Competitive metabolism of polycyclic aromatic hydrocarbon (PAH) mixtures in porous media biofilms

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Abstract Complex mixtures of polycyclic aromatic hydrocarbons and other organic compounds are usually present in contaminated sites. This may influence biodegradation patterns and changes in biofilm growth and development. Biodegradation studies of naphthalene, phenanthrene and pyrene were conducted in sole-substrate systems and in binary and tertiary mixtures to examine substrate interactions on biofilms in porous media systems. It was shown that phenanthrene and pyrene could not be degraded as sole carbon sources in the system, but binary systems of the 3- and 4-ring PAHs with acetate and naphthalene supplements stimulated their degradation, with up to 87.9% and 70.1% removal efficiencies respectively. However, in the tertiary systems the presence of phenanthrene inhibited pyrene degradation. Adsorption of PAHs to sand media was determined to be negligible. Biofilm growth, development and changes in composition were analyzed over time; these showed increases in both firmly and loosely attached viable biomass, as well as extracellular polymeric substance production that formed a complex matrix. Heterogeneous surface films and a variety of biological aggregate structures and growth patterns were observed by confocal microscopy.

Keywords Biodegradation; biofilm; cometabolism; CLSM; inhibition; polycyclic aromatic hydrocarbons

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Introduction

The contamination of soil and groundwater at Superfund sites by Polycyclic Aromatic Hydrocarbons (PAHs) is very common. PAHs are a group of aromatic compounds, usually produced by the incomplete combustion of coal, oil and gas, garbage and other organic substances. The main concern with PAHs is based on their unique characteristics: high levels of toxicity, microbial recalcitrance, high potential for bioaccumulation and high occurrence in the environment (Ryan *et al.*, 1991).

Substrate interactions complicate the biodegradation kinetics in PAH-contaminated environments. When in mixtures, PAHs can influence positively or negatively the rate and extent of biodegradation of other PAH components. Sometimes, cometabolic interactions enhance degradation of higher molecular weight PAHs, when soil consortia utilize inter-metabolites. In other cases, competitive inhibition was observed when PAHs were metabolized by a common enzyme system, with the degradation of 2- or 3-ring compounds preferred, and delay or decrease of the degradation of more persistent PAHs. Tiehm and Fritzsche (1995) showed that compounds such as anthracene, fluorene and pyrene do not support the growth of an aerobic mixed culture as a sole carbon source, but necessarily depended on the presence of more soluble PAHs. On the other hand, inhibition was reported by Stringfellow and Aitken (1995), who found that phenanthrene degradation was decreased in the presence of naphthalene, methylnaphthalene or fluorene. Both cometabolic and inhibitory effects have frequently been observed and widely reported in the literature (McNally *et al.*, 1999; Bouchez *et al.*, 1999; Yuan *et al.*, 2001; Dean-Ross *et al.*, 2002).

The objective of this study was to examine the cometabolism and competitive inhibition effects of multisubstrate utilization of PAHs by biofilms in porous media.

Naphthalene, phenanthrene and pyrene were studied in varying mixture combinations under aerobic conditions in simulated sandy aquifers. This study contributes to expanding the information on PAH biodegradation from single to multisubstrates in contaminated sites, and the role of the biofilm matrix in transporting nutrients and PAH-contaminants to biofilm microorganisms.

Materials and methods

Lab-scale reactors were constructed from 30-cm long, 3.8-cm diameter glass columns. The columns were equipped with five sample ports for pore water samples along the length of the column, as well as four flow cell ports used for examination of biofilm growth. A schematic diagram of the experimental setup can be found in Ebihara (1999). A nutrient solution was pumped through PAH feed generation columns to produce dissolved PAH concentrations; sufficient contact time for feed solution components was allowed in the generating columns to achieve the desired feed PAH concentrations of approximately 10 mg/L naphthalene, 1.0 mg/L phenanthrene and 0.1 mg/L pyrene. Linear pore water velocity through the soil column was maintained at 5 m/d, and a sodium acetate solution (10 mg/L) was used in the system for development and enhancement of biofilm. Aerobic conditions were always maintained in the reactors with the aid of a small dose of hydrogen peroxide (3.5% v/v added at a rate of 0.25 mL/h with a syringe pump).

The enrichment culture for inoculating the soil columns was started with a mixed liquor sample from an activated sludge aeration tank, taken from a domestic wastewater treatment plant. Mixed cultures have been shown to degrade several PAHs at significantly higher rates than individual bacterial strains because of their broader enzymatic capabilities (Yuan *et al.*, 2000). The columns were operated for a period of 6 weeks with acetate as a primary substrate, prior to PAH addition, in order to develop a suitable biofilm.

The soil used in the column was sand, so that the effects of sorption of the PAHs by organics in the soil were reduced. The media selected for growth was rounded medium silica sand (ASTM C-190) of approximately 0.38 mm median grain size. Sieve analyses were performed to describe in detail the size distribution of the sand.

Feed solution was added to each column at a rate of 2.03 mL/min (2.93 L/d) by a 1–100 rpm Masterflex pump and LS/14 pump heads (Cole-Parmer Instrument Company, Vernon Hills, IL). It consisted of essential nutrient salts and excess nitrate so that nutrient and electron acceptor concentrations were not limiting biological growth and activity. The composition of the macronutrients in the feed solution (excluding the organic substrate) was 32 mg/L $NaNO_3$, 10 mg/L NH_4Cl, 40 mg/L $Na_2HPO_4.7H_2O$, 10 mg/L KH_2PO_4, 1.4 mg/L $CaCl_2.H_2O$, 3.8 mg/L $MgSO_4$ and 0.65 mg/L $FeCl_3.6H_2O$, and the composition of the micronutrients in the feed solution was 0.0112 mg/L $MnSO_4$, 0.0007 mg/L $CuSO_4$, 0.0004 mg/L $Na_2MoO_4.2H_2O$ and 0.012 mg/L $ZnSO_4.7H_2O$. All columns were operated at room temperature.

Pore water samples were taken weekly at the five ports along the length of the columns and analyzed for the contaminant concentration, dissolved oxygen (DO) and pH. DO was measured by using a MI-730 Micro-Oxygen Electrode with an OM-4 Oxygen Meter (Microelectrodes, Inc., Bedford, NH). Due to the small volume of sample, the pH was measured by using pHydrion Papers 7–11 (Micro Essential Laboratory, Brooklyn, NY). PAHs were analyzed using a Hewlett-Packard 5890 gas chromatograph (GC) equipped with a Flame Ionization Detector (FID) and a Supelco PTA-5 column (30 m length, 0.53 mm ID). The column temperature profile during analysis was 100°C for 2 minutes, 30°C per minute to 260°C and then maintained at 260°C for 2 minutes. The injection port and FID temperatures were 300°C and 310°C, respectively. The carrier gas (pre-purified helium) flowrate was 6.11 mL/min, and the make-up gas (helium) flowrate

was 26.2 mL/min. Triplicate samples of 2 mL each were taken from each sample port and extracted with methylene chloride at a ratio of 1:1 (for naphthalene) and 4:1 (for phenanthrene and pyrene). The samples were compared to a six-point standard curve to determine the PAHs concentration.

Biofilm components such as volatile solids, lipid-phosphates, carbohydrates and proteins, were obtained in duplicate for sampling events at 1, 14 and 20 weeks to describe the chemical composition of the biomass present in the sand column. Viable biomass was measured by lipid phosphate analysis, using a modified method by Findlay *et al.* (1989). Sodium chloride (343 mg/L) was added to wet sand (0.2–0.5 g per sample), and 2 mL were taken before they were transferred to a new reaction vial, in order to quantify firmly and loosely attached biomass. Samples were extracted with chloroform and methanol, digested with potassium persulfate and dyed with malachite green. The absorbances at 610 nm were compared to a potassium calibration standard curve to determine the moles/dry sand mass. Total carbohydrates were determined by the phenol reaction method of Daniels *et al.* (1994). Wet sand (0.2–0.5 g per sample) was mixed with 1 mL MilliQ water, phenol and sulfuric acid. The absorbances at 488 nm were compared to a glucose calibration standard curve to determine the mass/dry sand mass. Protein concentrations were determined using the Coomassie® Plus Protein Assay Reagent (#23236) (Pierce Biotechnology, Rockford, IL). Wet sand (0.2–0.5 g per sample) was mixed with MilliQ water and the reagent, and sonicated for 10 minutes. The absorbances at 595 nm were compared to a bovine serum albumin calibration standard curve to determine the mass/dry sand mass. Total and volatile solids analyses (0.5–1.0 g per sample) were performed according to Standard Methods (1998).

A scanning confocal laser microscope (Zeiss LSM 510) was used to observe the physical structure, heterogeneity and growth patterns of biofilm in the flow cells. Samples were stained with 3 μL/mL BacLight® fluorescent stain (Molecular Probes, Eugene, OR) to determine viability of cells within the biofilm. Excitation was obtained using a 488 nm line of krypton–argon laser, and the emission signal for viable cells was obtained using a 522 nm filter set.

Results and discussion

Two main columns were used in this study (Columns 2 and 4) to test the biodegradation of sole-substrate, binary and tertiary mixtures of PAHs. Killed control columns (Columns 1 and 3) were operated simultaneously and at the same conditions as the main columns,

Table 1 Summary of sequence of experiments

	Pyrene studies		
	Column 1 (control)	**Column 2**	**Days**
Binary mixture 1 (BM1)	PYR, AC + Az	PYR, AC	0–71; 78–98
Sole-substrate (SS)	PYR + Az	PYR	71–78
Binary mixture 2 (BM2)	PYR, NAPH + Az	PYR, NAPH	98–146
Tertiary mixture (TM)	PYR, NAPH, PHE + Az	PYR, NAPH, PHE	146–157
	Phenanthrene studies		
	Column 3 (control)	**Column 4**	**Days**
Binary mixture 1 (BM1)	PHE, AC + Az	PHE, AC	0–64; 87–98
Sole-substrate (SS)	PHE + Az	PHE	64–87
Binary mixture 2 (BM2)	PHE, NAPH + Az	PHE, NAPH	98–146
Tertiary mixture (TM)	PHE, NAPH, PYR + Az	PHE, NAPH, PYR	146–157

PHE: phenanthrene, PYR: pyrene, NAPH: naphthalene, AC: acetate, AZ: azide

but with the addition of sodium azide (1 g/L), to minimize biomass growth. Table 1 summarizes the sequence of the experiments over a period of 22 weeks. Influent DO was maintained at 8–9 mg/L, whereas the effluent ranged between 0.5 and 1.0 mg/L, so it can be inferred that aerobic conditions were not limiting biological processes. The pH was maintained between 7.2 and 7.5 during the run.

Phenanthrene biodegradation studies

Biodegradation of phenanthrene was initially enhanced by the addition of acetate for the first 9 weeks, during which 87.9% removal of phenanthrene was achieved (Figure 1). Phenanthrene as a sole-substrate was then tested for the following 3 weeks and a sudden decrease in its biodegradation (to 8.5%) was observed; the metabolism of the contaminant appeared to be strongly dependent on the more easily degradable carbon source. Similar behaviors on the transformation of phenanthrene with addition of acetate, yeast extract, glucose and pyruvate were reported by Yuan *et al.* (2000). Reapplication of acetate for a period of 1 week then allowed the system to recover back to the initial phenanthrene removal efficiency (around 92.3%).

A binary mixture of phenanthrene and naphthalene was then added into the columns between weeks 13 and 20. Two main points were observed for the two PAHs during this phase: first, a transition stage of 2 weeks occurred where the biofilm was getting acclimated to utilizing the 2-ring PAH and, therefore, a decrease in the 3-ring PAH degradation efficiency was observed, down to 31.7%.

Second, naphthalene was later readily biodegraded, and its removals were measured to be as high as 99.9%, while phenanthrene degradation slowly increased up to 75.6%, indicating the need of a co-substrate as in the first binary mixture. Previous reports have documented that PAH-degrading bacteria utilize common enzymes for the degradation of two or more PAHs, while cometabolism is occurring. It has been shown that enzymes for naphthalene degradation in certain strains were found to be involved in the degradation of phenanthrene and anthracene, especially in *Pseudomonas* (Menn *et al.*, 1993).

Tri-component experiments were then carried out in the final 2 weeks of the run. Naphthalene degradation did not appear to be affected by the presence of phenanthrene or pyrene, and phenanthrene degradation was maintained at 77.8%. On the other hand,

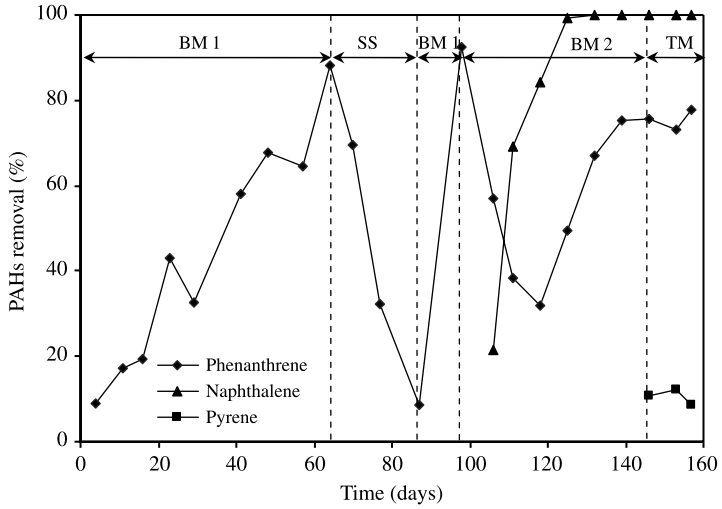

Figure 1 Removal efficiencies for sole-substrate and binary and tertiary mixtures of PAHs during phenanthrene studies (Column 4)

pyrene failed to biodegrade, probably due to competition for the common enzymes in the system; the final biodegradation percentage of pyrene was around 11.2% in the tertiary mixture, indicating a preference for more soluble PAHs. Similar inhibition behaviors have been reported by Stringfellow and Aitken (1995), Dean-Ross *et al.* (2002) and McNally *et al.* (1999) in suspended culture.

Naphthalene, phenanthrene and pyrene adsorption on the column supporting media played only a minor role, since final killed biomass concentrations of the three compounds were measured in the sand and were found to be negligible, as low as 0.24 ± 0.03 μg/g dry sand.

Pyrene biodegradation studies

Column experiments with pyrene and acetate were conducted for 10 weeks, as shown in Figure 2. Two important stages were observed: a lag phase of 7 weeks, when pyrene degradation was delayed, probably due to an acclimation period of the mixed culture to utilize the enzymes needed for the metabolism of the 4-ring PAH, and the subsequent 3 weeks, when it was successfully biodegraded (up to 54.8%). Degradation of pyrene was then measured when it was used as a sole-substrate for the mixed culture, but removal significantly decreased to 14.8% in less than 3 days. Pyrene degradation increased to 62.2% after once again amending with acetate for another 2.5 weeks.

A binary mixture of pyrene and naphthalene was later evaluated between weeks 13 and 20; it showed similar behavior as in the acetate addition experiment. Naphthalene was easily degraded within 2 weeks, up to 99.7%, while pyrene experienced a lag phase, with a decrease of its degradation down to 13.1% followed by an increase to 70.1%, due to cometabolism. Tertiary mixtures run over the remaining 2 weeks showed the 3-ring PAH significantly inhibiting pyrene from degrading. Phenanthrene reached 73.4% removal, while pyrene achieved only 16.1% in the three-component system.

Biofilm composition

Biofilm development and changes in composition were obtained at 1, 14 and 22 weeks. Highest total biomass values were observed in the first 7.5 cm of the column at 145 (at 1 week), 801 (at 14 weeks) and 2,375 μg/g TS (at 22 weeks) during the experimental run (Figure 3). Biomass seemed to increase more than 3 times during the period when binary

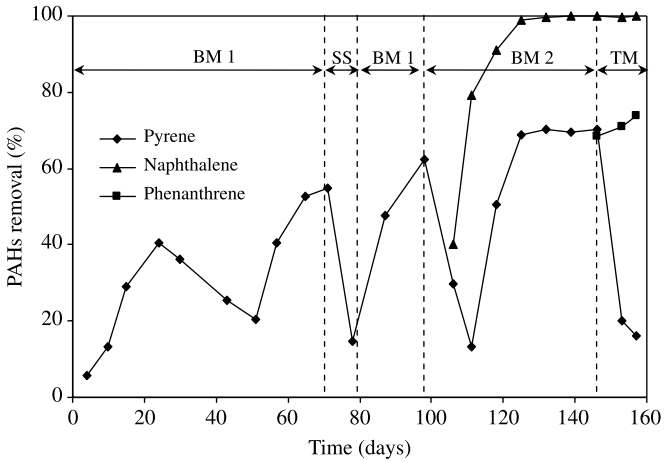

Figure 2 Removal efficiencies for sole-substrate and binary and tertiary mixtures of PAHs during pyrene studies (Column 2)

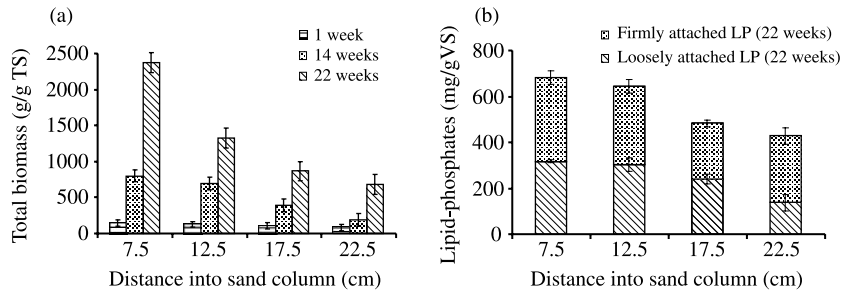

Figure 3 Total biomass profile (A) and viable biomass profile (B) across Column 4. Error bars indicate standard deviations

mixtures were fed, indicating the ability of cells to increase their numbers and polymeric substances production in the presence of low-molecular weight PAHs. Lipid-phosphates were used to determine the viable biomass as either firmly attached biomass (monolayer of surface biofilms directly attached to sand grain surfaces) or loosely attached biomass (cell aggregates and protrusion bodies easily separated from the media). LP profiles indicated that viable biomass increased over time, from 131 ± 20 (at 1 week), to 486 ± 16 (at 14 weeks), to 682 ± 22 mg/g VS (at 22 weeks), at 7.5 cm. Similar trends of progressive production of new biomass were observed across the column, and the total LP remained relatively constant, with an average of 555 mg/g VS (at 22 weeks). Firmly attached biomass (monolayer of surface biofilms directly attached to sand grain surfaces) ranged from 53.6% to 67.7% of total LP, but loosely attached biomass (cell aggregates and protrusion easily separated from the media) was also found to be an important fraction of total LP.

Extracellular polymeric substances (EPS) were characterized by measuring carbohydrate and protein contents (Figure 4). Carbohydrate profiles showed an increase from 54 ± 5 to 133 ± 7 mg/g VS in the first 14 weeks (at 7.5 cm), explained by both binary mixtures 1 and 2. During this time, the average carbohydrate concentration along the column was 111 mg/g VS, followed by a 12 (at 7.5 cm) to 36% (at 22.5 cm) increase over the next 8 weeks, meaning a spatial redistribution of biomass occurred with only a small amount of new carbohydrate production. Protein concentrations were at an average of 7 (at 1 week), 33 (at 14 weeks) and 47 mg/g VS (at 22 weeks). The maximum biomass and exopolymeric substance contents were achieved in the first 7.5 cm of depth, where most of the nutrients and contaminant were available, especially when binary mixtures were present; their production, though, was not found to be enhanced in a significant way

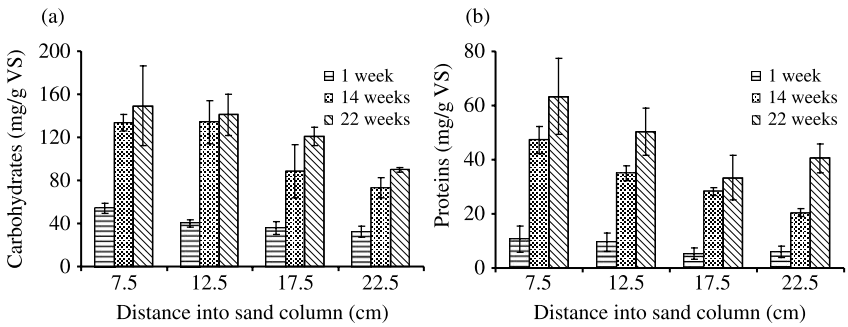

Figure 4 Carbohydrate profile (A) and protein profile (B) across Column 4. Error bars indicate standard deviations

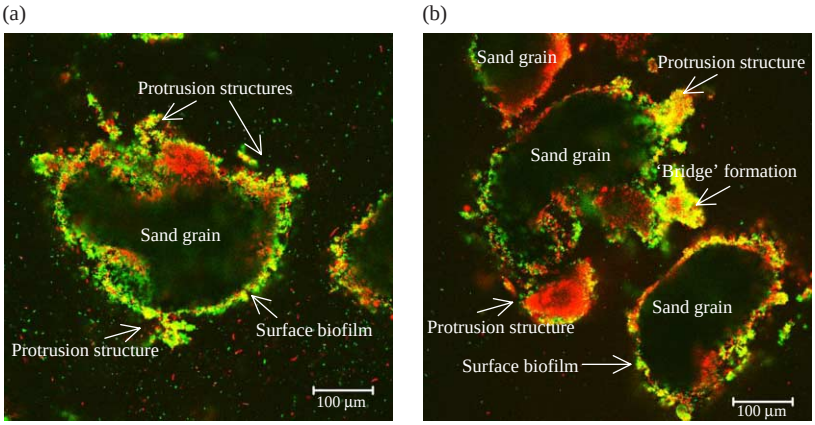

Figure 5 Optical sections of viable cells on sand grains at 7.5 cm into sand column (Column 4), after 22 weeks. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

by the tri-component PAHs. Similar results on biofilm composition were found for Column 2.

Biofilm structure

Biofilm structure examination by CSLM (Figure 5) was conducted at the end of the experimental run, in the active zone of the sand columns (0–7.5 cm). Dense biofilm coverage was observed on sand grain surfaces, indicating the presence of heterogeneous surface biofilms of approximately 15 to 30 μm in thickness. Surface biofilms consisted not only of monolayer biofilms, but also of small cluster-type structures in the range 15–20 μm in diameter. Binary mixtures appeared to increase the biomass per unit sand medium, improving biodegradation rates of PAH mixtures over time.

A large variety of biological aggregate structures ranging from 30 to 60 μm in thickness were also observed, including large cluster-and-protrusion-type structures, cell aggregate bridging and a thick bioweb-type growth containing viable cells developing in the pore spaces between soil grains. Similar biofilm physical structures were observed in Column 2.

Conclusions

Multiple substrate utilization is a very important process, especially in bioremediation of contaminated soils, where complex mixtures of pollutants are often present. The potential interactions of three PAH-based substrates were shown in this study: positive effects, resulting in an increase in biodegradation of at least one of the components, and negative effects, such as inhibition of the higher molecular weight PAHs, were observed. Sole-substrate experiments indicated that phenanthrene and pyrene were not able to be biodegraded alone, but instead they required a co-substrate or degradation intermediates from a more soluble and easily degradable compound such as acetate or naphthalene. Biofilm composition and structure analysis suggested a heterogeneous web-like matrix, with various aggregate formations of bacteria and exopolymeric substances. More complex mixtures of PAHs in soils need to be investigated in the future, in order to give a better understanding of the potential interactions during PAH biodegradation by mixed cultures.

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References

APHA, AWWA, and WEF (1998). *Standard Methods for the Examination of Water and Wastewater*, 20th ed., American Public Health Association, Washington, DC.

Bouchez, M., Blanchet, D., Bardin, V., Haeseler, F. and Vandecasteele, J.P. (1999). Efficiency of defined strains and of soil consortia in the biodegradation of polycyclic aromatic hydrocarbons (PAH) mixtures. *Biodegradation*, **10**, 429–435.

Daniels, L., Hanson, R.S. and Philips, J.A. (1994). Chemical Analysis. In *Methods for General and Molecular Bacteriology*, Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. (eds), American Society for Microbiology, Washington DC, pp. 512–553.

Dean-Ross, D., Moody, J. and Cerniglia, C.E. (2002). Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. *Microb. Ecolog.*, **41**, 1–7.

Ebihara, T. (1999). *Characterization and enhancement of microbial biofilms in porous media for the biodegradation of polycyclic aromatic hydrocarbons*, PhD. Dissertation, University of Cincinnati, Cincinnati, OH.

Findlay, R.H., King, G.M. and Watling, L. (1989). Efficacy of phospholipids analysis in determining microbial biomass in sediments. *Applied and Environmental Microbiology*, **55**(11), 2888–2893.

McNally, D., Mihelcic, J.R. and Luenking, D. (1999). Biodegradation of mixtures of polycyclic aromatic hydrocarbons under aerobic and nitrate-reducing conditions. *Chemosphere*, **38**(6), 1313–1321.

Menn, F.M., Applegate, B.M. and Sayler, G.S. (1993). NAH-plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acid. *App. Environ. Microbiol.*, **59**, 1938–1942.

Ryan, J.R., Loehr, R.C. and Rucker, E. (1991). Bioremediation of organic contaminated soils. *J.Hazard.Mat.*, **28**, 159–169.

Stringfellow, W.T. and Aitken, M.D. (1995). Competitive metabolism of naphthalene, methyl naphthalenes and fluorine by phenanthrene-degrading pseudomonads. *Appl. Environ. Microb.*, **61**, 357–362.

Tiehm, A. and Friztsche, C. (1995). Utilization of solubilized and crystalline mixtures of polycyclic aromatic hydrocarbons by *Mycobacterium* sp. *Appl. Microb. Biotechnol.*, **42**, 964–968.

Yuan, S.Y., Wei, S.H. and Chang, B.V. (2000). Biodegradation of polycyclic aromatic hydrocarbons in a mixed culture. *Chemosphere*, **41**, 1463–1468.

Yuan, S.Y., Chang, J.S., Yen, J.H. and Chang, B.V. (2001). Biodegradation of phenanthrene in river sediment. *Chemosphere*, **43**, 273–278.