

Biodegradation of Polycyclic Aromatic Hydrocarbons by Inoculated Microorganisms in Soil

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Polycyclic aromatic hydrocarbons (PAHs) are considered priority pollutants due to their potential toxicity, mutagenicity, and carcinogenicity (Keith and Telliard 1979). Soil pollution in the form of tar oil from coal liquefaction and gasification is a major source of PAH contamination (Cerniglia 1984). In soil, PAHs are slowly degraded by local microorganisms, and the introduction of non-local microorganisms to polluted sites is a bioremediation strategy that has attracted a lot of attention (Atlas 1991). Several researchers have demonstrated the method's effectiveness in increasing xenobiotic pollutant biodegradation (Heitkamp and Cerniglia 1988; Brodtkorb and Legge 1992). However, little attention has been paid to scenarios where five PAHs are found at the same site. In addition, little is known about controlling PAH degradation in soils that have been amended with sludge from wastewater treatment plants.

We have previously described the successful establishment of a PAH-adapted consortium with stable degradation power from sediments collected at sites of long-term petrochemical pollutant discharge in southern Taiwan (Yuan et al. 2000). The consortium 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 cocci-shaped. Positive identifications have only been achieved for strains B1 (*Pseudomonas fluorescens*) and B2 (*Haemophilus sp.*). Each isolate is capable of degrading phenanthrene on its own, but degradation rates for a PAH-adapted consortium are significantly faster than those for individual strains. We therefore added known concentrations of phenanthrene, pyrene, anthracene, fluorene, and acenaphthene to samples of two soil types in order to measure the effects of changes in pH, temperature, PAH concentration, and the individual or simultaneous presence of PAHs on PAH degradation under aerobic conditions. After determining optimal parameters, we also compared the effects of adding sludge from two separate sources on PAH degradation in soil.

MATERIALS AND METHODS

Phenanthrene, acenaphthene, anthracene, fluorene, and pyrene—all with 99.0% analytical standards—were purchased from Aldrich Chemical (Milwaukee, WI). With the exception of solvents, which were purchased from Mallinkrodt (Paris, KY), all other chemicals were purchased from Sigma (St. Louis). Before being used in

experiments, stock solutions of PAHs dissolved in dimethyl sulfoxide were initially established at the concentration of 10,000 mg/L, then diluted to 500 mg/L.

Soil samples were collected from northern Taiwan's Guishan and Taida areas. The composition of the Guishan clay loam samples was 35.0% clay, 31.2% silt, and 32.5% sand (pH 6.8, organic matter 1.37%). For the Taida loam sample the composition was 20.2% clay, 55.3% silt, and 34.1% sand (pH 6.6, organic matter 2.93%). Dried samples were crushed and sieved through a 2-mm mesh screen to remove plant and other debris. Collection sites for sludge samples were as follows: municipal sludge was taken from Minshi municipal sewage treatment plants in Taipei, and petrochemical sludge was taken from the Chinese Petroleum Corporation's refinery in Taoyuan, approximately 100 miles south of Taipei.

To establish the mixed culture, sediment was collected with an Ekman grab sampler at several known sites of continuous petrochemical effluent discharge in southern Taiwan. Adaptation was performed as follows: phenanthrene (1 μ g/g concentration) was added to 500 g of sediment at 7-day intervals under static incubation at 30°C without light for three years, after which a combination of the five PAHs were added at 7-day intervals for one year. In this paper, the final product is referred to as an aerobic PAH-adapted consortium.

Our 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). Each experiment was performed using 40 mL vials containing 1.0 g soil, 1 mL culture sample, and 5 μ g/g each of phenanthrene, acenaphthene, anthracene, fluorene, and pyrene. At first we compared the effects of autoclaved controls with or without PAH-adapted consortium on PAH degradation in our soil samples. To establish these controls, we autoclaved soil-filled serum bottles at 121°C for 20 min. Our inoculated controls consisted of soil samples treated with PAH-adapted consortium.

We also studied the effects of changes in the following factors on the consortium's degrading power: pH (5.0, 6.0, 7.0, 8.0 and 9.0); temperature (20, 30 and 40°C); the five PAHs at concentrations (5 and 20 μ g/g); aerobic or anaerobic conditions; the simultaneous or individual presence of the five PAHs at a concentration of 5 μ g/g; and sludge source (Municipal sludge or petrochemical sludge). For a description of the anaerobic treatment procedure, see Chang et al. (1999). Sample bottles were incubated with shaking at 30°C in darkness. We periodically collected small amounts of each sample in order to measure residual PAH concentrations. All experiments were conducted in duplicate.

With slight modifications, PAH extraction and analysis was performed as described in Yuan et al. (2000). Residual PAHs were extracted twice with *n*-hexane. Briefly, we added 10 mL *n*-hexane to sample bottles, which we then placed in a rotary shaker for 24 hrs at 160 rpm. After removing the *n*-hexane layer and extracting the water layer with 10 mL *n*-hexane, we centrifuged the extracts for 10 min at 12,000 g before filtering through 0.45 μ m filters. Our next step was to perform an analysis using a

high performance liquid chromatograph (HPLC) equipped with a fluorescence detector (model FL-1), pump (model 125), autosampler (model 502), system gold (all from Beckman) and a polymeric-bound silica column (Phenomenex). Fluorescence detector excitation level was set at 254 nm; emission level was set at 390 nm. A mixture of acetonitrile and water (40:60) was used for the mobile phase. The recovery percentage for phenanthrene, acenaphthene, fluorene, anthracene, and pyrene were 94.5, 89.1, 87.4, 90.3, 91.5 %, respectively.

Measurements of nitrate, ammonia, sulfate, and phosphate were taken using procedures described by the American Public Health Association (APHA 1992). The PAH degradation data collected during this experiment fit well with first order kinetics, $S = S_0 \exp(-k_1 t)$, where S_0 is the initial concentration, S the substrate concentration, t equals time period and k_1 represents the degradation rate constant.

RESULTS AND DISCUSSION

In the Taida soil samples, remaining PAH amounts after a 40-day incubation period were measured as follows: a) in the autoclaved control bottles, from 92.6 to 96.9%; b) not autoclaved and without the PAH-adapted consortium, from 90.1 to 94.6%; and c) not autoclaved and with the PAH-adapted consortium added, from undetectable to 13.5% (Table 1). This is strong evidence in support of the argument that PAH degradation in soil is the result of microbial action, and that such action is enhanced by treating the soil with the PAH-adapted consortium. Similar results were found for the Guishan soil samples.

Biodegradation rate constants for acenaphthene, fluorene, phenanthrene, anthracene, and pyrene in the Taida soil samples were measured as 0.039, 0.057, 0.410, 0.315 and 0.365 1/day, with respective half-lives of 17.8, 12.2, 1.7, 2.2 and 1.9 days. Biodegradation rate constants for acenaphthene, fluorene, phenanthrene, anthracene, and pyrene in the Guishan samples were measured as 0.021, 0.041, 0.390, 0.303 and 0.324 1/day, with respective half-lives of 38.7, 16.1, 1.8, 2.3 and 2.1 days. The high-to-low order of degradation rates for both soil samples was observed as phenanthrene > pyrene > anthracene > fluorene > acenaphthene. Our observed PAH half-lives in soil were much shorter than the 16 to 200 days reported by Howard et al. (1991). In each case, PAH degradation was faster in Taida soil than in Guishan soil, bioavailability of PAHs may be increased with greater organic content of soil, since apparent solubility of PAHs increase with hydrophobicity of soil. For this reason, we will restrict our discussion to the results from the Taida soil experiments.

Data on the effects of variation in incubation temperature and pH on PAH degradation in Taida soil are presented in Table 2. According to these results, optimal values were 30°C and 7.0, respectively. The optimal pH value was similar to that reported for adapted sediment consortia collected from known sites of continuous petrochemical effluent discharge in southern Taiwan (Yuan et al. 2001). It was found that the higher the concentration of combined PAHs is the slower the degradation rate becomes perhaps a reflection of increased levels of toxicity. Furthermore, we found that PAH degradation was inhibited under anaerobic conditions, evidence that the primary

degradation factor in the PAH-adapted consortium consisted of aerobic microorganisms and not facultative or anaerobic microorganisms.

In these experiments, phenanthrene was degraded faster than any other PAH. Overall, degradation rates were faster for PAHs with higher molecular weights (pyrene and anthracene) than those with lower molecular weights (acenaphthene and fluorene). Walton and Anderson (1988) reported that PAH bioavailability and biodegradability are primarily dependent upon the complexities of their chemical structures and corresponding physicochemical properties. Bauer and Capone (1988) suggested that enzymes with broad substrates were responsible for PAH biodegradation. In this experiment, the PAH-adapted consortium was enriched with phenanthrene for three years and with a combination of the five PAHs for one year. Enzymatic action may be responsible for the noted differences in PAH degradation tied to molecular weight. However, this conflicts with Park et al.'s (1990) finding that PAHs with higher molecular weights are more resistant to biotransformation than those with lower molecular weights.

Table 3 presents data on degradation rates for PAHs added individually or in combination to medium with or without Taida soil. Degradation rates were lower in culture samples containing soil than in soil-free samples. One possible explanation for this difference is the tendency of PAHs to adsorb to soil particles, thus reducing the biodegrading effectiveness of microorganisms or PAH bioavailability. These results are similar to those reported in Yuan et al. (2000). In addition, it was found that degradation activity was more vigorous when all five PAHs were simultaneously rather than individually present, perhaps because the presence of all five compounds provides more carbon source for use by the microorganisms, or cross acclimation may enhance the biodegradation rate of PAHs.

Table 1. Comparison of PAHs degradation with and without the addition consortium to selected soil samples within a 40-day incubation period.

Treatment	Remaining percentage ^a				
	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
Taida soil					
Sterile control	95.5±1.4	92.6±2.3	95.7±1.6	94.4±2.7	96.9±2.9
Without consortium	93.4±2.2	90.1±1.1	94.2±1.6	93.5±2.5	94.6±2.2
With consortium	13.5±1.5	11.6±1.7	— ^b	— ^b	— ^b
Guishan soil					
Sterile control	95.6±2.3	95.7±1.8	90.5±2.5	94.5±2.6	95.9±1.6
Without consortium	94.2±2.9	95.4±2.9	94.6±2.7	92.3±1.6	92.2±2.2
With consortium	30.4±2.2	18.4±1.6	— ^c	— ^c	— ^c

^a Values represent means ± standard deviation.

^b Phenanthrene, anthracene, pyrene were completely degraded within 7, 15 and 7 days, respectively.

^c Phenanthrene, anthracene, pyrene were completely degraded within 7 days.

Table 2. PAH degradation rate constants (k_i) under various incubation conditions^a.

pH	Temp	Degradation rate constant (k_i) ^b				
		Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
7	20°C	0.017±0.006	0.040±0.003	0.116±0.061	0.060±0.021	0.080±0.011
7	30°C	0.039±0.009	0.057±0.005	0.410±0.012	0.315±0.021	0.365±0.012
7	40°C	0.011±0.003	0.016±0.002	0.033±0.002	0.021±0.009	0.029±0.003
5	30°C	0.032±0.004	0.053±0.012	0.267±0.062	0.154±0.032	0.248±0.043
6	30°C	0.036±0.009	0.069±0.022	0.347±0.089	0.180±0.023	0.257±0.032
8	30°C	0.024±0.009	0.038±0.008	0.180±0.033	0.090±0.008	0.116±0.022
9	30°C	0.013±0.004	0.032±0.006	0.100±0.032	0.035±0.002	0.050±0.007
7 ^c	30°C ^c	0.014±0.005	0.015±0.003	0.020±0.003	0.016±0.001	0.018±0.005
7 ^d	30°C ^d	0.014±0.002	0.015±0.004	0.020±0.001	0.016±0.001	0.018±0.004

^a PAH concentrations 5 μ g/g for all the groups except 7^c.^b Values represent means \pm standard deviation.^c PAH concentrations 20 μ g/g.^d Incubated under anaerobic conditions.**Table 3.** PAH degradation rate constants (k_i) when present individually or simultaneously.

Treatment	Degradation rate constant (k_i) ^a				
	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
Individually present					
Medium ^b	0.050±0.002	0.054±0.009	0.301±0.014	0.105±0.008	0.161±0.002
Soil ^c	0.033±0.005	0.036±0.005	0.210±0.019	0.092±0.006	0.131±0.003
Simultaneously present					
Medium ^b	0.094±0.011	0.064±0.012	0.693±0.022	0.385±0.024	0.462±0.011
Soil ^c	0.039±0.009	0.057±0.005	0.410±0.012	0.315±0.021	0.365±0.012

^a Values represent means \pm standard deviation.^b Soil-free culture.^c Soil-containing culture.

After establishing optimal conditions, we looked at the effects of adding sludge on degradation rates; results are presented in Table 4. For all five compounds, degradation in soil amended by either sludge type was faster than in samples containing soil only, with rates ranging from 0.165 to 0.770 1/day, this represents a five to twenty-five fold increase. When measuring the effects of different suspended solids (SS) concentrations of municipal sludge, the high-to-low order of PAH degradation rates was noted as 5 g/L > 1 g/L > 0.1 g/L. A likely explanation for this order is the increased availability of carbon and other nutrients. However, when measuring degradation rates according to the level of SS in the soil samples, high-to-low order was 1 g/L > 5 g/L > 0.1 g/L for petrochemical sludge. Increased toxic potential may explain why degradation decreased as amendment concentrations increased from 1 to 5 g/L. Following the addition of petrochemical sludge, the high-to-low order of degradation rates was observed as acenaphthene > fluorene > phenanthrene > anthracene > pyrene. Perhaps a reflection of Park et al.'s (1990) observation that the lower the PAH molecular weight, the faster the degradation rate.

However, the high-to-low order following the addition of municipal sludge was observed as phenanthrene > pyrene > anthracene > fluorene > acenaphthene, perhaps as the results of addition of phenanthrene to the consortium during the three-year adaptation process. In a previous study (Chang et al. 2001), we reported that the PAH-adapted consortium was the primary degrading agent in municipal sludge samples, but autochthonous microorganisms were most likely the primary degrading agents in petrochemical sludge samples—with or without the addition of the consortium.

Table 4. PAH degradation rate constants and half-lives in two sludge types.

SS(g/L)	Degradation rate constant (k_1) ^a				
	Acenaphthene	Fluorene	Phenanthrene	Anthracene	Pyrene
Inoculated control	0.039±0.009	0.057±0.005	0.410±0.012	0.315±0.021	0.365±0.012
Municipal sludge					
0.1	0.165±0.003	0.277±0.022	0.462±0.022	0.330±0.016	0.385±0.019
1	0.217±0.011	0.289±0.021	0.495±0.014	0.433±0.018	0.462±0.022
5	0.365±0.034	0.462±0.024	0.770±0.022	0.578±0.027	0.693±0.029
Petrochemical sludge					
0.1	0.433±0.002	0.385±0.009	0.385±0.008	0.347±0.021	0.315±0.016
1	0.866±0.021	0.693±0.021	0.558±0.022	0.500±0.018	0.462±0.021
5	0.578±0.012	0.433±0.019	0.433±0.028	0.365±0.021	0.277±0.018

^a Values represent means ± standard deviation.

Changes in nitrate, ammonia, sulfate, and phosphate concentration during PAH degradation in soil amended with petrochemical sludge (SS 1 g/L) are described in Figure 1. After 15 days of incubation, nitrate content decreased from 365 to 165 mg/L, ammonia content decreased from 245 to 115 mg/L, sulfate content decreased from 220 to 120 mg/L, and phosphate content decreased from 120 to 78 mg/L. A possible explanation is that some of the organic compounds found in sludge provide nutrients, which support the growth of microorganisms. 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 consortium tested in this research uses these nutrients as part of the PAH degradation process.

Our conclusion is that the aerobic PAH-adapted consortium used in this study is capable of biodegrading phenanthrene, acenaphthene, anthracene, fluorene, and pyrene in soil. Results from batch experiments show a 70-100% PAH reduction within 40 days of initial treatment. Our results indicate that the consortium's ability to degrade 2-, 3-, and 4-ring PAHs makes it a potential candidate for cleaning up PAH-contaminated sites, under the optimal conditions of pH7.0, 30°C, and the simultaneous presence of the five PAHs. The addition of sludge can also enhance the PAH degradation process. For future projects we will study identification of intermediate PAH degradation products, and use a bioreactor to determine operating parameters for soil bioremediation under aerobic conditions.

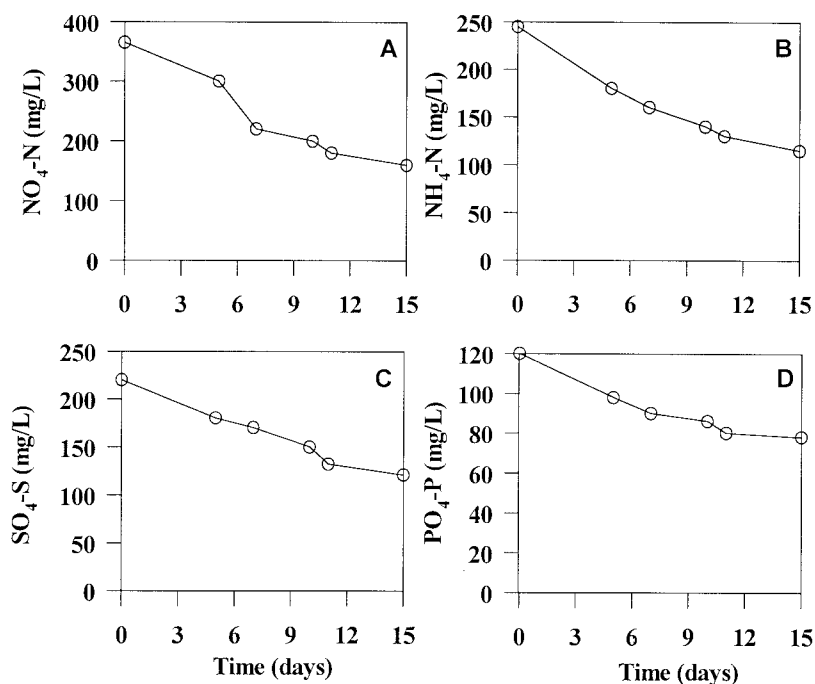


Figure 1. Changes in nitrate (A), ammonia (B), sulfate (C) and phosphate (D) concentrations during PAH degradation in soil containing amended petrochemical sludge.

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