rate (μ g/g/day)									
	Acenaphthene		Fluorene		Phenanthrene		Anthracene		Pyrene	
	T	M	T	M	T	M	T	M	T	M
Individual	0.631	0.014	0.530	0.017	0.490	0.113	0.225	0.033	0.136	0.076
Simultaneously	0.992	0.017	0.905	0.042	0.821	0.131	0.426	0.099	0.318	0.117

T and M represent petrochemical and sewage sludge, respectively.
The standard deviation was less than 10%.

Table 2 also presents the degradation rates for the five PAHs when present individually in the treated sludge samples. PAH degradation was found to be enhanced when the five compounds were present simultaneously in both petrochemical and sewage sludge. Trzesicka-Mlynarz and Ward (1995) reported that assemblages of bacterial species with a variety of enzymatic capabilities have greater capacities for degrading complex PAH mixtures. The enhanced degradation rates measured in the combined PAH group were likely to be due to the cross acclimation of the degradation enzymes. These results are consistent with those reported in a previous study (Chang et al. 2002).

The discussion here is restricted to the results of experiments on PAH degradation after addition of the phenanthrene-adapted bacterial mixture to sewage sludge. Table 3 presents PAH degradation rates at various pH values, temperatures and total solids concentrations in the sewage sludge samples. The results show that the optimal conditions for degradation were pH 7, 30℃. This is again consistent with the results from a study of PAH degradation in soil (Yuan et al. 2002). PAH degradation rates when the total solids concentration was 5 g/L were higher than those for 10 g/L. One possible explanation is that PAHs are generally hydrophobic compounds with higher sorption capacities in sludges with higher total solid concentrations, and therefore are not available to be degraded by microorganisms. A higher total solids concentration thus represents a higher level of adsorption.

Table 4 presents data on the effect of using different nitrate and carbon sources on PAH degradation in sewage sludge. No significant differences in degradation rates were observed when the concentration of ammonium chloride was reduced from 2.0 to 0.2 g/L. When potassium nitrate was substituted for ammonium chloride, degradation rates were found to be lower than those in the presence of ammonium chloride. Banat et al. (2000) reported that a reduction in ammonium ion concentration was associated with the stripping of nitrogen in ammonia, and the oxidation of ammonium ion into nitrate. The bacterial mixture may preferentially use ammonium as a nitrogen source. PAH degradation was enhanced by the addition of yeast extract. We measured an increase in microbial numbers from 2.6 $\times 10^6$ CFU/mL to 4.25×10^7 CFU/mL following 20-day incubations. This finding supports previous reports that yeast extract supplementation also enhances the transformation of PAHs in soil (Yuan et al. 2000).

Several other researchers have also shown that the presence of co-substrates enhances PAH degradation (Bossert and Bartha 1986). In the present study no significant differences in PAH degradation were observed following treatment of the sludge samples with carbon sources such as acetate, pyruvate and lactate. This agrees with findings reported by Chang et al. (1999). It is possible that some of the organic compounds present in the sludge provide carbon source that support the growth of the bacterial mixture. PAH degradation was also enhanced by the addition of hydrogen peroxide at a concentration of 1.0%. Cytochrome c is a protein ubiquitous to all eukaryotic organisms, which is involved in the electron transport system, and able to catalyze peroxidase-like reactions in the presence of an electron acceptor, such as hydrogen peroxide (Vazquez-Duhalt 1999).

Table 3. Effects of changes in various incubation factors on PAH degradation in sewage sludge.

TS	pH	Temp	PAH degradation rate (μ g/g/day)				
(g/L)		(℃)	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
5	6	30	0.012	0.018	0.076	0.042	0.052
5[a]	7[a]	30	0.017	0.042	0.131	0.099	0.117
5	8	30	0.012	0.018	0.084	0.040	0.058
5	9	30	0.010	0.020	0.057	0.030	0.017
5	7	20	0.009	0.023	0.091	0.059	0.078
5	7	40	0.012	0.031	0.107	0.081	0.091
10	7	30	0.015	0.024	0.115	0.058	0.076

Each degradation rate for the inoculated control and treatment were significantly different ($P<0.05$).
The standard deviation was less than 10%.
[a] Inoculated control.

Table 4. Effects of treatment with different carbon sources, nitrogen sources or hydrogen peroxide on PAH degradation in sewage sludge.

Treatment	PAH degradation rate (μ g/g/day)				
	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
Inoculated control	0.017[a]	0.042[a]	0.131[a]	0.099[a]	0.117[a]
Lactate	0.019[a]	0.039[a]	0.126[a]	0.097[a]	0.111[a]
Acetate	0.018[a]	0.043[a]	0.132[a]	0.098[a]	0.109[a]
Pyruvate	0.015[a]	0.038[a]	0.127[a]	0.099[a]	0.113[a]
Yeast extract	0.057	0.082	0.180	0.126	0.149
NH_4Cl[b]	0.018[a]	0.044[a]	0.129[a]	0.098[a]	0.113[a]
KNO_3[c]	0.012	0.036	0.079	0.047	0.062
H_2O_2	0.106	0.160	0.236	0.181	0.217

The standard deviation was less than 10%.
[a] Data not significantly different at $P = 0.05$; for all others a significant difference as found at $P = 0.05$ vs. control.
[b] NH_4Cl concentration: 0.2 g/L.
[c] KNO_3 concentration: 2 g/L.

Table 5 presents data on the effects of surfactants on the degradation of the five PAHs in the sewage sludge samples. In all cases, PAH degradation was delayed by the addition of Triton N101, SN70 or Brij30 at a concentration of 0.1%. The decreasing order of degradation rate was: Brij30 → Triton N101 → SN70. Cellular toxicity can result from the interaction of surfactant molecules with cell membranes or directly with membrane-bound proteins (Laha and Luthy 1992). Surfactants can directly inhibit enzymes involved in the catabolic pathway either by association with the enzyme or with the substrate (Chan et al. 1991).

Table 5. Effects of treatment with various surfactants on PAH degradation in sewage sludge.

Treatment	PAH degradation rate (μ g/g/day)				
	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
Inoculated control	0.017	0.042	0.131	0.099	0.117
Triton N101	0.007	0.010	0.015	0.012	0.014
Brij30	0.008	0.014	0.025	0.018	0.022
SN70	0.002	0.004	0.010	0.006	0.009

Each degradation rate for the inoculated control and treatment were significantly different ($P<0.05$).
The standard deviation was less than 10%.

The conclusion for this study is that the five PAHs examined can be degraded by autochthonous microorganisms in petrochemical sludge samples and by the phenanthrene-adapted bacterial mixture in sewage sludge samples. The decreasing order of degradation rate in sewage sludge was: phenanthrene → pyrene → anthracene → fluorene → acenaphthene; in petrochemical sludge the order was acenaphthene → fluorene → phenanthrene → anthracene → pyrene. PAH degradation rates were found to be higher in petrochemical sludge than in sewage sludge. The optimal conditions for PAH biodegradation were determined as 30°C and pH 7. It was also found that when the five PAHs were added to samples simultaneously, all of their individual degradation rates were enhanced. Adding yeast extract or hydrogen peroxide was also found to enhance PAH degradation, but addition of a carbon source did not significantly affect PAH degradation. Addition of SN70, Brij30 or Triton N101 was found to delay PAH degradation. Future research will focus on the feasibility of using the phenanthrene-adapted bacterial mixture and operation parameters examined in this study for large-scale removal of PAHs from sludge.

Acknowledgments. This research was supported with funds from the National Science Council, Republic of China (No. NSC89-2313-B-031-003).

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