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# Enhancing the Intrinsic Bioremediation of PAH-Contaminated Anoxic Estuarine Sediments with Biostimulating Agents

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**Estuarine sediments are frequently polluted with hydrocarbons from fuel spills and industrial wastes. Polycyclic aromatic hydrocarbons (PAHs) are components of these contaminants that tend to accumulate in the sediment due to their low aqueous solubility, low volatility, and high affinity for particulate matter. The toxic, recalcitrant, mutagenic, and carcinogenic nature of these compounds may require aggressive treatment to remediate polluted sites effectively. In petroleum-contaminated sediments near a petrochemical industry in Gwangyang Bay, Korea, *in situ* PAH concentrations ranged from 10 to 2,900 μg/kg dry sediment. To enhance the biodegradation rate of PAHs under anaerobic conditions, sediment samples were amended with biostimulating agents alone or in combination: nitrogen and phosphorus in the form of slow-release fertilizer (SRF), lactate, yeast extract (YE), and Tween 80. When added to the sediment individually, all tested agents enhanced the degradation of PAHs, including naphthalene, acenaphthene, anthracene, fluorene, phenanthrene, fluoranthene, pyrene, chrysene, and benzo[*a*]pyrene. Moreover, the combination of SRF, Tween 80, and lactate increased the PAH degradation rate 1.2-8.2 times above that of untreated sediment (0.01-10 μg PAH/kg dry sediment/day). Our results indicated that *in situ* contaminant PAHs in anoxic sediment, including high molecular weight PAHs, were degraded biologically and that the addition of stimulators increased the biodegradation potential of the intrinsic microbial populations. Our results will contribute to the development of new strategies for *in situ* treatment of PAH-contaminated anoxic sediments.**

***Key words***: biodegradation, bioremediation, anoxic sediment, PAH, biostimulation

Polycyclic aromatic hydrocarbons (PAHs) are artificial fused-ring compounds that enter sediment systems from coke and petroleum-refining industries, accidental spills and leakages, and rainwater runoff from roadways (Kanaly and Harayahama, 2000; Karthikeyan and Bhandari, 2001; Wang *et al.*, 2001; Rothermich *et al.*, 2002; Chang *et al.*, 2003). PAHs pose an environmental threat primarily because of their toxicity, low volatility, resistance to microbial degradation, and ability to sorb to sediments. Documented impacts on critical habitats include contamination of benthic ecosystems, with subsequent harm to the marine food chain (Coates *et al.*, 1997; Elliott, 2001; Wang *et al.*, 2001).

Researchers have studied the anaerobic biodegradation of PAHs in sediment since the late 1980s (Lovley *et al.*, 1989; Coates *et al.*, 1997; Hayes *et al.*, 1999; Rothermich *et al.*, 2002). For anaerobic processes, PAH degradation coupled to sulfate reduction is the most relevant to coastal marine sediments, because sulfate is abundant in seawater, whereas nitrate concentrations are typically low and Fe(III) is often only slightly available (Coates *et al.*, 1997; Rothermich *et al.*, 2002). Apart from a study on the methanogenic degradation of benzene (Weiner and Lovley, 1998), few reports on the methanogenic transformation of PAHs have been published to our knowledge.

PAHs are generally slowly degraded in the environment, particularly in marine systems (Kanaly and Harayahama, 2000; Nieman *et al.*, 2001; Han *et al.*, 2003). The low degradation rates of PAHs may be due to their stable structures, adsorption onto particulate matter, or nutrient deficits in the environment (Gibbons, 1991; Thomas *et al.*, 1998; Elliott, 2001; Wang *et al.*, 2001). The bioremediation of PAH-contaminated anoxic sediments has been restricted to the application of alternative electron acceptors (Mihelcic and Luthy, 1988; Lovley *et al.*, 1989; McFarland and Sims, 1991; Lovley *et al.*, 1994; Lovley *et al.*, 1996; Coates *et al.*, 1997; Nieman *et al.*, 2001). However, little information is available on the effects of applying surfactants, inorganic or organic nutrients, or growth factors on PAH biodegradation in anoxic

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sediments.

The objective of this study was to examine the degradation of PAHs in anaerobic estuarine sediments to investigate the possible role of microbial activity. We also evaluated the effects of adding biostimulating agents (surfactants, nutrients, carbon sources, and growth factors) on the extent and rate of PAH degradation.

## Materials and Methods

### *Sample collection*

PAH-contaminated sediments were collected as grab samples from an estuary immediately adjacent to a petrochemical factory in the industrial area of Gwangyang Bay, Korea. The site was selected because of its size and the duration of hydrocarbon contamination from fuel spills and industrial wastes. We collected sediments and seawater from the site. We discarded the top 1cm of light-brown oxic sediment and packed the remaining sulfide-blackened anoxic sediment into canning jars. The sediments and seawater were kept at 4°C during transportation and were stored in the dark prior to use. The physicochemical characteristics of the sediment were determined according to standard methods (APHA, 1998) after extracting the water-soluble fractions from the sediment using deionized water. Total organic carbon was determined in terms of weight loss on ignition (LOI) at 650°C using methods described by Carter (1993).

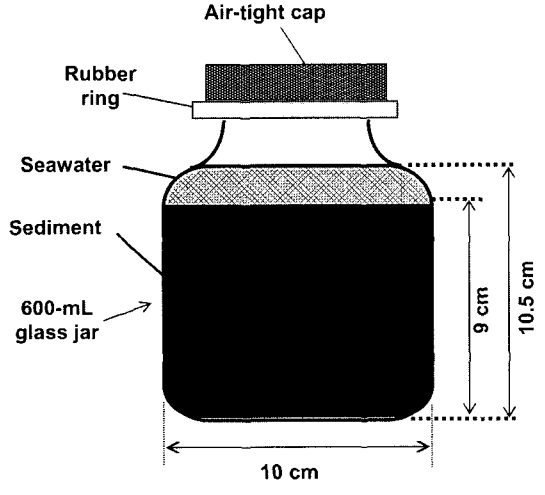


**Fig. 1.** Schematic representation of packed sediment in a 600 ml air-tight jar.

### *Experimental design*

We used sealed 1 L air-tight jars as packed-sediment microcosms to investigate the degradation of PAHs in the sediment (Fig. 1). Aliquots of sediment (600 ml) were packed into the jars after homogeneously mixing them in a glove box with the various treatments. Seawater collected *in situ* was added to cover the top 1-1.5 cm of sediment and then the headspace was filled with nitrogen gas. The treatments consisted of an abiotic control (AC), a no-treatment control (NTC), slow-release fertilizer (SRF), Tween 80, lactate, yeast extract (YE), SRF + Tween 80, and SRF + Tween 80 + lactate (Table 1). One gram of SRF (Chobi Ltd., Korea) consists of 148.9 mg urea-N, 18.0 mg phosphate-P, and 500 mg clay filler, with silica and latex added as support material.

At each sampling time, the overlying surface seawater was siphoned off so that the sediment could be sampled without disturbing the surface water. We sampled 10 ml sediment aliquots in triplicate using 10 ml blunt-ended syringe corers. The sediment in the jars was then topped with the same seawater again. The sediments were incubated at 20°C in the dark without shaking.

### *PAH extraction*

The dry weight of each sediment core was measured after drying at 60°C for 10 h and finely grinding in a mortar. We added 100 μl of $d_{10}$-phenanthrene solution (Sigma, 200 mg $d_{10}$-phenanthrene in 1 L $CH_2Cl_2$) to the sediment as a surrogate standard. The sediments were extracted three times using 30 ml of $CH_2Cl_2$ in a sonic bath (Bransonic, USA) for 30 min at 30°C. The $CH_2Cl_2$ phase was decanted after each extraction cycle and filtered

**Table 1.** Treatments of PAH-contaminated sediments

| Treatment | Chemical agent | Concentration (in pure water) |
|---|---|---|
| Abiotic control | Glutaraldehyde | 5% (v/v) |
| Unamended control | None | |
| Inorganic nutrients | Slow-release fertilizer (SRF)[a] | 9 mg |
| Surfactant | Tween 80 | 10x CMC[b] |
| Carbon source | Lactate or dextrin | 1% (w/v) |
| Growth factor | Yeast extract | 0.1% (w/v) |
| Surfactant + Nutrients I | Tween 80 + SRF | 10x CMC[b] + 9 mg |
| Surfactant + Nutrients II | Tween 80 + SRF + Lactate | 10x CMC[b] + 9 mg + 1% (w/v) |

[a]1 g SRF (Chobi Ltd., Korea) consists of 148.9 mg urea-N, 18.0 mg phosphate-P, and 500 mg clay filler, with silica and latex added as support material.
[b]1x CMC, 0.075% (v/v)

through filter paper. The composite extracts were evaporated using a rotary vacuum evaporator at 30°C to <1 ml in volume. After adding 50 μl of decafluorobiphenyl (DFB) solution (200 mg DFB in 1 L $CH_2Cl_2$) as an internal standard, the total volume of the extract was adjusted to 1 ml with $CH_2Cl_2$, filtered through a PTFE 0.2 μm syringe filter (Advantec, USA), and stored in amber glass vials sealed with Teflon-butyl rubber caps. The samples were refrigerated before GC analysis.

***PAH analysis***

PAH concentrations in the extracts were analyzed using a Hewlett-Packard 6890 series gas chromatograph with an Agilent HP-5 fused silica capillary column (60 m × 0.32 mm i.d., film thickness 1.0 μm) and flame ionization detector (FID). Helium was used as the carrier gas at a flow rate of 1.5 ml/min and average velocity of 26 cm/sec. The temperature was ramped from 150°C to 190°C at 8°C/min with 5 min holding, then to 220°C at 2°C/min, then to 300°C at 15°C/min, and finally to 310°C at 2°C/min with 20 min holding. Each PAH was identified using PAH standard (Sigma, USA) and confirmed by GC/MS (Varian, USA) analysis.

## Results and Discussion

***Sediment characteristics***

As shown in Table 2, the organic matter content of the Gwangyang sediments was 9.5% by dry weight. Compared to values reported for most coastal sediments (3-10%), these sediments were highly enriched in organic matter, suggesting that particulate organic inputs to Gwangyang Bay are very high (Khim *et al.*, 2001; Wang *et al.*, 2001). Total N and P concentrations were 42.5 and 0.43 μg/g dry sediment, respectively. These values are very low compared to those reported for other coastal sediments, such as those from Boston Harbor, MA, USA (Wang *et al.*, 2001). The optimal recommended C:N:P ratio for most bioremediation applications is approximately 100:10:1 (Alexander, 1999; Maier *et al.*, 2000; Rittmann and McCarty, 2001). Our analysis showed that the sediments lacked an adequate supply of inorganic nutrients, such as nitrogen and phosphorus, for the biodegradation of PAHs. Thus supplying inorganic nutrients could be the most important step to enhance PAH biodegradation. Concentrations of sulfate, nitrate, and carbonate were not high enough to support anaerobic respiration (Table 2), which indicates that these electron acceptors can limit anaerobic respiration processes. Genthner *et al.* (1997) reported that nitrate and sulfate concentrations in the 15-20 mM range were required to biodegrade 2-ring or 3-ring PAHs in anaerobic sediments. The sediment pH of 7.92 was in the optimal range for mineralization of petroleum hydrocarbons (Gibbons, 1991; Chang *et al.*, 2002).

**Table 2.** Physicochemical characteristics of Gwangyang Bay sediments

| Parameters | Units | Value |
|---|---|---|
| pH | _ | 7.92 |
| Moisture | g$H_2O$/g wet sediment | 0.51 |
| Organic matter | g/g dry sediment | 0.095 |
| Salinity | part per thousand | 9.20 |
| Total-N | μg/g dry sediment | 42.50 |
| Nitrate-N | μg/g dry sediment | 31.30 |
| Total-P | μg/g dry sediment | 0.43 |
| Phosphate-P | μg/g dry sediment | 0.32 |
| Sulfate | mg/g dry sediment | 0.51 |
| Sulfide | mg/g dry sediment | 1.00 |
| Carbonate | μmol/g dry sediment | 29.10 |
| Iron | μg/g dry sediment | 18.70 |

The concentrations of the 14 detected PAHs ranged from 9.3 to 2,889 μg/kg dry sediment (Table 3), which is significantly higher than those reported in other studies (Prahl and Carpenter, 1983; Huntley and Bonnevie, 1995; Lim, 1998). The PAH distribution was characteristic of the signature of asphaltenic petroleum products, i.e., very high levels of fluoranthene, pyrene, and chrysene (Rothermich *et al.*, 2002).

***Degradation of PAHs under various conditions***

To evaluate the anaerobic degradation of *in situ* PAHs in

**Table 3.** Concentrations of PAHs in Gwangyang Bay sediment

| PAH | Concentration (ng/gds[a]) |
|---|---|
| Naphthalene | N.D.[b] |
| Acenaphthene | 60.4 |
| Acenaphthylene | 9.3 |
| Fluorene | 18.4 |
| Phenanthrene | 76.9 |
| Anthracene | 80.8 |
| Fluoranthene | 2,888.8 |
| Pyrene | 1,171.5 |
| Benzo[*a*]anthracene | 651.8 |
| Chrysene | 1,644.2 |
| Benzo[*e*]fluoranthene | 210.0 |
| Benzo[*k*]fluoranthene | 66.7 |
| Benzo[*a*]pyrene | 104.3 |
| Indeno[*1,2,3-cd*]pyrene | N.D. |
| Dibenz[*a,h*]anthracene | 47.8 |
| Benzo[*ghi*]perylene | 25.3 |
| Total | 7,056.2 |

[a]gds, g dry sediment
[b]N.D., Not detected

the contaminated sediments, we monitored GC-detectable concentrations of PAHs for 120 days after amending with possible biostimulating agents. Compared to the PAH concentrations in the biologically active sediments, those in the sediment treated with glutaraldehyde remained virtually constant during 120 days of incubation (Table 4). This clearly demonstrates that PAH degradation is a biological process and that indigenous microorganisms in the sediment have a high potential of degrading 2- to 4-ring PAHs. Compared to the amended sediments, there was little loss of *in situ* PAHs in the unamended sediment. Additions of electron acceptors such as sulfate or carbonate also did not enhance the degradation of PAHs (data not shown). However, Tween 80, SRF, lactate, and yeast extract significantly enhanced the degradation of PAHs, which indicates that these factors were absent from or deficient in the native sediments.

Surfactants are useful for the bioremediation of environments contaminated with PAHs because they enhance the desorption and solubility of hydrophobic compounds. Many studies have sought to stimulate PAH biodegradation with surfactants that enhance the solubility and desorption rates of PAHs from soil particles under aerobic conditions (Shin *et al.*, 1999; Joo *et al.*, 2001; Garon *et al.*, 2002; Prak and Pritchard, 2002). Until recently, little attention had been paid to enhancing PAH degradation in anaerobic sediments by the use of surfactants. In this study, the addition of Tween 80 at a final concentration of 10x CMC increased the solvent extraction recovery of *in situ* PAHs from sediment samples up to 2.5 times (data not shown). The results demonstrated that when micelles were formed, PAH molecules were partitioned to their hydrophobic cores, leading to an apparent increase in the solubility of the PAHs. The increased availability of PAHs resulted in enhanced PAH biodegradation (Table 4).

A major difference between remediating aquifer and marine sediments by nutrient amendment is that dissolved nutrients can be added to groundwater, whereas nutrients should be added to marine sediments in a less soluble form for slow release via dissolution into the sediments over several years (Adegbidi *et al.*, 2003). The addition of SRF could maintain nutrient supplements for microorganisms for a long time, as well as prevent nitrogen and phosphorus compounds from being lost by dilution at high tide (Choi *et al.*, 2003).

Although *in situ* marine sediments contain quantities of macronutrients and trace elements used by microorganisms, certain compounds essential for PAH degradation could be deficient because the microorganisms quickly use them up. We chose lactate, a readily utilizable substrate for sulfate-reducing bacteria under anaerobic conditions, and dextrin, a less favorable but long-lasting substrate, as amending agents. Yeast extract was chosen to supply essential nutrients, such as amino acids, vitamins, and trace elements, for the growth of microorganisms. As shown in Table 4, lactate and yeast extract significantly enhanced the PAH degradation rate, whereas dextrin did not. Previous studies have also reported that the presence of co-substrates such as acetate, lactate, pyruvate, yeast extract, and glucose enhance PAH degradation (Yuan *et al.*, 2000; Chang *et al.*, 2002). We predict that a preference for the type of organic nutrients exists that could be site specific.

During the 120 days of incubation, the total *in situ* PAH concentrations were determined to verify the effects of the various biostimulators on the remediation of PAH-polluted marine sediments (Fig. 2). The desorption of PAHs by the addition of Tween 80 could enrich the bioavailable PAHs for PAH-degrading microorganisms in contaminated marine sediments. The amendment of contaminated sediments with nitrogen and phosphorus in the form of SRF enhanced *in situ* PAH degradation, which indicated the significance of inorganic nutrients as limiting factors of PAH degradation in sediments. Lactate, a preferred carbon and energy source for sulfate reducers, also enhanced PAH degradation significantly. The combination of inorganic nutrients ($SRF_S$), a surfactant (Tween 80), and an organic nutrient (lactate) increased PAH degradation rates more than did the individual compounds

**Table 4.** Effects of biostimulating agents* on PAH degradation in sediments

| PAH | Degradation rate of PAHs (μg-PAH/kg-dry sediment/day) | | | | | | |
|---|---|---|---|---|---|---|---|
| | GAL | NTC | T80 | SRF | LAC | DEX | YE |
| Phenanthrene | 0.0 | 0.0 | 1.3 | 2.0 | 2.1 | 0.0 | 1.8 |
| Anthrathene | 0.0 | 0.3 | 2.9 | 3.5 | 5.1 | 0.0 | 0.2 |
| Fluoranthene | 0.0 | 0.8 | 1.6 | 0.7 | 11.1 | 0.2 | 3.8 |
| Pyrene | 0.0 | 0.0 | 0.4 | 0.1 | 7.5 | 0.5 | 1.6 |
| Chrysene | 0.0 | 0.8 | 4.4 | 2.1 | 2.4 | 0.7 | 2.3 |
| Benzo[*a*]pyrene | 0.0 | 1.7 | 2.1 | 6.1 | 1.4 | 0.0 | 0.0 |
| Total | 0.0 | 3.6 | 12.7 | 14.5 | 29.6 | 1.4 | 9.7 |

*GAL, glutaraldehyde abiotic control; NTC, unamended control; T80, Tween 80; SRF, slow-release fertilizer; LAC, lactate; DEX, dextrin; YE, yeast extract. Table 1 lists the concentration of each agent.

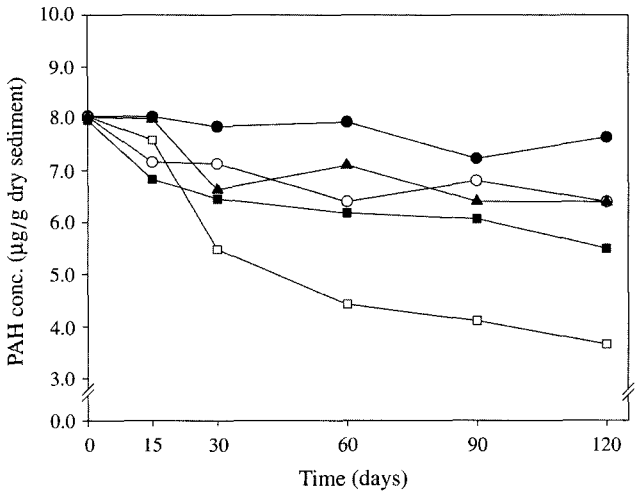


**Fig. 2.** The time course of PAH degradation in sediment with various treatments. ●, control; ○, SRF; ▲, Tween 80; ■, lactate; □, SRF + Tween 80 + lactate.

(Fig. 2). Thus, amending the PAH-contaminated sediment with biostimulators increased the intrinsic biodegradation potentials of these PAHs. Our results will contribute to the development of new strategies for *in situ* treatment of PAH-contaminated anoxic sediments in Gwangyang Bay.

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