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Biodegradation of Selected PAH from Sediment in Bioslurry Reactors

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in environments impacted by fossil fuels. They may be introduced directly or by the combustion of fossil fuels (Manoli and Samara 1999). Due to their physical properties, namely low water solubility and high adsorption potential, they tend to accumulate in sediments. They have been identified in contaminated environments, their concentrations depending on distance from sources of industrial activity. They are of concern to human health because the four- and five-ringed aromatic compounds have been identified as genotoxicants in short-term mutagenicity assays and as carcinogens in long term rodent bioassays (Menzie et al. 1992).

One of the most promising methods of removal of PAHs from contaminated environments is that of bioremediation In this method, the biodegradation potential of microorganisms is maximized in order to achieve rapid removal of the contaminants with minimum disturbance and negative impact to the environment. Biodegradation has been used to remove PAHs with three or fewer aromatic rings from contaminated soil while removal of higher molecular weight PAHs is more difficult to achieve (Wilson and Jones 1993).

The goal of the research project reported here is to define conditions which maximize the biodegradation of PAHs in laboratory sediment slurries. Factors such as slurry loading rate, presence of detergents, aging and strain selection were identified as contributing to efficiency of biodegradation. In this paper, conditions to maximize biodegradation rates are described and applied to contaminated sediments from the Grand Calumet River, in western Indiana.

MATERIALS AND METHODS

The microorganisms used in this research were isolated from sediments in the Grand Calumet River, in northwestern Indiana. One organism, identified as a *Mycobacterium flavescens*, was capable of using pyrene as sole source of carbon and energy (Dean-Ross and Cerniglia 1996). The second organism was isolated using anthracene as sole source of carbon and energy, and has been identified as a

Rhodococcus species (Dean-Ross et al. 2001). The medium used for growth of the bacterial strains and for the soil slurry experiments was the mineral salts medium of Cohen-Bazire et al. (1957).

In order to determine whether the addition of detergents to bacterial cultures would have an effect on the rate of biodegradation, a screening test as described by Dean-Ross and Cerniglia (1996) was employed. Serum vials were inoculated with 1 ml (containing approximately 10^7 cells) of a growing culture of the test microorganism, and supplemented with 50 µl of a solution containing 100 µg of ^{14}C-labeled pyrene or anthracene and sufficient unlabeled PAH to bring the concentration in the vial to 50 µg/ml. Vials were fitted with well containing a 0.2 mL 1 M KOH to absorb ^{14}C-CO_2. Stock solutions of detergents were added to bracket the CMC for the detergent in the vial. After an appropriate time interval, the reaction was stopped by the addition of 1 mL 3 M H_2SO_4. Wicks were removed and placed in Ecolume.^{14}C-CO_2 was determined by liquid scintillation counting (Tracor Analytic Delta 300 Liquid Scintillation System, Elk Grove, IL).

Pristine sediment was obtained from Crooked Lake Biological Station and contained 75% sand, 16% silt, 9% clay, 1.7% organic matter and no detectable levels of PAH as determined by GC. Contaminated sediment was obtained from the Grant Calumet River at Bridge Street. This sediment contained 89% sand, 5% silt, 6% clay and 5.1% organic matter. PAH analysis is reported in the Results and Discussion section. The sediment suspension was sieved through a 2 mm sieve and kept under refrigeration in the dark until use. Soil slurries were prepared using the dry weight of the sediment, supplemented with concentrated mineral salts medium to make up the required loading rate.

In order to standardize conditions for bioremediation of contaminated sediments, a model slurry system was developed. Sediment slurries (500 mL) were prepared and spiked with unlabeled pyrene to achieve a concentration equal to the adsorptive capacity of the sediment as determined by Liu et al. (1991) plus 1.08 µg of [4,5,9,10]-C^{14}-pyrene per flask (ChemSyn, Lenexa, KS, >98% radiopurity). Flasks were placed on a rotary shaker and attached to a gas train. Air was passed through concentrated KOH to remove carbon dioxide, then a sterile cotton filter to prevent contamination of the contents. After passing through the slurry, the air was passed through two traps in series, each containing 20 ml of 1 M KOH to trap radiolabeled carbon dioxide. At appropriate intervals, the first trap was removed for sampling, the second trap was put in its place and another trap was placed in the second position. Treatments were prepared in quadruplicate. One flask of the four was treated with 2.5% (w/v) $HgCl_2$ to serve as a killed control. Samples removed from the KOH traps were added to an appropriate volume of Ecolume and assayed for radioactivity by liquid scintillation counting as described above. The amount of radiolabeled CO_2 produced by the killed control was subtracted from the average of the viable replicates to correct for abiotic release of radioactivity (<2% total added radioactivity). For aging experiments, a stock solution containing a mixture of unlabeled and ^{14}C-labeled pyrene calculated as

above was added to the sediment, which was mixed thoroughly and incubated in a brown bottle under refrigeration for four months prior to use.

Once conditions were standardized, a bioremediation experiments were conducted. In one, pristine sediment was supplemented with weighed amounts of the following unlabeled PAHs: phenanthrene, anthracene, pyrene and fluoranthene. In a second, contaminated sediment from the Grand Calumet River was used. Ten ml samples of the slurries were withdrawn at appropriate intervals and were extracted using 3-10 ml volumes of methylene chloride, which was subsequently dried over anhydrous sodium sulfate and evaporated using a Buchi rotary evaporator, (Model R-114, Brinkmann Instruments, Westbury, NY). The residue was dissolved in 100 µl of methylene chloride containing eicosane as an internal standard. Quantitation of PAHs was performed by GC using a Model 8500 gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a flame ionization detector using the method of Dean-Ross and Cerniglia (1996). Using this method, the limit of detection was determined to be 2 µg/ml of the original mineral salts medium. ANOVA was performed on the results using MINITAB; statistical differences were determined at the 0.05 significance level.

RESULTS AND DISCUSSION

Sediment slurries offer advantages in bioremediation projects by provide sufficient oxygen to maintain aerobic conditions, under which PAH degradation rates will be maximized. Furthermore, they provide an opportunity to control nutrient levels and temperature (Wilson and Jones, 1993). While slurry reactors have been used in soil bioremediation projects (Jee et al. 1998; Rutherford et al. 1998; White et al. 1999;), their use for PAH-contaminated sediments has been limited (Launen et al. 2002). In order to determine the optimum sediment loading rate, three sediment loadings were used : 5%, 10% and 20%, based on w/v of dry sediment weight. Sediments were prepared in quadruplicate, with one replicate serving as sterile control. Results are shown in Fig. 1. There were no significant differences between the 5 and 10% loading rate, while a lower degradation rate was observed using a 20% loading rate. Consequently, the 10% loading rate was used in subsequent experiments.

Tests were conducted to determine whether addition of surfactant would have an effect on the biodegradation rate of PAHs by the test microorganisms indicated that all of the detergents selected except Brij 35 and Tween 80 inhibited bacterial activity at concentrations below and slightly above the critical micelle concentration for the respective detergents. Due to the lack of inhibitory effects at high concentration, Tween 80 was selected to determine whether the presence of a surfactant in a sediment slurry would have a stimulatory effect on the rate of biodegradation. It was added to sediment slurries to achieve final concentrations of 50 and 130 ppm, selected as noninhibitory concentrations from results of the screening test. Results (not shown) indicated that neither concentration of surfactant had a significant effect on PAH degradation by *M. flavescens*. Both

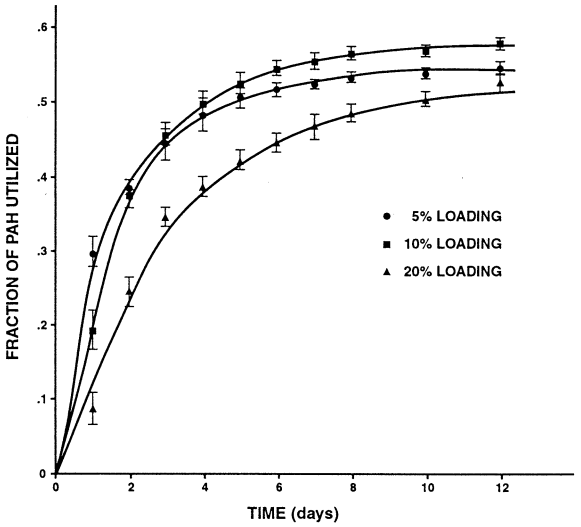

Figure 1. Effect of loading rate of mineralization of pyrene by *M. flavescens* in bioslurry reactors. Data points are the average of three replicates and error bars represent the standard error of the mean.

stimulation and inhibition of biodegradation rates of water-insoluble organic compounds have been observed in the presence of surfactants (Rouse et al., 1994). The use of surfactants to aid in solubilization and therefore increase the availability of PAHs in soils and sediments has been demonstrated (Guha and Jaffe, 1996; Liu et al. 1995; Willumsen and Arvin 1999). On the other hand, lack of stimulation has also been observed (Tsomides et al. 1995). These results suggest that the interaction between PAH, surfactant, and microorganism is complex, and does not result in the stimulation of biodegradation suggested by the increase in solubilization.

An additional factor affecting the biodegradation of PAHs in contaminated sediments is that of contact time between the PAH and the sediment (aging). An experiment was set up similarly to the previous experiments, except that sediment aged as described in the Methods section was used. Results (not shown) indicated that pyrene in aged sediments was utilized at the same rate as freshly spiked sediment, regardless of the presence of Tween 80. While the length of the aging period has been demonstrated to affect the biodegradation rate in contaminated soils the magnitude of the observed aging effect has been shown to vary with the type of microorganisms present (Guthrie and Pfaender 1998; Sandoli et al. 1996) and the water regime during aging (White et al. 1997). In the present study, no significant difference in the rate and extent of pyrene degradation was observed between freshly contaminated and aged sediments, even in the presence of a surfactant.

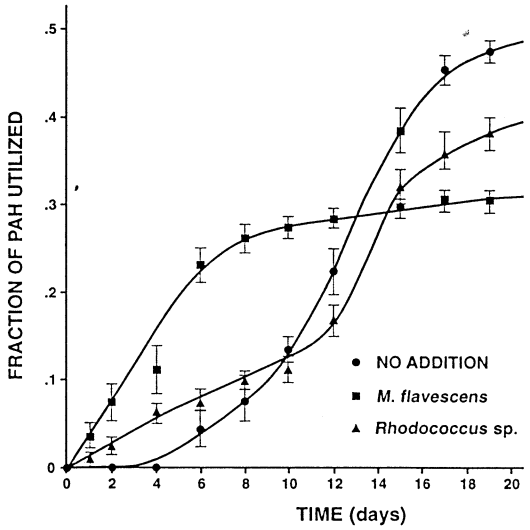

Figure 2. Mineralization of pyrene in the absence of added bacterial strains, in the presence of *M. flavescens* and in the presence of *Rhodococcus* sp. Data points are the average of three replicates; error bars represent the standard error of the mean.

The use of an inoculum of a PAH-degrading culture often increases the rate of degradation of the PAH in contaminated soils and sediments. An experiment was conducted to compare the ability of the two strains of bacteria to utilize the test substrate. Results are shown in Fig. 2. It can be seen that one organism, *M. flavescens*, rapidly degraded pyrene, while the *Rhodococcus* species utilized pyrene at a rate comparable to that of the uninoculated control. On the other hand, while the degradation of pyrene by *M. flavescens* leveled off after 10 days, the degradation by *Rhodococcus* and the uninoculated sediment continued to increase at a slow rate. Similar enhancements of biodegradation by inoculation have been observed in the case of phenanthrene in soil (Madsen and Kristensen 1997). This suggests that although PAH-degrading microorganisms may be present in the sediment, addition of a PAH-degrading strain may bring about an enhancement of the rate of biodegradation.

In order to simulate a bioremediation project, four PAHs were added to pristine sediment in slurry bioreactors. Aliquots were removed for GC analysis at appropriate time intervals. Results for pyrene, anthracene, phenanthrene and fluoranthene are shown in Fig. 3. The presence of the added bacteria strain had a significant stimulatory effect on utilization of the PAH in comparison to uninoculated sediment. It is noteworthy, however, that the indigenous population was capable of adapting to degrade the four PAHs, but required a longer time period for degradation of the added PAHs.

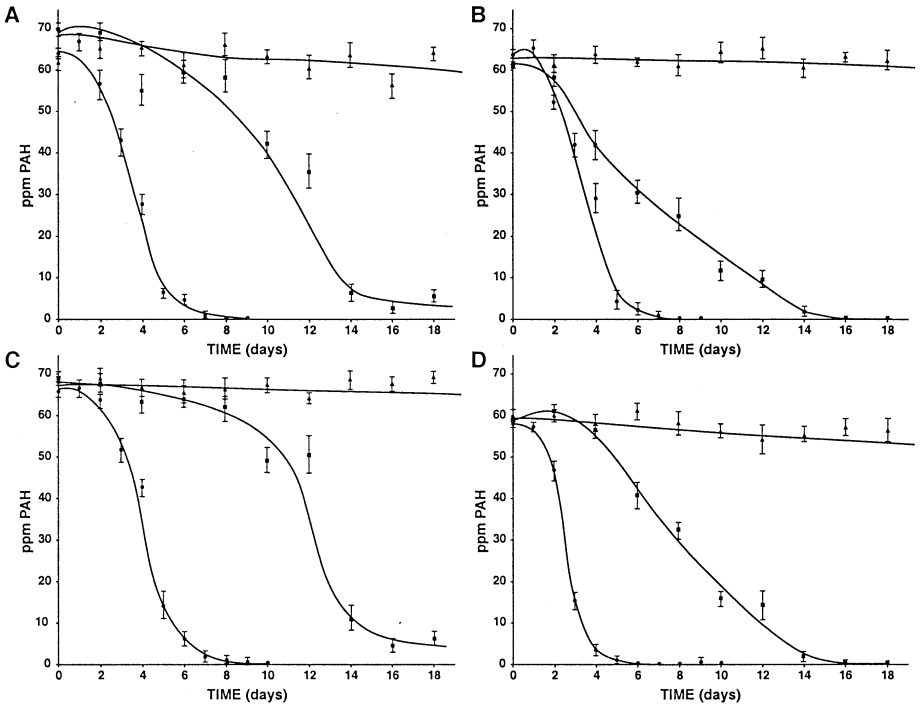

Figure 3. Utilization of fluoranthene (A), anthracene (B), pyrene (C) and phenanthrene (D) in a bioslurry reactor under the following conditions: no added bacteria (squares), added bacteria (circles), sterile control (triangles). Data points are the average of three replicates; error bars are the standard error of the mean.

Contaminated sediment from the Grant Calumet River was used in the bioslurry reactor to determine the effectiveness of the above conditions. Disappearance of the same four PAHs was followed over time as shown in Fig. 4. Phenanthrene (present initially at a concentration of 36.5 ppm) disappeared by the first sampling day and anthracene (present initially at a concentration of 110.2 ppm) disappeared by day 4 regardless of the presence of *Rhodococcus* sp., so they are not shown in Fig. 4. Disappearance of fluoranthene in the presence of *Rhodococcus* sp.was not significantly different from the disappearance in the presence of the bacterial strain after the first day, which may indicate the presence of bacterial strains capable of degrading fluoranthene in the contaminated sediment. The disappearance of pyrene was significantly different in the presence of the bacterial strain until day 15, indicating that the indigenous bacteria initially utilized the pyrene at a slower rate than the added bacterial strain. By day 15, however, pyrene had been removed to the detection limit regardless of the presence of the *Rhodococcus* sp.

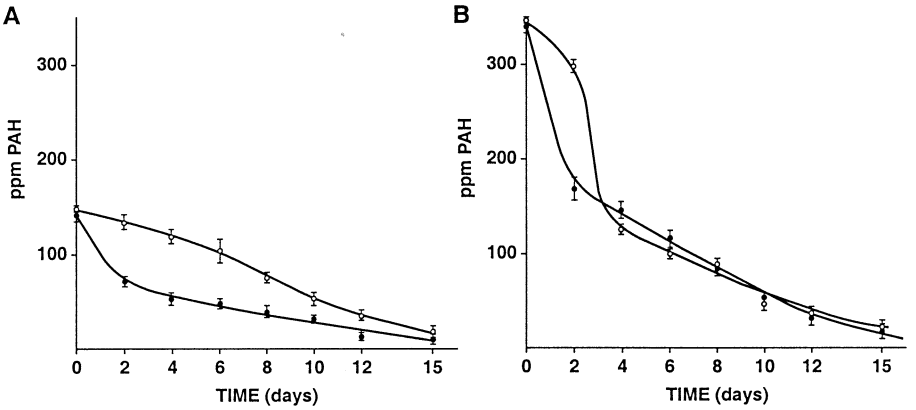

Figure 4. Utilization of fluoranthene (A) and pyrene (B) in a bioslurry reactor under the following conditions: no added bacteria (open circles), added bacteria (filled circles). Data are expressed in terms of dry weight of sediment. Data points are the average of two replicates; error bars are the standard error of the mean.

In summary, slurry bioreactors provide an efficient means of removal of contaminants such as PAHs from contaminated sediments. Addition of bacterial strains of known PAH-degradative capabilities may speed up the rate of biodegradation. Indigenous bacteria present in sediments appear to be capable of removing PAHs within acceptable time frames in the bioslurries, and in some cases within the same time frame as PAH-degrading strains.

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