USE OF PAH-DEGRADING BACTERIA IN BIOREMEDIATION OF PAH-CONTAMINATED SEDIMENTS

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ABSTRACT
Sediments in the Grand Calumet River in northwestern Indiana are heavily contaminated with polycyclic aromatic hydrocarbons (PAHs). Enrichment culture was used to obtain strains of bacteria which could degrade multiple PAHs. One strain, a *Mycobacterium flavescens*, was isolated using pyrene as sole source of carbon and energy, while a second, a *Rhodococcus* species, was isolated on anthracene. Several nonionic detergents were screened for the ability to enhance biodegradation of PAHs in liquid culture. Tween 80 was selected as producing the greatest stimulation of the PAH biodegradation rate. A sediment assay system was developed to optimize conditions for bioremediation. In this slurry system, bioremediation potential was assessed by measuring production of radiolabeled carbon dioxide from labeled PAHs. A sediment-water slurry ratio of 10% was found to produce the highest biodegradation rate of the three loading ratios used (5%, 10% and 20%). In the presence of sediment, a statistically significant effect of detergent on PAH biodegradation rate was not observed. Inoculation of the sediment with the *M. flavescens* stimulated the biodegradation rate in comparison to that observed in the unsupplemented sediment, although the extent of removal of the PAH was greatest in the uninoculated control. It is concluded that sediment slurries provide a means of effectively removing PAHs from contaminated sediment.

RÉSUMÉ
Les sédiments de la rivière Grand Calumet en Indiana du nord-ouest sont fortement contaminés d'hydrocarbures aromatiques polycycliques (HAP). On a trouvé deux espèces de bactéries dan des cultures d'enrichessement aux HAP à partir des sédiments contaminés. L'une des espèces, le *Mycobacterium flavescens*, a été isolée au pyrene comme source unique de carbone et d'énergie. L'autre, une espèce de *Rhodococcus*, a été trouvée à l'anthracene. On a mis en oeuvre plusieurs surfactants nonioniques pour déterminer leur capacité de faire accroître la biodégradation des HAP. Tween 80 a produit la plus grand intensification de vitesse de biodégradation des HAP en culture liquide. On a mis en oeuvre un bioréacteur biphasique (bioslurry) pour optimizer les conditions experimentales de la biodégradation. Dans ce système, on a déterminé le potential pour la dépollution des sediments en mésurant la production de ^{14}C-CO_2 de HAP radiolabele par ^{14}C. On a obtenu la plus grande production de ^{14}C-CO_2 avec une proportion sédiment/eau de 10%. En presence de sédiment, on n'a observé aucun effect significatif de surfactant sur la vitesse de biodégradation. La bioaugmentation du sédiment par *M. flavescens* a accéléré la vitesse de biodégradation par compariaison avec celle qu'on a observé au sédiment sans inoculum, bien que la dépollution du HAP ait été la plus importante dans le traitement sans inoculum. Pour conclure, la méthode « bioslurry » fournit un moyen efficace de dépolluer des sédiments contaminés.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons are ubiquitous contaminants in environments impacted by fossil fuels 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 compounds have been identified as genotoxicants in short-term mutagenicity assays and as carcinogens in long term rodent bioassays (Menzie et al. 1992). Sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) are frequent consequences of industrialization in the Great Lakes Region, including Hamilton Harbour, Ontario (Murphy, 2000), and the Grand Calumet River and Indiana Harbour, Indiana (Simmers et al. 1991). Due to the widespread distribution of PAHs in the Great Lakes, and their potential as a human health hazard, the remediation of PAH-contaminated sediments has assumed a high priority for regulatory agencies in the Great Lakes region.

One of the most promising methods of removal of PAHs from contaminated environments is that of bioremediation (Atlas and Cerniglia, 1995). 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 (Wang et al., 1990; Gray et al., 1994), while removal of higher molecular weight PAHs is more difficult to demonstrate (Wilson and Jones, 1993).

The goal of the research project reported here is to maximize biodegradation of PAHs in sediment slurries. Factors such as slurry loading rate, presence of detergents, aging and strain selection were identified as contributing to efficiency of bioremediation. In this paper, results of studies to maximize biodegradation rates are presented.

2. MATERIALS AND METHODS

2.1 Chemicals

Pyrene, ^{14}C-labeled, was obtained from ChemSyn Laboratories (Lenexa, KS) and was >98% radiopurity. Unlabeled PAHs were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were >98% purity. The following detergents were supplied by Sigma Chemical Co. (St. Louis, MO) at the highest purity available : Brij 30, Brij 35, Tween 80, Triton N-101, Triuton X-100,Tergitol NP-10, and polyoxyethylene 10 lauryl ether. Ecolume (ICN Pharmaceuticals, Aurora, OH) was used as a scintillation cocktail.

2.2 Microorganisms and culture conditions

The microorganisms used in this research were isolated by enrichment culture 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 at al. (1957).

2.3 Detergent effects on biodegradation

In order to determine whether the addition of detergents to bacterial cultures would have an effect on the rate of biodegradation, a screening test similar to that utilized 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 50,000 dpm of ^{14}C-labeled PAH and sufficient unlabeled PAH to bring the concentration in the vial to 50 µg/ml. Stock solutions of detergents were added to bracket the CMC for the detergent in the vial. After an appropriate time interval, $^{14}CO_2$ was determined by liquid scintillation counting (Tracor Analytic Delta 300 Liquid Scintillation System, Elk Grove, IL).

2.4 Sediment slurries

Sediment was obtained from Crooked Lake Biological Station and contained no detectable levels of PAH contamination as determined by GC. The sediment 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 and to ensure that each treatment contained the same amount of nutrients.

In order to standardize conditions for bioremediation of contaminated sediments, a model slurry system was developed. Sediment slurries 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 0.25 µCi radiolabeled pyrene per flask. Flasks were placed on a rotary shaker and hooked up to a gas train. Air was passed through concentrated KOH to remove carbon dioxide, then a sterile cotton filter to maintain sterility in the flasks. After passing through the flask, 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 $HgCl_2$ to serve as a killed control. For aging experiments, a weighed amount of pyrene (calculated as above) supplemented with ^{14}C-labeled pyrene 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 experiment was conducted in which sediment slurries were supplemented with weighed amounts of unlabeled PAHs. The contaminated sediment slurry was incubated overnight prior to initiation of the experiment. 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.

2.5 Analytical

Samples removed from the KOH traps were added to an appropriate volume of Ecolume and assayed for radioactivity using a Tracor Analytic Delta 300 Liquid Scintillation System (Elk Grove, IL). The killed control was subtracted from the average of the viable replicates to correct for abiotic release of radioactivity. 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). The column was a fused silica capillary column, 30 m long with an internal diameter of 0.24 mm, coated with 1.0 µm of a bonded and cross-linked stationary phase consisting of 5% phenyl-substituted polymethylsiloxane (DB-5, J&W Scientific, Rancho Cordova, CA). Initial column temperature was 150 ^{o}C ; column temperature was increased to 275 ^{o}C at a rate of 5 ^{o}C/min, and was held at the final temperature for 5 min. The injector and detector temperatures were 250 ^{o}C. Using this method, the limit of detection was determined to be 2 µg/ml of the original mineral salts medium.

3. RESULTS

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

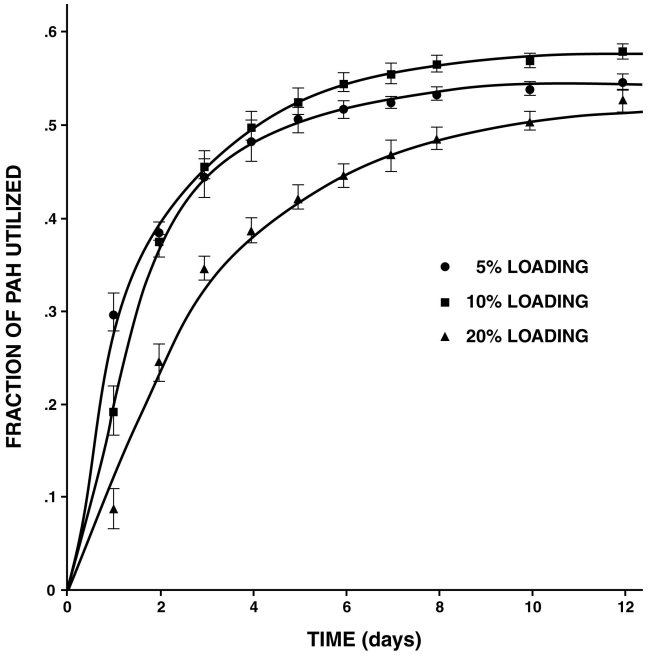

Fig. 1. Effect of loading ratio on biodegradation of pyrene in bioslurry reactors.

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.

Screening tests were conducted to determine whether addition of surfactant would have an effect on the biodegradation rate of PAHs by the two test microorganisms. It was found that all of the detergents selected except Brij 35 and Tween 80 inhibited bacterial activity at concentrations below and slightly above the CMC for the respective detergents. In the presence of Brij 35, utilization of pyrene by *M. flavescens* was increased by 175% at 50 ppm, 177% at 80 ppm and 155% at 130 ppm, while the presence of Tween 80 increased pyrene utilization 137% at 30 ppm, 150% at 50 ppm, 157% at 70 ppm and and 160% at 130 ppm. Utilization of anthracene in the presence of Brij 35 by the *Rhodococcus* sp. was increased by 300% in the presence of 50 ppm, 120% at 80 ppm, and was inhibited 75% at 130 ppm while the presence of Tween 80 increased utilization by 170% at 30 ppm, 160% at 50 ppm, 310% at 70 ppm and 198% at 130 ppm.

Due to the lack of inhibitory effects at high concentration, Tween 80 was selected to determine whether the presence of a surfactant in a soil slurry would have a similar stimulatory effect on the rate of biodegradation. Tween 80 was added to soil slurries to achieve final concentrations of 50 and 135 ppm, selected as noninhibitory concentrations from results of the screening test. Results are shown in Fig. 2, and indicate that neither concentration of surfactant had a significant effect on PAH degradation by *M. flavescens*.

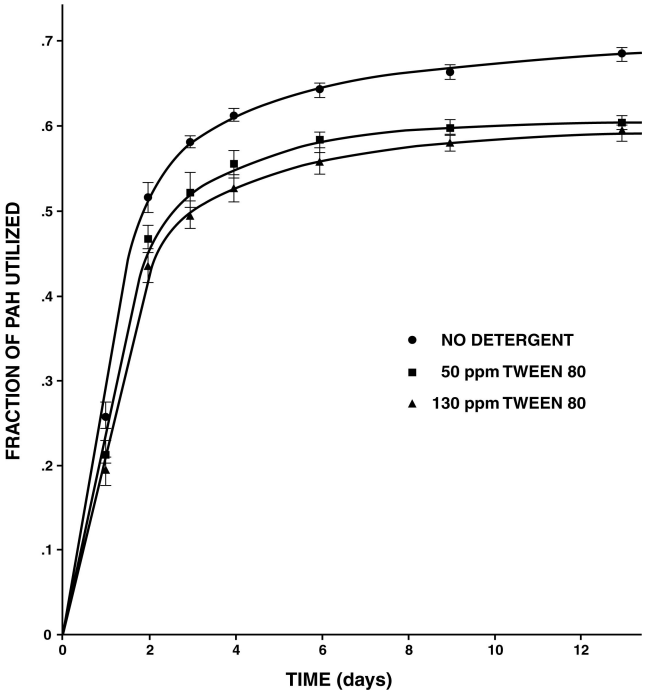

Fig. 2. Effect of surfactant on biodegradation of pyrene in bioslurry reactors.

Because in contaminated environments, the sediments have been in contact with PAHs for long periods of time, the effect of aging was investigated. This experiment was set up similarly to the previous experiments, except that sediment aged as described in the Methods section was used. Results are shown in Fig. 3, and indicate pyrene in aged sediments was utilized at the same rate as freshly spiked sediment. Furthermore, biodegradation rate was unaffected by the presence of surfactant in this system also.

An experiment was conducted to compare the two strains of bacteria in terms of their effect on the ability of the bacterial strain to utilize the test substrate. Results are shown in Fig. 4. It can be seen that the *M. flavescens* gave the most rapid mineralization of pyrene under the test conditions, with the mineralization rate of the *Rhodococcus* sp. being similar to that of uninoculated sediment.

In a final study, four PAHs were added to sediment to simulate a bioremediation project using contaminated sediment. Aliquots were removed for GC analysis at appropriate time intervals. Results for pyrene, anthracene, phenanthrene and fluoranthene are shown in Fig. 5. The results confirm that addition of detergent had no significant effect on biodegradation rate. In addition, the presence of the added bacterial strain had a stimulatory effect on utilization of the PAH in comparison to uninoculated sediment.

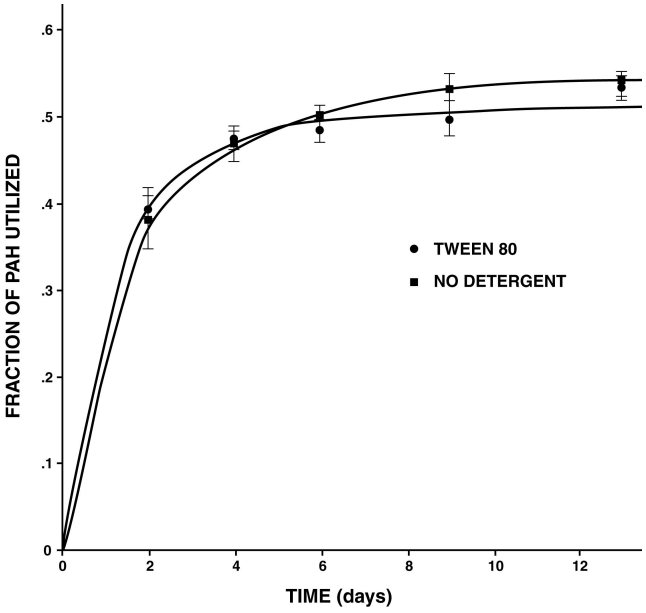

Fig. 3. Biodegradation of pyrene in aged sediment in the presence and absence of Tween 80.

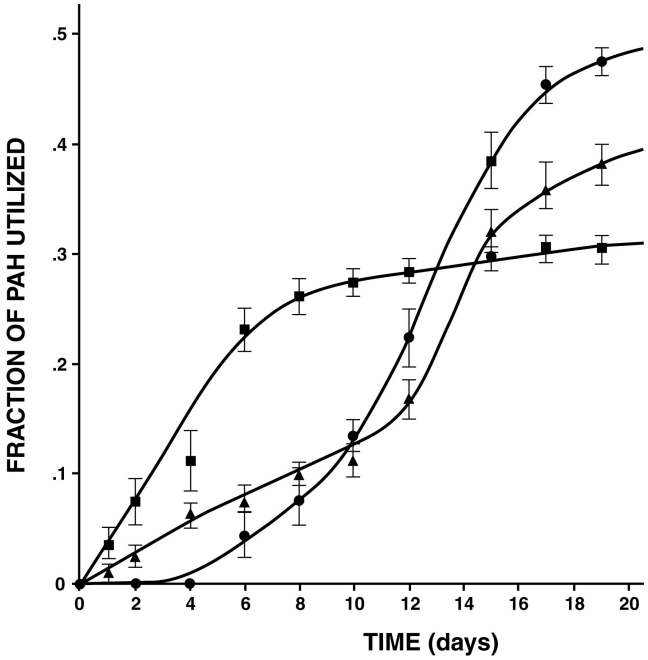

Fig. 4. Effect of inoculum on biodegradation of pyrene: no inoculum (circles), *M. flavescens* (squares) and *Rhodococcus* (triangles).

4. DISCUSSION

Sediment slurries offer advantages in bioremediation projects. They provide sufficient oxygen to maintain aerobic conditions, under which PAH degradation rates will be maximized, and they offer opportunities to control nutrient levels, temperature and aeration. In addition, they provide an opportunity to supplement the sediment with microbial strains known to degrade PAHs (Wilson and Jones 1993). Several studies have indicated the feasibility of adapting a soil slurry bioreactor for bioremediation of contaminated soils and sediments. Jee et al. (1998) demonstrated phenanthrene removal in a slurry reactor. In addition to PAHs, slurries have been shown to be effective in removal of many low solubility contaminants such as creosote (Rutherford et al. 1998), trinitrotoluenes (Boopathy and Manning 1999; Zhang et al. 2000), pentachlorophenol and creosote (Mueller at al. 1991), and chlorobenzenes (Brunsbach and Reineke 1994).

Both 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 availability of PAHs in soils and sediments has been demonstrated (Liu et al. 1995). Studies on the biodegradation of fluoranthene by *Sphingomonas paucimobilis* demonstrated an increase in mineralization rates in the presence of low levels of a Triton X-100, but the observed increase was not proportional to fluoranthene solubilization (Willumsen and Arvin 1999).

Guha and Jaffe (1996) observed that micellar-phase phenanthrene was available to microorganisms, but the bioavailable fraction decreased with increasing surfactant concentration. On the other hand, lack of stimulation has also been observed. Tsomides et al. (1995) observed that many non-ionic surfactants were inhibitory to PAH-degrading organisms. Triton X-100, which was not inhibitory, did not have a significant stimulatory effect on phenanthrene mineralization and concentrations above the critical micellar concentration. 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). It has been noted in numerous studies that as the period of aging lengthens, PAHs and other organic contaminants become progressively less available for uptake and therefore microbial degradation (Hatzinger and Alexander 1995; Alexander, 2000). However, the magnitude of the observed aging effect has been shown to vary with the type of microbial community present (Sandoli et al., 1996; Guthrie and Pfaender 1998) 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.

The use of an inoculum of a PAH-degrading culture often increases the rate of degradation of the PAH in contaminated soils and sediments. In the current study, one organism, *M. flavescens*, rapidly degraded pyrene, while addition of a *Rhodococcus* species showed no difference in biodegradation rate in comparison to the uninoculated control. Similar enhancements of biodegradation by

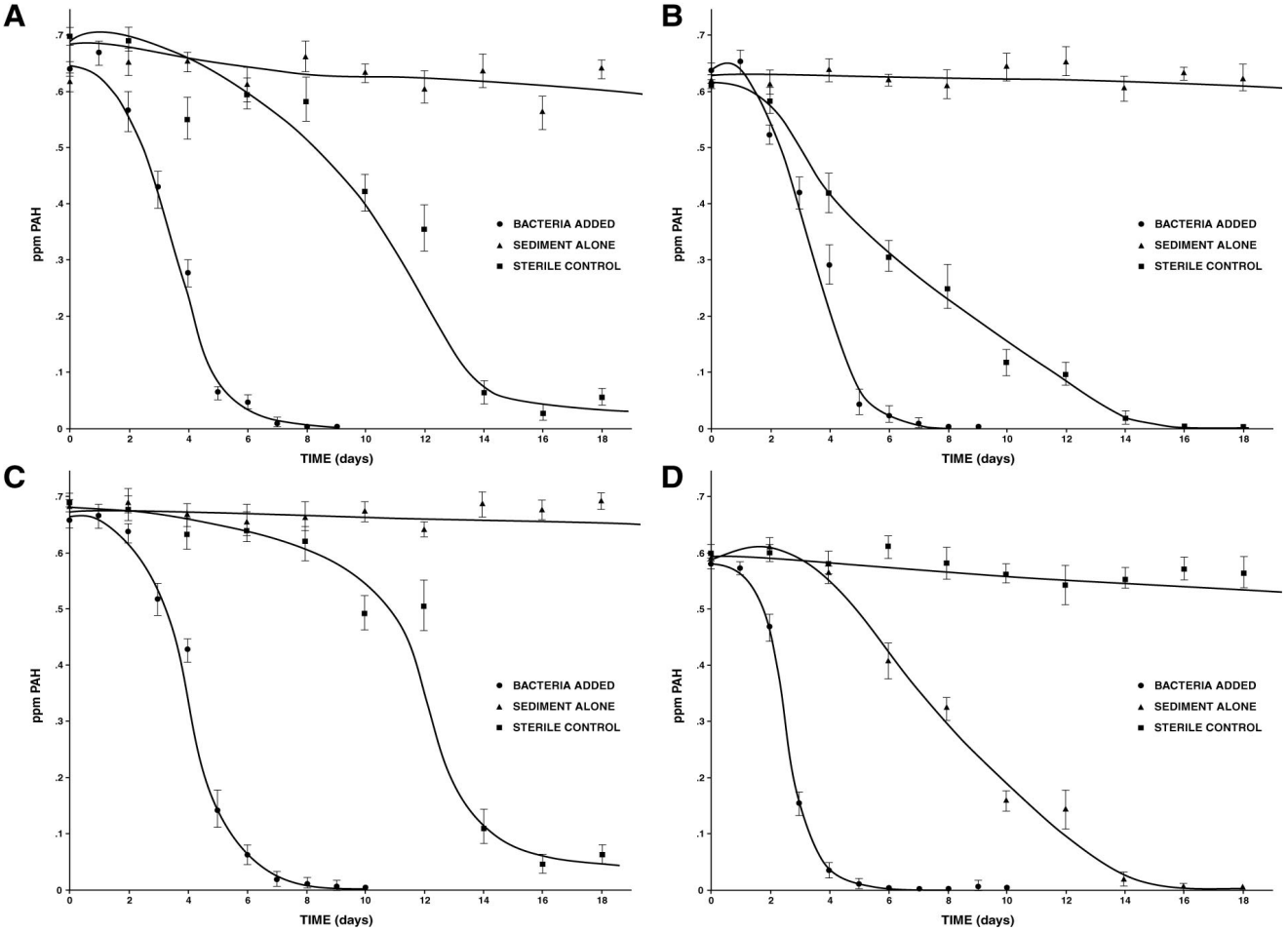

Fig. 5. Utilization of fluoranthene (A), anthracene (B), pyrene (C), and phenanthrene (D) in a bioslurry reactor.

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 biodegradation rates.

Results reported here indicate that PAH levels can be reduced to below the detection limit by a soil-slurry using an inoculum within 8 to 10 days. Reductions of at or close to the detection limit were achieved in uninoculated soil slurries within 16 to 18 days. A reduction in phenanthrene of 95% was observed within 14 days in a sediment slurry bioreactor, while higher molecular weight PAHs were removed over longer time periods (Launen et al. 2002). Complete removal of phenanthrene was observed in a slurry bioreactor over a 7 day period (Jee et al. 1998). Use of a slurry bioreactor for creosote contaminated sediment brought about a reduction of >50% of the targeted PAH within 3-5 days (Mueller et al. 1991). Slurrying was shown to enhance the rate of utilization of both aged and unaged phenanthrene in contaminated soils (White et al. 1999).

In conclusion, the use of slurry bioreactors represents an effective means of removal of PAHs from contaminated sediments. The presence of surfactants is not needed to achieve optimal removal rates. While inoculation will increase the speed of removal of select PAHs, satisfactory removal of PAHs can be achieved using the indigenous microbial population.

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