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Biodegradation of Bicyclic and Polycyclic Aromatic Hydrocarbons in Anaerobic Enrichments

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Although many polycyclic aromatic hydrocarbons (PAHs) are known to be biodegraded under aerobic conditions, most contaminated sediments are anaerobic. With recent results demonstrating that some bicyclics and PAHs can be degraded without oxygen, information on specific biodegradation rates and electron acceptor stoichiometry is lacking. A fluidized bed reactor (FBR) enrichment approach was used to enrich for bacteria from creosote-contaminated marine sediments with nitrate or sulfate as the sole potential terminal electron acceptors and with naphthalene, biphenyl, dibenzofuran, and phenanthrene as the sole source of carbon and energy. Influent and effluent analysis showed removal of naphthalene, biphenyl, and phenanthrene in the FBRs but not dibenzofuran after 100−200 days. Batch incubations of FBR cells, using strict anaerobic techniques, confirmed the transformation of naphthalene, biphenyl, and phenanthrene with stoichiometric removal of nitrate by the nitrate FBR enrichment. Similarly, phenanthrene, biphenyl, and naphthalene stimulated sulfide production in the sulfate-reducing enrichment and were removed, generally with stoichiometric production of sulfide. The specific PAH biodegradation rates in these cultures were 1−2 orders of magnitude lower than those reported for aerobic cultures. These results show that bicyclics and PAHs can be biodegraded under nitrate- and sulfate-reducing conditions and suggest that anaerobic treatment may provide a useful option for remediation of PAH-contaminated sediments.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous geoenvironmental pollutants (*1*−*3*). They result primarily from the combustion of fossil fuels and the distillation and use of crude oil and other petroleum products. In marine environments, PAHs are predominantly found in near-shore sediments around harbors and shore-side facilities that produce and store PAH-containing materials and near the wooden pilings of docks and retaining walls.

PAHs with less than five rings are usually biodegradable by bacteria under aerobic conditions. The aerobic microbial attack on PAHs can be summarized as follows: dioxygenase attack of a single (usually terminal) ring leading to a catechol-like structure, oxygenolytic cleavage of the catechol (either meta or ortho to the dihydroxyl groups) by the action of another dioxygenase, followed by further ring cleavage to metabolically utilizable substrates. These well-known capabilities make an aerobic approach to in situ sediment remediation seem attractive at first glance. However, in situ sediment treatment using aerobic methods would face many technical problems. The oxic zone of polluted sediments typically makes up only the surficial 1−10 mm, and compounds spend 90−99% of the time under anoxic conditions (*4*−*6*). Consequently, treatment below this zone would require the introduction of oxygen into the sediment, which is technically difficult due to the buoyancy of air and the low solubility of oxygen in water. Therefore, our present understanding of PAH degradation in aerobic environments is not generally applicable to sediment bioremediation.

Anaerobic biological treatment may provide solutions to some of these problems. Anaerobic electron acceptors such as nitrate, sulfate, and Fe(III) can be used by bacteria to biodegrade aromatic contaminants such as benzene, toluene, ethyl benzene, and xylene (BTEX) (*7, 8*). However, under anaerobic conditions, PAHs tend to be highly stable to anaerobic attack. This stability makes it difficult to cleave the ring without oxygen, and until recently unsubstituted aromatic rings were thought to be recalcitrant to biodegradation under anaerobic conditions. However, recent research has shown that unsubstituted mono- and polyaromatic compounds can be degraded under nitrate-reducing (*9*), iron-reducing (*10*), sulfate-reducing (*11*−*15*), and methanogenic conditions (*7, 8*).

If anaerobic biodegradation of PAH were found to be inducible under a variety of electron acceptor conditions, it may be possible to stimulate biodegradation of these compounds in contaminated sediments through the addition of anaerobic electron acceptors, such as nitrate or sulfate salts. In the research discussed here, we describe attempts to enrich for anaerobic naphthalene, biphenyl, dibenzofuran, and phenanthrene biodegradation in sediment-free cultures using fluidized bed reactor (FBR) enrichment approaches. An FBR enrichment approach was chosen because their operation results in long cell mass residence times with a high mass loading of dissolved substrate (*16*). This is a desirable characteristic for enriching slow growing organisms on sparingly soluble substrates such as PAHs.

Materials and Methods

The FBRs were inoculated with coal tar creosote-contaminated sediment obtained from Eagle Harbor in Puget Sound, WA (47°37.23″ N latitude, 122°29.30″ W longitude) as described (*17*). Sediment from the core and overlaying seawater was used to fill glass bottles (500 mL) for transport to the laboratory and storage at 4 °C prior to the setup of the FBRs.

Enrichment Strategies. The FBRs consisted of a glass reactor with a recycle pump to fluidize a bed of diatomaceous earth biocarrier and a feed pump to supply organics, electron acceptor, and nutrients. The FBRs were supplied with either sulfate or nitrate as the sole potential electron acceptor and a mixture of biphenyl, naphthalene, phenanthrene, and dibenzofuran as the sole carbon sources (no other organic compounds were added).

Subsamples (500 g) of subsurficial sediment from the boxcores were placed in a stoppered Erlenmeyer flask (500 mL) with a deoxygenated N_2 headspace and homogenized by shaking on a shaker table (24 h, 180 rpm). Approximately 25 g of the homogenized sediment was placed in an

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Erlenmeyer flask (1 L) and diluted with 1 L of deoxygenated artificial seawater (ASW) medium leaving no headspace. The ASW recipe was based on a formulation by Rockne et al. (*18*) modified by the addition of NH_4Cl (0.425 g L^{-1}) as the nitrogen source. Either $NaNO_3$ (3.5 mM, nitrate-reducing enrichments) or Na_2SO_4 (28 mM, sulfate-reducing enrichments) was added as a potential electron acceptor. The media was made anaerobic using standard methods (*19*).

The sediment/ASW mixtures were placed on a shaker table (100 rpm) at room temperature (20−22 °C) and incubated. After 2 weeks, the flasks were removed from the table and allowed to settle for 20 min, and the supernatants were used to fill the FBRs.

The FBRs were approximately 3 cm i.d. × 30 cm tall with an empty bed volume of about 200 mL. The FBRs contained a bed of Celite, particles of silica/alumina (size R-633, Celite Corp., Lompoc, CA), as a support medium for bacterial growth. The biocarrier was fluidized by recirculating ASW media as described previously (*16*) and maintained at room temperature (20−27 °C).

The ASW feed for the FBRs was prepared as described above with the addition of resazurin (0.1 mg L^{-1}) redox indicator and sulfide (2 mg S L^{-1} final concentration) to poise the E_h of the ASW feed (*20*). The feed solution was pumped through an in-line glass column (50 mL empty bed volume) filled with glass beads (3 mm diameter, Fisher Scientific, Bellafonte, PA), which were previously coated with phenanthrene, biphenyl, dibenzofuran, and naphthalene. All of these compounds are components of coal-tar creosote and were present in the sediment that was the source of the inoculum. The PAH mixture was dissolved in methylene chloride (1 g L^{-1} each compound), and the solution was placed in the glass column with the glass beads. The methylene chloride was allowed to evaporate under a stream of air sparged through the column (<0.1 m^3 h^{-1} at standard temperature and pressure), leaving the beads coated with the PAHs. The PAHs dissolved into the influent medium as it was pumped through the column at a rate that resulted in a 1-day hydraulic residence time in the FBRs. A N_2 headspace was kept in the feed flasks using a manometric gas apparatus as described previously (*18, 21*).

Samples (5 mL) were taken periodically from ports in the influent feed line, the effluent line, and through a side port in the FBRs with a N_2-purged syringe for analysis of PAH and electron acceptor as described below.

PAH Degradation Experiments. Experiments with subsamples of the FBR biomass were performed using cultures in serum bottles under strictly anaerobic conditions. Cell mass and biocarrier (30 mL) were anoxically transferred from the FBR through a side port to a N_2-filled serum bottle (30 mL). The mass of biocarrier and cells removed from the FBR was determined by measuring the volatile and total suspended solids (VSS and TSS) of the suspension (*22*). Cells were removed from the carrier by vigorous shaking on a vortexer for approximately 1 min. Following settling of the biocarrier (1 min), the supernatant was resuspended in fresh medium in a serum bottle (30−60 mL) and used to inoculate the degradation experiments described below. All operations were carried out under oxygen-free N_2.

Cell suspensions were added after the PAHs were sorbed to the bottle walls using a methylene chloride delivery system. The PAHs were dissolved in methylene chloride in variable concentrations depending on the target concentration. Aliquots of the solution (50 or 100 μL) were added to the test bottles and distributed evenly around the inside of the bottles. The methylene chloride was allowed to evaporate (1−2 min), leaving the PAHs adsorbed to the vial wall. Identical amounts of PAH were added to bottles with filter-sterilized (0.2 μm), degassed, deionized water (DIW) to determine the initial PAH concentration. PAHs were quantified following incubation for 1 week to allow for complete solubilization of the PAHs in the DIW. Formaldehyde solution (5% v/v) was added to killed control bottles.

Anaerobic ASW was prepared as described above except the reductant was titanium citrate (0.2 mM) to ensure that no trace oxygen was present. The test bottles (30 mL) were inoculated with the cell mass suspension from the FBR by anoxic transfer from the serum bottle and prepared using Hungate's technique (*19*). The medium was sparged for an additional 10−20 min, and the vials were capped with Teflon-lined, thick butyl rubber stoppers (20 mm, West Biodirect, Lionville, PA). N_2 was deoxygenated by passage through a column of reduced copper filings at 300 °C. The bottles were placed in an anaerobic glovebox (Forma Scientific Inc., Marietta, OH) containing a gas composition of 80% N_2, 18% CO_2, and 2% H_2 for incubation.

The test bottles were sampled periodically with a syringe for determination of PAHs, nitrate, and sulfide. The sampling syringes were flushed with deoxygenated N_2, which was transferred into the glovebox in a large serum bottle (160 mL) fitted with a thick butyl rubber stopper immediately prior to sampling. The N_2 supply bottle was pressurized (5−10 psi) with deoxygenated N_2 to prevent the glovebox atmosphere from seeping into the bottle when a volume of gas was removed by the sampling syringe. The N_2 supply bottle also contained fresh $TiCl_3$ solution (2 M, 20 mL) to further scrub any trace oxygen that may have entered during the sampling procedure.

Samples (1 mL) for PAH and nitrate analysis were placed in vials (4 mL) and diluted 1:1 with methanol for transport out of the glovebox. Samples for sulfide analysis (1 mL) were drawn from the experimental bottles into a N_2-purged syringe. The needle of the syringe was closed by partial insertion into a butyl rubber stopper. The syringe was transferred out of the glovebox, and its contents was analyzed immediately allowing no exposure to oxygen.

Determination of Biodegradation Rate and Reaction Stoichiometry. Specific PAH removal rates were determined as described previously (*18*). The rate was normalized to the initial biomass (as VSS) at the beginning of the experiment. The use of an initial measurement of cell mass was valid because the amount of PAH was insignificant as compared to the concentration of cell mass (PAH to VSS less than 6% wt/wt). Therefore, even complete utilization of PAH for cell mass production would result in at most a 6% increase in biomass.

The stoichiometry of PAH removal and electron acceptor transformation was determined from the ratio of the mass of nitrate removed (nitrate-reducing subcultures) or sulfide produced (sulfate-reducing subcultures) to the mass of PAH removed at the end of the experiment. The theoretical stoichiometry was calculated assuming complete oxidation of the PAH to CO_2, as in the following reaction equations for naphthalene (*23*):

$$1/48C_{10}H_8 + 1/5NO_3^- + 1/5H^+ \rightarrow 5/24CO_2 + 1/10N_2 + 11/60H_2O$$

$$1/48C_{10}H_8 + 1/8SO_4^{2-} + 3/16H^+ \rightarrow 5/24CO_2 + 1/16H_2S + 1/16HS^- + 1/12H_2O$$

Chemical Analysis. PAHs in the FBR influents and effluents were analyzed by gas chromatography (GC); all other PAH analyses were by high-pressure liquid chromatography (HPLC). For GC analysis, aliquots of the sample (2 mL) were placed in a 4-mL HPLC vial (Sunbrokers, Charlotte, NC), and 2 mL of hexane was added. The tubes were sealed with Teflon-lined caps, vortexed on a high setting for 90 min, and centrifuged at 1500*g* for 10 min. The hexane was withdrawn

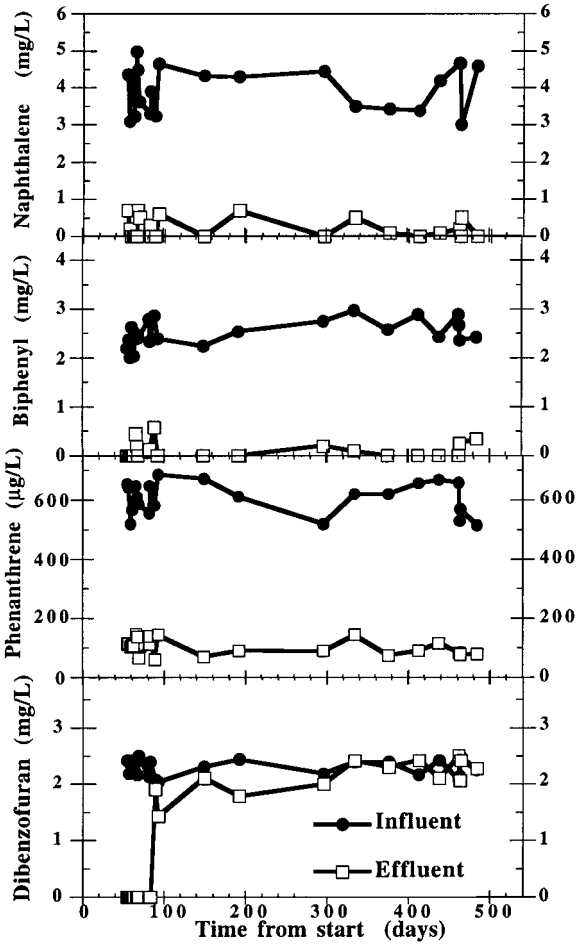

FIGURE 1. Influent (closed symbol) and effluent (open symbol) PAH concentration in the nitrate-reducing FBR showing removal of biphenyl, naphthalene, phenanthrene, and dibenzofuran.

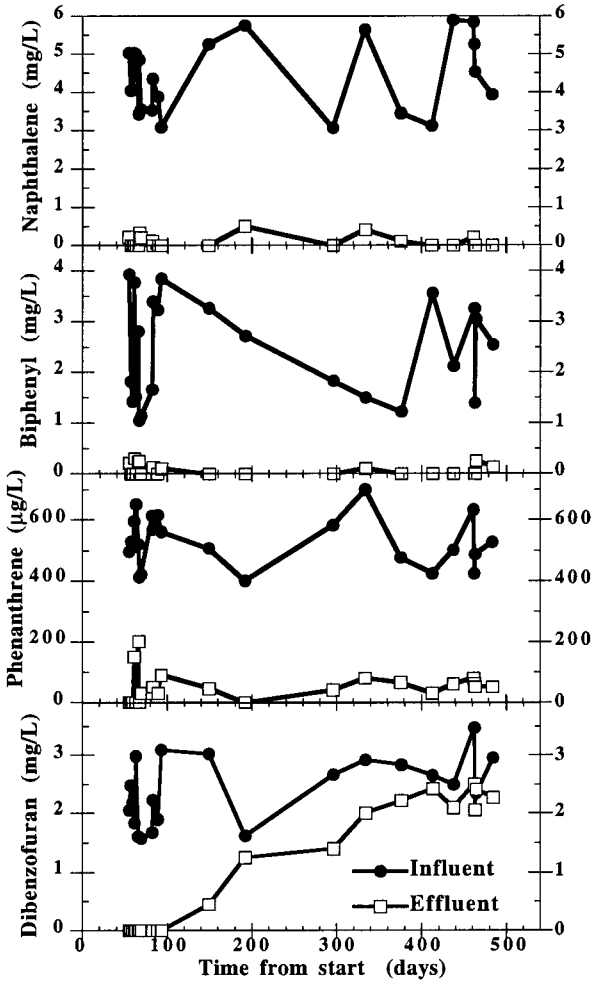

FIGURE 2. Influent (closed symbol) and effluent (open symbol) PAH concentration in the sulfate-reducing FBR showing removal of biphenyl, naphthalene, phenanthrene, and dibenzofuran.

and placed in a 2-mL GC vial, and PAHs were quantified on a Perkin-Elmer Autosystem GC as described previously (*18*). For HPLC analysis, the methanol-diluted samples (described above) were vortexed on a high setting for 90 min and centrifuged (10 min, 1500*g*). An aliquot was transferred to an HPLC vial insert (0.5 mL) for analysis on an HPLC (Waters model 501) fitted with a C-18 column (25 cm) and a UV absorbance detector at 210 nm (dibenzofuran and naphthalene) or 247 nm (biphenyl and phenanthrene). The isocratic mobile phase was 85% methanol/15% H_3PO_4 (0.1%). Peak quantification was performed by a Maxima 820 chromatographic workstation software. Sulfide was measured by iodometric titration (*22*), modified for small sample volumes. Samples (1 mL) were placed in a beaker with 20 mL of DIW, 2 mL of HCl (6 N), three drops of potato starch (20 g L^{-1}) indicator, and 250 μL of 0.025 N KI/I_2 solution. The sample was back-titrated to clear with 0.01 N $Na_2S_2O_3 \cdot 5H_2O$ solution, and sulfide was determined as the difference between titration with and without sample. Nitrate was measured on filtrates (0.45 μm) of the methanol-diluted sample (described above) by the method of Schroeder (*24*).

Results and Discussion

FBR Enrichment Studies. The FBRs were inoculated and were run for 4 weeks prior to initial sampling. Concentrations of phenanthrene, biphenyl, naphthalene, and dibenzofuran in the FBR effluents were substantially decreased relative to the influent concentrations at the start (Figures 1 and 2). After 100−200 days of operation, dibenzofuran concentrations in the effluent increased and eventually reached the influent concentration, indicating that no further dibenzofuran removal occurred. Possible mechanisms for the initial losses in the FBRs include biodegradation, adsorption, and abiotic transformation. The dibenzofuran data suggest a saturation−adsorption mechanism. Initially, dibenzofuran losses in the FBR may have been due to adsorption to biosolids, tubing, and bed material. The available adsorption sites would eventually have become saturated, leading to breakthrough of dibenzofuran, as was observed. Biomass concentrations were significantly higher in the sulfate-reducing FBR, as suggested by VSS concentrations (2.4−6 g of VSS L^{-1} vs 0.25−0.8 g of VSS L^{-1} for the sulfate- and nitrate-fed FBR, respectively). The higher biomass would result in more potential adsorption sites in the sulfate FBR and also likely result in an increased time before dibenzofuran breakthrough in the sulfate-reducing FBR (compared to the nitrate-reducing FBR), in agreement with the observed results (400 and 100 days, respectively).

Because dibenzofuran is intermediate in solubility and hydrophobicity as compared to the other test compounds, similar adsorption behavior would be expected with biphenyl, naphthalene, and phenanthrene. However, these compounds continued to be substantially removed in both FBRs through 500 days of operation, suggesting the involvement of additional loss mechanisms such as biodegradation. Both nitrate loss and sulfide production was observed in the nitrate and sulfate FBRs, respectively (data not shown). There are no known abiotic oxidation reactions between nitrate or sulfate and the PAHs. Thus, continued removal of biphenyl,

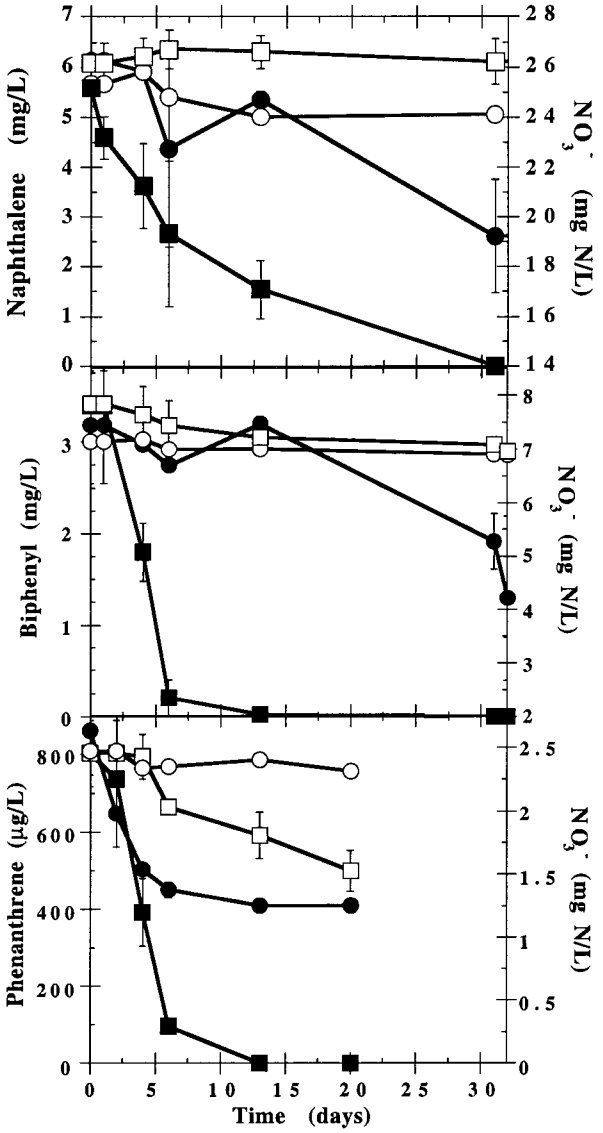

FIGURE 3. Anaerobic biodegradation of PAHs (squares) with concomitant reduction of nitrate (circles) by a nitrate-reducing enrichment culture. Concentrations in corresponding killed controls are shown by open markers. Incubated in separate bottles with corresponding PAH. Average of triplicates ± SEM.

naphthalene, and phenanthrene in the nitrate and sulfate FBRs beyond 100−200 days was likely due to biodegradation under nitrate- or sulfate-reducing conditions. Due to the adsorption effects, the acclimation period (if any) for naphthalene, biphenyl, and phenanthrene biodegradation could not be determined.

The amount of nitrate removal and sulfide production in the nitrate- and sulfate-reducing FBRs was approximately stoichiometrically equivalent to the total amount of PAH removed in each FBR. The observed removal of biphenyl, naphthalene, and phenanthrene would be stoichiometrically equivalent to 5.5−6.5 mg of NO_3-N L^{-1} removal in the influent feed, comparing well with the observed range of 3−6 mg of NO_3-N L^{-1} removal in the nitrate FBR. Similarly, biphenyl, naphthalene, and phenanthrene removal in the sulfate-reducing FBR could account for 9−12 mg of S L^{-1} sulfide production, in agreement with the observed sulfide concentrations in the FBR effluent (6−10 mg of S L^{-1}). Much of the sulfide used to poise the ASW feed (2 mg of S L^{-1} initial concentration) was probably oxidized during preparation of the media and was thus neglected from the calculation.

TABLE 1. Ratio of PAH Removal to Electron Acceptor Reduction[a]

enrichment/PAH	e-acceptor/PAH stoichiometry (actual)[b]	e-acceptor/PAH stoichiometry (theoretical)[c]
nitrate reducers		$NO_3^- \rightarrow N_2$
phenanthrene	14.5	13.2
biphenyl	10.8	11.6
naphthalene	10.7	9.6
sulfate reducers		$SO_4^{2-} \rightarrow HS^-$
phenanthrene	50.4	8.25
biphenyl	8.8	7.25
naphthalene	6.2	6.0

[a] Actual versus theoretical. [b] Moles of nitrate transformed or moles of sulfide produced per mole of PAH removed during incubation. [c] Moles of nitrate transformed or moles of sulfide produced per mole of PAH removed assuming oxidation of PAH to CO_2.

The higher biomass concentrations in the sulfate-reducing FBR may have reflected the greater initial biomass addition from the sediment inoculum and the higher rate of cell mass loss in the nitrate reactor effluent. Cell mass in the nitrate-reducing FBR was continually lost in the effluent, thus keeping the standing cell mass concentration lower in the FBR. In contrast, the sulfate-reducing FBR did not have large quantities of cell mass in the effluent and appeared to maintain a more solid biofilm on the biocarrier, which may have included substantial quantities of inactive biomass.

After 400 days of operation, biomass from the FBRs was harvested for PAH biodegradation experiments using strict anaerobic techniques to determine whether losses observed in the FBRs were due to biological activity, and if so, to establish that anaerobic PAH degraders were present in the FBR. Individual PAHs (phenanthrene, biphenyl, naphthalene, or dibenzofuran) and the appropriate electron acceptor were added to the FBR biomass in serum bottles and incubated anaerobically.

PAH Degradation by the Nitrate-Reducing FBR Biomass. Naphthalene, biphenyl, and phenanthrene were completely removed by the nitrate-reducing culture in individual bottle tests (Figure 3). Both naphthalene and phenanthrene losses (corrected for killed controls) were concomitant and nearly equivalent stoichiometrically with nitrate removal, assuming oxidation of PAH to CO_2 and denitrification to N_2 (Table 1). Nitrate reduction was nearly simultaneous with naphthalene and phenanthrene removal, consistent with a coupled reaction hypothesis. Only minor losses of nitrate were observed in killed controls. Biphenyl was also removed, but a lag occurred before nitrate was consumed (Figure 3). After the lag, nitrate removal was approximately stoichiometrically equivalent to biphenyl and naphthalene removal (Table 1). These results could be explained by production and accumulation of a metabolic intermediate similar in oxidation state to the parent compound followed by delayed oxidation to CO_2. Dibenzofuran was not degraded relative to killed controls (data not shown), as expected from the FBR results. Nitrate removal with cell mass in the absence of PAH was not statistically significant ($p = 0.01$) during triplicate incubations (up to 66 days, data not shown). Thus, nitrate reduction by the culture was dependent upon the presence of PAH.

PAH Degradation by the Sulfate-Reducing Biomass. To demonstrate dependence of sulfate reduction on PAH biodegradation, preliminary experiments were performed with PAHs in excess of solubility. Sulfide was produced when phenanthrene, naphthalene, or biphenyl were provided to the sulfate-reducing FBR biomass in duplicate batch incubations (data not shown). In contrast, no sulfide was produced (up to 72 days) during duplicate incubations of

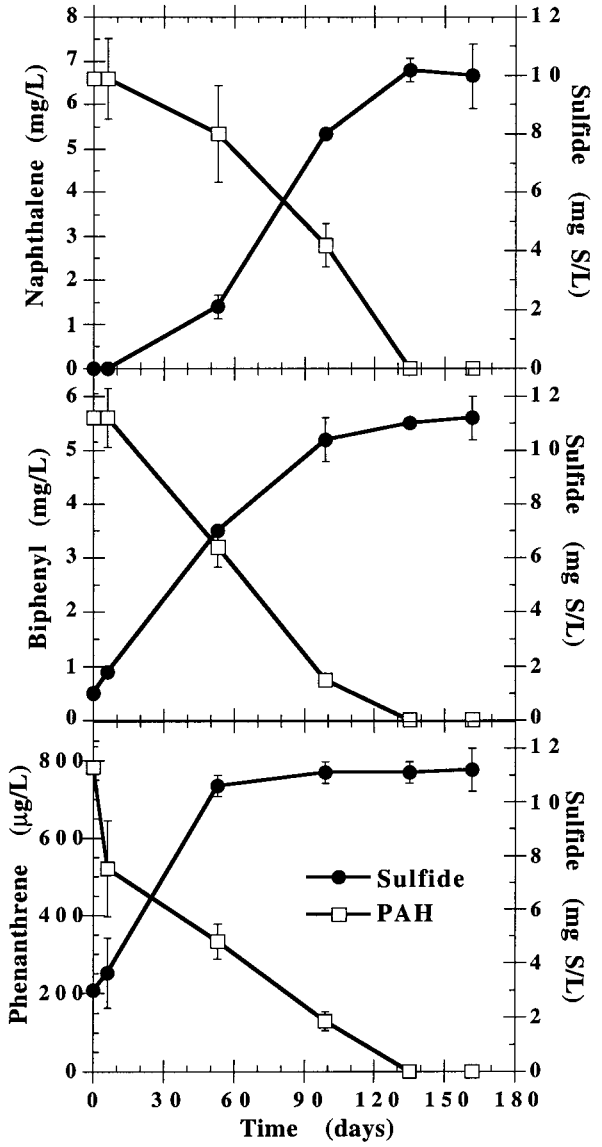

FIGURE 4. Anaerobic biodegradation of PAHs (squares) with concomitant production of sulfide (circles) by a sulfate-reducing enrichment. Incubated in separate bottles with corresponding PAH. Average of duplicates ± SEM.

cell mass in the absence of PAH (data not shown). Sulfide production was greatest in cultures supplied with phenanthrene or biphenyl (11−13 mg of S L^{-1}), whereas the naphthalene-fed biomass exhibited a lag (>35 days) before sulfide production commenced, reaching 4.5 mg of S L^{-1} after 72 days.

After exposure to excess PAH the culture was resuspended in fresh media and fed PAH at concentrations below solubility to determine the stoichiometry of PAH degradation and sulfide production. Both biphenyl and naphthalene were removed over a period of 20 weeks (Figure 4). Biphenyl and naphthalene transformation was concomitant with and nearly stoichiometric with sulfide production (Table 1). Phenanthrene was also removed, but sulfide was produced in amounts more than five times in excess of stoichiometry. It is not clear why excess sulfide was produced in the degradation of phenanthrene, while degradation of the other compounds was nearly stoichiometric with sulfide production. Some of the excess could have been due to the presence of phenanthrene that was sorbed to biomass carried over from the previous experiment and not quantified by the extraction procedure. This explanation is consistent with

TABLE 2. Comparison of Specific PAH Utilization Rates for Various Enrichment and Pure Cultures

	specific PAH removal rate mg (g of VSS day)$^{-1}$		
culture	naphthalene	phenanthrene	biphenyl
nitrate reducers			
this study	2.4 ± 0.5	1.1 ± 0.2	5.3 ± 0.1
sulfate-reducers			
this study	0.43 ± 0.06	0.12 ± 0.03	0.52 ± 0.03
aerobic			
marine[b] (31)	260 ± 19	18 ± 4.0	ND[a]
marine (32)	ND	ND	16[c]
freshwater (31)	91 ± 13	74 ± 21	ND
marine[b] (18)	ND	98 ± 2	ND

[a] ND, not determined. [b] Culture enriched from Eagle Harbor sediment. [c] Assuming cell weight = 10^{-13} g/cell.

the observation that, after day 55 of the batch experiments, sulfide production was stoichiometrically equivalent to the remaining phenanthrene removal (Figure 4). No transformation of dibenzofuran was observed in the batch experiments (data not shown), consistent with the FBR results.

Anaerobic PAH Removal Rates. Specific PAH utilization rates in the nitrate-reducing subculture were 1−5 mg (g of VSS day)$^{-1}$, 1−2 orders of magnitude lower than that of aerobic marine and freshwater enrichments reported in the literature (Table 2). Specific PAH removal rates in the sulfate-reducing enrichment were 0.1−0.5 mg (g of VSS day)$^{-1}$; significantly slower than the nitrate-reducing enrichment. A consortium may be required to degrade PAHs under sulfate-reducing conditions, necessitating intercellular transport of metabolites between bacteria. Nitrate-reducing bacteria may be able to completely degrade PAHs without intercellular transport of metabolites within a consortium, perhaps resulting in more rapid degradation.

The PAH utilization rates in Table 2 allow estimates to be made of the total cell mass that was present in the FBRs. The highest PAH mass removal rate in both FBRs was for naphthalene; 0.6−1 and 0.7−1.2 mg d^{-1} for the nitrate-reducing FBR and sulfate-reducing FBR, respectively (Figures 1 and 2). Based on these removal rates and the PAH utilization rates in the batch experiments (Table 2), the minimum cell mass required in the FBRs to account for the observed PAH removal was 0.3−0.6 g of VSS in the nitrate FBR and 1.6−2.8 g of VSS in the sulfate FBR. These estimates agree with the measured total bioreactor biomass.

Some unknown factors may have limited the growth rate of these enrichments. PAH was the sole carbon source available to the enrichment; no vitamins or other growth cofactors were added to the ASW medium, as had been done in a recent study of anaerobic PAH biodegradation by sulfate reducers (*15*). With further understanding of anaerobic PAH biodegradation metabolism, the PAH utilization rates may be susceptible to improvement by the addition of a limiting component.

The use of Hungate's technique, coupled with a strong reductant and incubation in an anaerobic glovebox precluded the involvement of molecular oxygen in these reactions, a mechanism that has been suggested as an explanation for previous results showing anaerobic degradation of unsubstituted mono- and polyaromatics (*25*, *26*). Both the nitrate to PAH transformation ratio and the dependence of nitrate transformation on the presence of PAH are consistent with the hypothesis that PAH degradation was coupled to denitrification. Similarly, sulfide production to PAH degradation ratios were consistent with the hypothesis of anaerobic PAH biodegradation coupled to sulfate reduction.

The inclusion of cell growth in the theoretical stoichiometric calculation [using the energetics equations of McCarty

(*27*)] would result in a minor decrease (4%) in the predicted sulfide production to PAH utilization ratio (*23*). The decrease in the predicted nitrate to PAH utilization ratio would be more substantial (approximately 28%), suggesting that the assumption of complete mineralization was generally accurate for the sulfate-reducing culture, but may have resulted in an overestimate of nitrate removal by the nitrate-reducing culture.

Although anaerobic specific PAH utilization rates were lower than reported aerobic rates, these rates may be acceptable given the time scale envisioned for an anaerobic treatment approach involving capping or for natural attenuation. For example, maximum naphthalene mineralization rates were reported in the range of 1−4 ppm day^{-1} in aerobic intertidal sediments (*28*). At these degradation rates, the time required for removal of naphthalene in Eagle Harbor sediment (35 ppm, assumed 100% bioavailable) would be on the order of 10−40 days. At degradation rates 1−2 orders of magnitude slower than this, the cleanup time could be increased to 1−10 years, a time frame that may be acceptable for a sediment environment given that the sediment surface is covered with a clean cap.

The results presented in this paper add to the growing body of research demonstrating that aromatic compounds once thought to be recalcitrant to biodegradation under anaerobic conditions are degraded anaerobically. In previous studies, naphthalene, acenaphthene, fluorene, phenanthrene, and fluoranthene were reported to be biotransformed under anaerobic conditions (*9*, *13*, *15*, *29*, *30*). In all of these studies, undefined sediment enrichments were used. No anaerobic PAH biotransformations have been reported for pure or mixed cultures free of sediment or soil. This report is the first to show phenanthrene biodegradation under nitrate-reducing conditions and to show clear evidence of anaerobic biphenyl biodegradation. Furthermore, these are the first enrichment cultures shown to sustain biodegradation of PAHs under nitrate-reducing conditions over long periods of time. Finally, these are the first measurements of specific rates of anaerobic PAH degradation. These rate data will permit comparisons to be made between different degradative processes and for assessments of methods to enhance anaerobic degradation rates.

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