

Succession of Phenotypic, Genotypic, and Metabolic Community Characteristics during In Vitro Bioslurry Treatment of Polycyclic Aromatic Hydrocarbon-Contaminated Sediments

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Dredged harbor sediment contaminated with polycyclic aromatic hydrocarbons (PAHs) was removed from the Milwaukee Confined Disposal Facility and examined for in situ biodegradative capacity. Molecular techniques were used to determine the successional characteristics of the indigenous microbiota during a 4-month bioslurry evaluation. Ester-linked phospholipid fatty acids (PLFA), multiplex PCR of targeted genes, and radiorespirometry techniques were used to define in situ microbial phenotypic, genotypic, and metabolic responses, respectively. Soxhlet extractions revealed a loss in total PAH concentrations of 52%. Individual PAHs showed reductions as great as 75% (i.e., acenaphthene and fluorene). Rates of ¹⁴C-PAH mineralization (percent/day) were greatest for phenanthrene, followed by pyrene and then chrysene. There was no mineralization capacity for benzo[a]pyrene. Ester-linked phospholipid fatty acid analysis revealed a threefold increase in total microbial biomass and a dynamic microbial community composition that showed a strong correlation with observed changes in the PAH chemistry (canonical r^2 of 0.999). Nucleic acid analyses showed copies of genes encoding PAH-degrading enzymes (extradiol dioxygenases, hydroxylases, and meta-cleavage enzymes) to increase by as much as 4 orders of magnitude. Shifts in gene copy numbers showed strong correlations with shifts in specific subsets of the extant microbial community. Specifically, declines in the concentrations of three-ring PAH moieties (i.e., phenanthrene) correlated with PLFA indicative of certain gram-negative bacteria (i.e., *Rhodococcus* spp. and/or actinomycetes) and genes encoding for naphthalene-, biphenyl-, and catechol-2,3-dioxygenase degradative enzymes. The results of this study suggest that the intrinsic biodegradative potential of an environmental site can be derived from the polyphasic characterization of the in situ microbial community.

It is estimated that approximately 10% of all dredged materials (an estimated 14 to 28 million cubic yards annually from U.S. waterways) are impacted with organic and/or inorganic contaminants (22). Polycyclic aromatic hydrocarbons (PAHs) are frequently encountered in the sediments of navigation channels. Due to the fact that a single dredging operation can involve the removal of thousands of cubic yards of sediment, the physical handling of this material must be kept to a minimum for any remediation strategy to be economically feasible. From this perspective, bioremediation is an attractive treatment alternative.

For biotreatment efforts to be effective, however, it is essential that indigenous microorganisms be present that are capable of degrading the PAH mixtures under technically and economically sustainable physicochemical conditions (i.e., within the confined disposal facilities). Current treatment technologies do not allow for the economical reuse of dredged materials as reclaimed soils (15). Bioremediation may fulfill this need, but in order for bioremediation to work efficiently and successfully we need to learn more about and monitor the in

situ interactions that occur between the extant microbiota and the contamination.

Microbiological processes can reduce hydrocarbon concentrations in sediments to levels that no longer pose an unacceptable risk to the environment or to human health (16). The microbial biodegradation of two- and three-ring PAHs has been extensively reviewed (2, 7, 8, 11) and, more recently, a variety of microorganisms have been isolated and shown to metabolize PAHs with up to four rings (18). Although individual species of bacteria and bacterial consortia have been shown to metabolize PAHs in laboratory culture, identifying such a potential in a community of microorganisms in situ is more difficult. Biodegrading organisms may or may not be the predominant species, which directly affects our ability to identify and quantify their presence. In addition, the physicochemical properties of the immediate environment can have a major influence on microbial physiology as well as contaminant bioavailability.

To fully identify the nature of a contaminant's impact on an extant microbiota, a polyphasic approach that combines phenotypic and genotypic measurements is necessary (14). The analysis of ester-linked phospholipid fatty acids (PLFA) provides an estimate of the "viable" microbial biomass (assuming rapid degradation of intact phospholipids upon cell death), as well as a 'fingerprint' of the in situ microbial community struc-

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ture (20). However, shifts in microbial community composition can also be induced by changes in other environmental factors such as temperature, pH, moisture content, nutrient levels, etc. One way to minimize misinterpretation of in situ microbial community shifts (by PLFA) is to tie these shifts to the abundance of genes related to the biodegradation of target contaminants. This can be accomplished by use of a multiplex PCR approach designed to determine the presence and abundance of several different biodegradative genes in a single sample.

In this work we hypothesized that the intrinsic biodegradative potential of dredged sediment could be derived from a polyphasic characterization of the in situ microbial ecology. We used PLFA and DNA analyses to track microbial community biomass and gene presence over time from untreated and biotreated PAH-contaminated sediments. Biotreatment of 16 EPA priority pollutant PAHs was measured via bioslurry and microcosm tests. The reduction in PAH levels was correlated with fluctuations in total microbial community biomass, changes in the potential rates of ^{14}C -PAH mineralization, changes in PLFA-defined microbial taxa, and changes in the genetic catabolic potential of the in situ microbiota.

MATERIALS AND METHODS

Study material. The sediment used in this study was obtained from the Jones Island Confined Disposal Facility (CDF) (commonly known as the Milwaukee CDF) operated by the Milwaukee Harbor Port Authority. The CDF is a 44-acre facility located in the South Milwaukee Harbor. The CDF was constructed in 1975 and has a maximum thickness of 10 meters. It serves as a disposal facility for maintenance-dredged materials unsuitable for open-lake disposal (6). These sediments originated from the Milwaukee Harbor and Port Washington Harbor, located 25 miles north of Milwaukee, Wis. In December 1998 sediment from the facility (56.5% sediment by weight and 43.5% water) was mixed, later wet-sieved using a 2-mm sieve to promote homogeneity, stored in 55-gallon drums for 2 months at 4°C to allow for settling of fines, and then remixed before use in the bioslurry studies. Although disturbance and storage effects likely induced changes in the indigenous microbiota, the focus of the study was on the bioslurry manipulation of a contaminated material and not the characterization of the extant biota within the CDF.

Bioslurry reactors. The study consisted of five bioslurry reactors operated over a 4-month period. Two reactors served as controls (anaerobic and poisoned), and the remaining three served as active (aerobic) reactors. Each bioreactor received 5.3 kg (wet weight) of dredged material and 3.5 liters of modified Stanier's Basal Medium (1) minus any carbon source. Reactors were continuously stirred (by two impellers) at 350 rpm. Reactors contained three diffuser stones that bubbled either argon (control reactors) or air (aerobic reactors) supplied from compressed gas cylinders after passing through double charcoal filters. All reactors were sealed to prevent the ingress of atmospheric air.

The anaerobic reactors were treated with sodium azide and mercuric chloride (each at 500 ppm) to inhibit microbial growth. Additional treatments were applied (500 ppm at day 18, 1,000 ppm at 1 week, and 750 ppm every 3 weeks thereafter) to enhance the effectiveness of each compound. Both control and active reactor sediments were routinely monitored for colony formation on nutrient agar (72 h at 30°C) as a partial measure of the effectiveness of the treatments on inhibiting microbial growth.

Bioreactors were kept at room temperature in the dark (except for sampling events) and monitored daily for pH, dissolved oxygen, oxidation-reduction potential, and temperature and every other day for nitrate and phosphate analysis (every week after 1 month of incubation). Dissolved oxygen was measured by Orion Model 840 oxygen meters, and pH and redox by Beckman F11 pH meters. Carbon/nitrogen/phosphorus ratios were maintained at 100:10:1, requiring the addition of phosphate (1 M Na_2HPO_4 and 1 M KH_2PO_4 ; 200 ml, total volume) 4 weeks into the study and every 2 to 3 weeks thereafter. Carbon content was based on sediment total organic carbon (4,000 to 38,000 mg/kg for the active reactors at study initiation). Dissolved oxygen was maintained in the range of 8 to 9 mg/liter for the aerobic reactors and at 0 mg/l for the control anaerobic reactors. pH was maintained at or near neutral conditions, i.e., pH ~7.0.

Initial PAH concentrations were determined immediately after homogenization (T_0). Sediment PAH concentrations were also determined for each biore-

actor after it was loaded. Once the reactors were operated for 18 h ($T_{0.025}$ month), initial ester-linked polar lipid fatty acids (PLFA), DNA, and radiorespirometry subsamples (22 g [wet weight] total) were collected. Thereafter, the reactors were sampled once a month for a total of 4 months (T_1 to T_4). PAH and radiorespirometry assays were performed on pelletized material ($5,000 \times \text{g}$ for 12 min).

Contaminant chemistry. Initial PAH concentrations (T_0) were determined by analysis of four replicates from the sieved and mixed 55-gallon drum. Three samples from each bioslurry reactor were collected at $T_{0.025}$, T_1 , T_2 , T_3 , and T_4 months. These samples underwent Soxhlet extraction and gas chromatography-mass spectrometry (GC-MS) analyses for determination of PAH concentrations according to Environmental Protection Agency method 3540.

Radiorespirometry. A modified version of the method described by Fulthorpe et al. (9) was used to assess microbial activity potentials in the bioslurry reactors. Two grams (wet weight) of slurry material from each respective reactor was placed into 15-ml Teflon-lined screw-cap test tubes, to which was added 2.7 ml of a modified Stanier's Basal Media and a tracer amount of the ^{14}C -labeled substrate. These secondary microcosms were run in triplicate and spiked with 20,000 dpm of either phenanthrene-9- ^{14}C (>95%) at a specific activity of 46.9 mCi/mmol (Sigma Chemical Co., St. Louis, Mo.), pyrene-4,5,9,10- ^{14}C (>95%) at a specific activity of 58.7 mCi/mmol (Sigma), chrysene-5,6,11,12- ^{14}C (98%) at a specific activity of 47.4 mCi/mmol (ChemSyn Laboratories, Lenexa, Kans.), benzo[a]pyrene-7- ^{14}C (>95%) at a specific activity of 16.2 mCi/mmol (Sigma), or acetic acid-1,2- ^{14}C (>95%) at a specific activity of 116 mCi/mmol (ICN Pharmaceuticals, Inc., Costa Mesa, Calif.). Glass fiber filters (Whatman, Maidstone, United Kingdom), 10 mm in diameter, saturated in 1 M barium hydroxide were used to trap evolved $^{14}\text{CO}_2$. Test tubes and bioslurry reactors were incubated at room temperature (20°C). The test tubes were placed on tube rollers inclined at 45° and rotated at 10 rpm. Microcosms derived from the aerobic reactors were sparged daily with filtered compressed breathing air. The BaOH-saturated filters were collected daily over a 15-day period (5 days for the acetate treatment) and placed into 1.5 ml of scintillation cocktail (Ultima Gold; Packard Instruments Co., Downers Grove, Ill.) before counting them on a top count microplate scintillation counter (Packard Instruments). Filters were counted twice, and counts were corrected for background and counting efficiency using the external standard method described by the manufacturer.

Ester-linked PLFA analyses. PLFA analysis has been detailed elsewhere (19). Briefly, a 2-g (wet weight) aliquot from 5-g total (per reactor) of slurry material was extracted for 3 h at room temperature in 6 ml of dichloromethane-methanol-water (1:2:0.8 [vol/vol/vol]). Amino-propyl solid-phase extraction columns (Supelco, Bellefonte, Pa.) were used to separate the total lipid into neutral lipid, glycolipid, and phospholipid fractions (17). Phospholipid fatty acid methyl esters (from the polar lipid fraction) were prepared for GC-MS by mild alkaline methanolic transesterification. The resulting phospholipid fatty acid methyl esters were dissolved in hexane containing methyl-nonadecanoate (50 pmol μl^{-1}) as an internal standard and analyzed using a gas chromatograph equipped with a DB-1 capillary column (50 m by 0.25 mm [inner diameter]; 0.1- μm film thickness; J&W Scientific, Folsom, Calif.) and a flame ionization detector. Peak identities were confirmed using a gas chromatograph-mass selective detector (Hewlett-Packard GC6890-5973 MSD) with electron impact ionization at 70 eV. Areas under the peaks were converted to concentrations, summed, and then normalized to the gram weight extracted for biomass determinations. For community comparisons, the percent contribution of each peak was calculated and then normalized using an arcsine square-root transformation.

DNA isolation from bioslurry reactors. Total DNA was isolated from triplicate 500-mg slurry samples using a Mini Bead Beater system essentially as described in Borneman et al. (5), using a Fast DNA SPIN Kit (Bio 101, Vista, Calif.). Typically, 1 to 10 μg of DNA was recovered, although DNA was not quantitated due to the small sample size and the coextraction of contaminants. Therefore, an undiluted sample was taken to represent 50 mg of bioslurry per μl of extract. Total DNA was suspended in 50 μl of Molecular Biology Grade H_2O (Five Prime-Three Prime, Boulder, Colo.) and stored at -20°C until further analysis. The emphasis of the study was on determining the number of copies of genes per milligram of slurry material and not per microgram of DNA recovered; therefore, the total DNA quantity was not determined. The reproducibility of the extraction efficiency was determined through the assay of triplicate independent extractions.

Primers and targets used in multiplex PCR. Details on the multiplex PCR assay and primers used will be published elsewhere (E. J. Perkins, unpublished data). Briefly, the primers target the following genes (written 5' to 3'): (i) toluene dioxygenase (*todC1*) from *Pseudomonas putida* F1 (23) (forward primer todC1F, GCGAGATAGAAGCGCTCTTG; reverse primer todC1R, GTATTGATACC TGGGAGGAAG; with an expected product size of 924 bp); (ii) toluene-4-

TABLE 1. PAH concentrations in the initial Milwaukee CDF sediments and in the control and active bioslurry reactors throughout a 4-month incubation period

PAH	Initial PAH concn ($\mu\text{g/g}$) ^a	PAH concn ($\mu\text{g/g}$) ^b in:											
		Control reactors ($n = 6$) ^c at time (mo):						Active reactors ($n = 9$) ^d at time (mo):					
		0.025	1	2	3	4	% Loss	0.025	1	2	3	4	% Loss
MW 128–143	3	2	2	2	2	2	55	2*	1	2*	2	1	55
MW 154–178	23	19	17	22	19	18	19	13*	6*	9*	7*	7*	67
MW 202–228	56	47	41	53	55	52	8	38*	19*	28*	23*	27*	52
MW 252	21	13	11	15	16	15	27	11*	8*	11*	10*	11*	48
MW 276–278	12	6	5	8	8	8	28	5*	6	9	8	9	26
Total	115	87	77	100	99	95	17	68*	40*	58*	50*	56*	51

^a That is, the initial PAH concentration in the Milwaukee CDF sediment after homogenization (T_i).

^b Control reactors were treated with sodium azide and mercuric chloride. Active reactors were supplemented with N and P (1 M) and sparged with breathing air. % Loss, percent loss in PAH relative to the initial (T_i) concentration. *, Significantly less than the corresponding control value ($\alpha = 0.05$).

^c Two reactors, three subsamples per reactor per time point.

^d Three reactors, three subsamples per reactor per time point.

monoxygenase (*tmoA*) from *Pseudomonas mendocina* KR1 (23) (forward primer *tmoAF*, GCTATGTTACCGAAGAGCAGC; reverse primer *tmoAR*, GGAATAGATCCCAGTACCAGG; with an expected product size of 900 bp); (iii) alkane hydroxylase (*alkB*) from *Pseudomonas oleovorans* TF4-1L (12) (forward primer *alkB-f*, TGGCCGGCTACTCCGATGATCGGAATCTGG; reverse primer *alkB-r*, CGCGTGGTGATCCGAGTGCCGCTGAAGGTG; with an expected product size of 869 bp); (iv) biphenyl dioxygenases designed from a multiple protein alignment of several extradiol dioxygenase enzyme large subunits (Perkins, unpublished) (forward primer *bph-f*, TGCAGTACCACGGCTGGCCCTA; reverse primer *bph-r2*, GCNGCRAAYTTCCARTTRCANGG; with an expected product size of 295 bp); (v) catechol 2,3-oxygenase (*xylE*) from *P. putida* mt-2 pWW0 (Perkins, unpublished) (forward primer *c2303f*, CAAGGCCACGACGTGGCNTT; reverse primer *c2303r*, CGGTTACCGGACGGGTGGAAGAAGT; with an expected product size of 202 bp); and (vi) naphthalene dioxygenase and 2-nitrotoluene dioxygenase (*ntdAc*) from *Pseudomonas* sp. strain JS42 (Perkins, unpublished) (forward primer *2NT-F*, TTTGTGTGCGGTYTACCACGGNTGGGG; reverse primer, *2NT-R*, TCTCACCTACAAAAGTTTCCGCAAAARSCTCCAGTT; with an expected product size of 321 bp).

Each subsample was analyzed by multiplex PCR in four dilutions (1/1, 1/10, 1/100, and 1/1,000). Quite often, PCR inhibitory compounds were present at too high a level to permit DNA detection in undiluted samples. Tenfold dilution was generally sufficient to permit successful PCR analysis. A 5- μl portion of each dilution was then added to each PCR reaction, so that the initial sample represents DNA from 5 mg of sediment. PCR reactions were composed of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 5% dimethyl sulfoxide, 250 μg of bovine serum albumin per ml, 200 μM concentrations of each deoxynucleoside triphosphate, 8 pmol of each primer, 0.8 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif.), and 5 μl of bioslurry DNA extract for a final reaction volume of 20 μl . PCR reactions were amplified in 200- μl thin-walled tubes using a PTC-200 thermal cycler (MJ Research, Watertown, Mass.). The thermal profile for amplification was 15 min of denaturation at 95°C followed by 35 cycles of 20 s of denaturation at 94°C, 1 min of annealing at 60°C, and 4 min of extension at 72°C. PCRs were finished by 10 min of extension at 72°C.

Next, 20- μl PCR reaction mixtures were precipitated with 5 μl of 10 M ammonium acetate and 75 μl of ethanol. DNA was recovered by centrifugation at 14,000 $\times g$ in a microcentrifuge. DNA pellets were washed with 70% ethanol to remove residual salts. Pellets were then resuspended into 2 μl of SYBR Green I loading dye containing 17 mg of Blue Dextran (Sigma) per ml, 8 mM EDTA, and a 1/1,000 dilution of SYBR Green I (Molecular Probes, Beaverton, Oreg.). Molecular weights of the PCR products were estimated by comparison to the Genescan 2500 ROX size standard (Applied Biosystems), which was stained with SYBR Green I loading dye. DNA was analyzed on a 5% Long Ranger HydroLink nondenaturing gel (FMC BioProducts, Rockland, Maine) using an ABI 377 automated DNA sequencer (Applied Biosystems). The gels (12 cm long and 0.4 mm thick) and running conditions were as described in the standard ABI protocols (Applied Biosystems).

Determination of minimum detectable gene copy number. A band was scored as either present or absent at each size (base pair length) and dilution (1/1 to 1/10,000). For each band, the point of dilution to extinction was then determined, and an estimation of the total number of gene copies present was calculated. If

we assume that the slurry samples have similar effects upon gene detection limits, the minimum detectable gene copy number would thus be equal to the maximum dilution factor at which the gene was detected in two or more replicates per microliter of sample DNA extract multiplied by the total volume (in microliters) of slurry DNA extract per gram of sample slurry.

Statistical analyses. Differences between treatments were assessed with a least-squared significant difference test. Correlations between contaminant and PLFA percentages and gene copy numbers were evaluated using Spearman rank order and canonical correlation analyses. Relationships among variables within a data set were examined by hierarchical cluster analysis (Wards method). All examinations were performed with the Statistica software package, version 5.0 (Statsoft, Inc., Tulsa, Okla.).

RESULTS

Extractable PAH. The active reactors showed a net loss in total extractable PAH of approximately 52% (Table 1). Total PAH concentrations decreased from the T_i average of $115 \pm 5.7 \text{ mg kg}^{-1}$ to $56 \pm 3.8 \text{ mg kg}^{-1}$ at $T = 4$ months. The decline in total PAH was statistically significant at an alpha value of 0.05. The greatest loss, at 67%, occurred in the molecular weight (MW) 154 to 178 compounds (acenaphthene, fluorene, phenanthrene, and anthracene), followed by a 52% loss in the MW 202 to 228 compounds (fluoranthene, pyrene, chrysene, and benzo [*a*]anthracene), a 48% loss in the MW 252 compounds (benzo [*b*]fluoranthene, benzo [*k*]fluoranthene, and benzo [*a*]pyrene), and a 26% loss in the MW 276 to 278 compounds (benzo [*g,h,i*]perylene, indeno [*1,2,3-c,d*]pyrene, and dibenzo [*a,h*]anthracene).

Total extractable PAH concentrations decreased 17% in the control reactors from an average T_i concentration of $115 \pm 5.7 \text{ mg kg}^{-1}$ to $95 \pm 3.8 \text{ mg kg}^{-1}$ at T_4 . The greatest reductions occurred in PAHs of MW 128 to 143 (55%), MW 252 (27%), and MW 276 to 278 (28%). The loss observed in the MW 128 to 143 moieties was equal to that observed in the active reactors. Reductions also occurred in PAHs of MW 154 to 178 and MW 202 to 228, but to a lesser degree. The difficulties encountered in killing the microorganisms in the control reactors and the presence of a viable microbial population, may have accounted for the 17% reduction in total PAHs observed over the 4-month period.

Microbial biomass and community composition (PLFA). The microbial biomass in the control reactors was always less than that in the active reactors. Active reactor biomass was

significantly greater (least significant difference [LSD] test at $\alpha = 0.10$) than that measured in the control reactors at 1 month ($20,883 \pm 5,810$ versus $3,832 \pm 1,367$), 2 months ($46,323 \pm 9,488$ versus $8,694 \pm 1,129$), and 3 months ($13,773 \pm 2,879$ versus $4,798 \pm 1,018$) of the incubation period. Microbial biomass in the active reactors also indicated a dynamic community, showing changes as a result of the nutrient addition. Although poisoning was initially effective in suppressing the microbiota in the control reactors, by the end of the study the viable biomass in these reactors reached a level comparable to that of the active reactors. However, none of this biomass was culturable on nutrient agar.

Although biomass in the control reactors increased through 4 months of the study, hierarchical cluster analysis showed the microbial community composition of the control reactors to be very different from that of the active reactors at each time sampled, including T_4 (Fig. 1a). This result is intuitive based on the differences in treatment regimes; however, the result confirms that the control reactors (with the lower biomass and higher PAH concentrations) contained a significantly different microbiota than that seen in the active reactors. Within the active reactor cluster, sample subclusters could be defined (k-means) for each time point sampled (T_1 month to T_4 months), indicating a continually shifting microbiota within this treatment. $T_{0.025}$ month active reactors clustered with $T_{0.025}$ month control reactors. The time points T_2 months, T_3 months, and T_4 months from the control reactors were distinguishable from the earlier time points, reflecting the steady increase in viable biomass within this treatment.

The data matrix used to generate the dendrogram provided in Fig. 1a was transposed and used to indicate which PLFA covaried across the active reactor samples. A k-means clustering algorithm was used to define three discrete microbial communities (i.e., four PLFA cluster groups) (Fig. 1b). Community 1 was comprised of 18:0, i15:0, a15:0, and cy 17:0, which are indicative of both gram-positive and gram-negative bacteria. Community 2 included the PLFA 16:0, 16:1w7c, and 18:1w7c, which are indicative of gram-negative bacteria. Community 3 included 14:0, 15:0, 17:0, i16:0, i17:0, a17:0, 16:1w5c, 16:1w9c, 17:1w8c, cy19:0, i15:1w7c, i18:1, br17:1, br19:1, n16:1w7t, 10me16:0, 10me18:0, 10me18:0, and 12me18:0, PLFA found in gram-positive and gram-negative bacteria, as well as in actinomycetes and some fungi. The final cluster group comprised a number of trace compounds, the sum total of which never exceeded 6%. Biomass contributions from the three defined communities are illustrated in Figure 2. Community 1 (a mixed community of gram-positive and gram-negative bacteria) remained relatively unchanged throughout the study period, community 2 (a predominantly gram-negative community) showed a slight decline in relative abundance, whereas community 3 (another mixed community also showing the presence of actinomycete PLFA biomarkers) showed a general increase.

Although polyunsaturated PLFA were detected, their contribution to the overall community dynamics in the active reactors was minimal, never exceeding 4% of the total PLFA detected. Linoleic acid (18:2w6) was the predominant dienoic acid detected ($3.6\% \pm 0.6\%$ at $T_{0.025}$), and eicosapentanoic acid (20:5w3) was the predominant polyunsaturated fatty acid detected ($1.1 \pm 1.1\%$ at $T_{0.025}$). Both of these acids are syn-

thesized by a number of eukaryotes and declined in relative percentage over the time course of the study.

Mineralization potentials (radiorespirometry). Potential rates of ^{14}C -PAH mineralization in the active slurries generally increased in the order of benzo[a]pyrene < chrysene < pyrene < phenanthrene (Table 2). For acetate and phenanthrene, the potential activities or mineralization extents in the active slurries were significantly greater than those measured in the control slurries at all time points sampled, other than the initial sampling ($T_{0.025}$). Mineralization extents for each of the PAHs examined were greatest at times T_1 months and T_2 months (Table 2), corresponding to the greatest biomass levels and the initial introduction of nutrients. Benzo[a]pyrene mineralization extents never exceeded the impurity level of the radiolabel (5%).

Genetic catabolic potential. Genes encoding enzymes associated with aromatic degradation—naphthalene dioxygenase, biphenyl dioxygenase, catechol 2,3-dioxygenase, toluene monooxygenase, and alkane hydroxylase—were present in the original soil in copy numbers below the detection limit of the assay ($<10^6$ copies per g of soil). Copy numbers for each of the genes assayed increased to the maximum detectable number (10,000 copies over the detection limit) within the first and second months of incubation. To identify trends in the data, the log was taken of the total number of gene copies detected in each of the three active slurry reactors and plotted against the time sampled (Fig. 3). Data below the detection limit were plotted at 0.01. The copy number of three of the catabolic genes assayed—naphthalene dioxygenase, biphenyl dioxygenase, and catechol 2,3-dioxygenase—increased to and remained at elevated levels from T_2 on through the duration of the study. The increase in copies of these genes corresponds with the increase in microbial biomass and phenanthrene, fluorene, and chrysene mineralization rates. In contrast, toluene monooxygenase and alkane hydroxylase decreased in gene copy numbers to below detectable limits after 3 and 4 months of incubation, respectively. Since the reactors were operated in batch mode, the reduction in gene copy numbers could not have been a result of washout but rather was a result of biological mechanisms.

Gene copies for toluene monooxygenase and alkane hydroxylase were never detected in the control reactor sediments. Only 10 copies of the naphthalene dioxygenase and catechol 2,3-dioxygenase genes were detected at time T_4 . Copies of the biphenyl dioxygenase gene were more prevalent in the control sediments, with 10,000 copies detected at T_1 , 100 copies detected at T_2 , and 10 copies detected at T_4 . Although more prevalent, distribution of the diphenyl dioxygenase gene copies was dissimilar to that observed in the active reactor sediments (Fig. 3).

Correlations among microbial parameters. Spearman's rank order coefficients were used to correlate PLFA k-means defined microbial communities (type 1 to type 3 as a relative percentage) to the log of the total number of gene copies detected in the three active reactors at each sampling period (Table 3). Toluene monooxygenase was positively correlated to community 1 (a mixed community of gram-positive and gram-negative bacteria). Naphthalene dioxygenase, biphenyl dioxygenase, catechol 2,3-dioxygenase, and alkane hydroxylase were positively correlated with community 3 (a mixed community

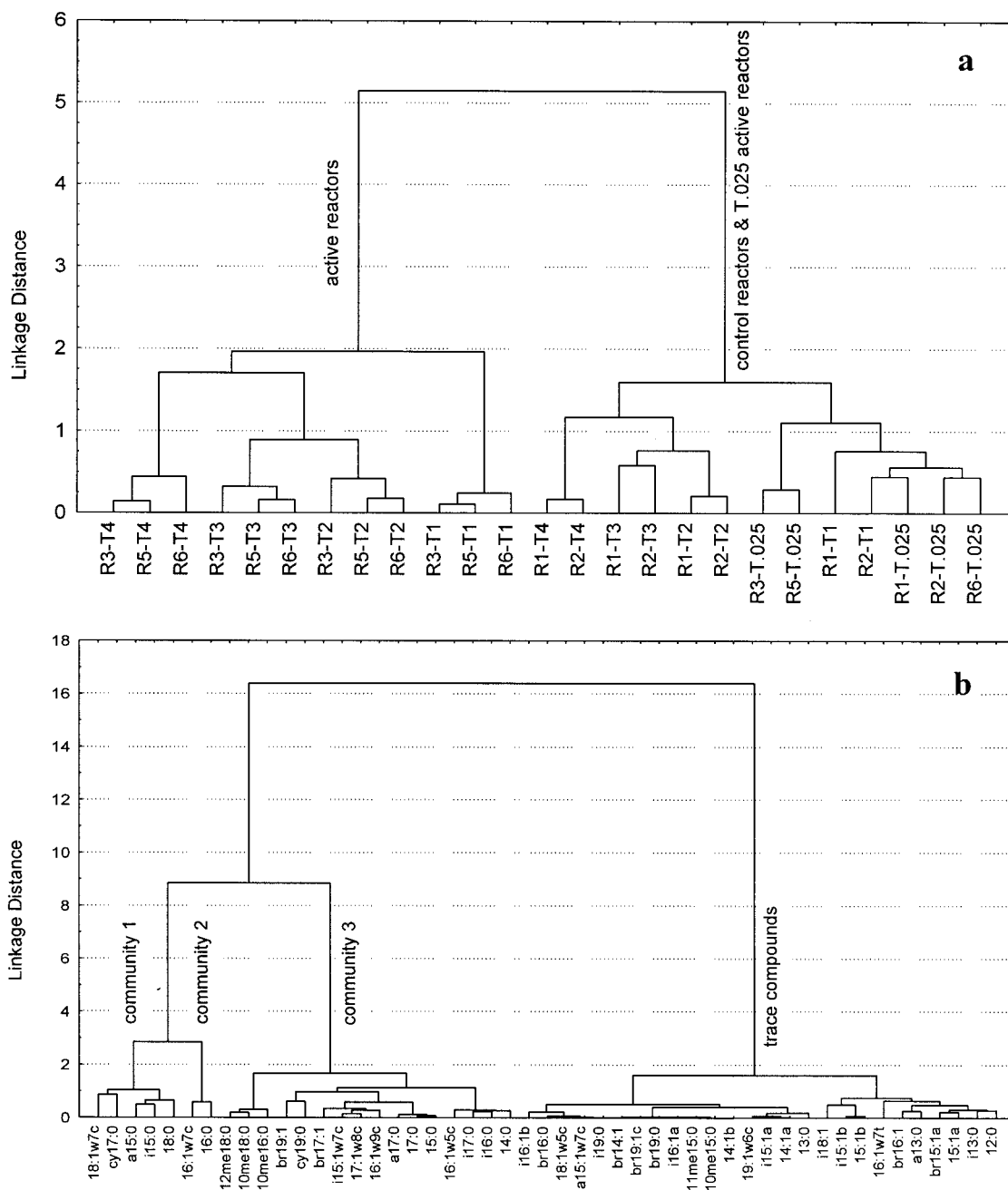


FIG. 1. Dendrogram illustrations of the results of hierarchical cluster analyses of arcsine square-root transformed PLFA mole percentages identified in control and active bioslurry reactor sediments. (a) Results when the algorithm (Ward's method) was run against variables (samples). (b) Results when the algorithm was run against cases (PLFA from the active reactors only). Active reactor samples in panel A are indicated by the prefixes R3, R5, and R6, and control reactors are indicated by the prefixes R1 and R2. Samples taken at 1-month intervals are indicated by the suffix T1 to T4. The T.025 suffix indicates an initial analysis. The robustness of the identified clusters in both panels was verified by k-means analysis. A linkage distance of 2.0 or less defines community types 1 to 3.

including actinomycete PLFA biomarkers). Community 2 (which comprised only of 16:0 and 16:1w7c) did not positively correlate with any of the catabolic genes measured, whereas the trace components showed a positive correlation with catechol 2,3-dioxygenase gene copies.

Correlations between microbial parameters and PAH profiles. Correlation analyses were used to determine significant

relationships between microbial biochemical characteristics and PAH chemical characteristics. PLFA and PAH concentrations were expressed as relative percentages (of the respective total identified) and compared by canonical (r^2) and Spearman rank order correlation (R) analyses. A high (r^2 of 0.999) canonical correlation coefficient indicated that individual PAH and PLFA concentrations covaried throughout the bioslurry

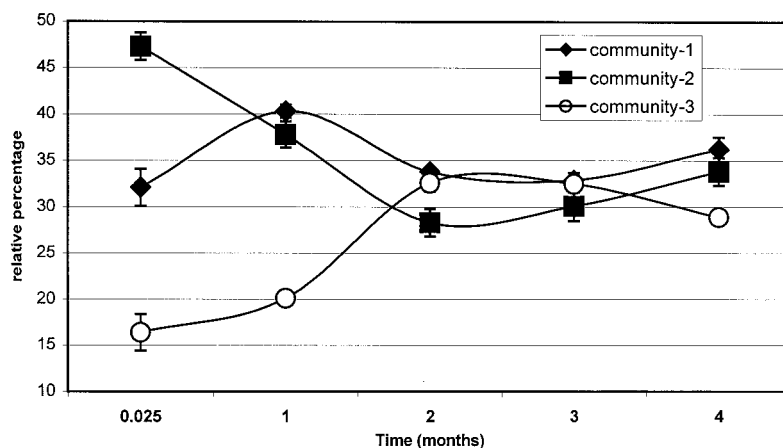


FIG. 2. Average distribution of the community types defined in Fig. 3 within the active bioslurry reactors throughout the 4-month incubation period. The relative percentage of each community type is derived from the sum total of the individual PLFA that make up the community.

time course. Spearman rank order analysis indicated a number of significant relationships between individual PAH and PLFA percentages (Table 4). Only those PLFA and PAH that showed a significant correlation ($P < 0.05$) are listed, and only negative correlations (i.e., as PLFA percentage increases the PAH percentage decreases) are highlighted. The PLFA showing a significant negative correlation with the three ring PAHs (MW 152 to 178) include 17:0, 18:0, i17:0, 16:1w9c, 16:1w5c, br17:1, 10me16:0, 10me18:0, and 12me18:0, the same fatty acids (except for 18:0) that defined community 3 illustrated in Fig. 1b. Those PLFA used to define community 2 (16:0 and 16:1w7c) showed significant negative correlations with only the five- and six-ring (MW 252 to 278) PAH moieties. Community 1 PLFA (cy17:0, a15:0, and i15:0) showed significant negative correlations with both three- and four-ring PAH moieties.

Three-ring PAH moieties declined in relative abundance as the PLFA describing community type 3 increased, whereas PLFA describing community type 2 decreased in relative abundance as the PAH relative abundance increased. These results imply that the three- and four-ring PAH moieties may serve as a carbon and energy source for one subset of the microbial community (i.e., one containing PLFA indicative of gram-positive and gram-negative bacteria, as well as actinomycetes and

some fungi, which produce dioxygenase catabolic enzymes). In contrast, the five- and six-ring PAH moieties in the MW range of 252 to 278, may be toxic to another subset of the microbial community (i.e., one containing PLFA indicative of gram-negative bacteria, which showed no correlation to any of the catabolic genes assayed for).

DISCUSSION

A net reduction in extractable levels of PAH, primarily in the MW class from 128 to 278, was realized after 4 months of incubation. Although the active reactors were supplemented with N and P, no carbon or energy source was added. The resident microbiota were able to use the carbon and energy sources liberated from the slurried sediment to increase their biomass fivefold during the first 2 months of incubation. The subsequent decrease in biomass after T_2 months likely resulted from a natural progression of biological mechanisms (i.e., parasitism and predation). The active reactors also showed a dynamic microbial taxon, one clearly evolving over the duration of the study. Thus, the observed fluctuations in viable biomass and community composition appear to have been driven by

TABLE 2. Average mineralization extents for ^{14}C -labeled acetate, phenanthrene, pyrene, chrysene, and benzo[a]pyrene in secondary microcosm examinations of Milwaukee CDF sediment under bioslurry treatment

Labeled substrate	% Cumulative $^{14}\text{CO}_2^a$									
	Control reactors ($n = 6$) ^b at time (mo):					Active reactors ($n = 9$) ^c at time (mo):				
	0.025	1	2	3	4	0.025	1	2	3	4
Acetate	23.3	43.7	4.4	1.1	1.6	28.5	33.3*	28.0*	30.0*	22.7*
Phenanthrene	7.2	0.2	0.7	0.4	0.6	6.3	22.9*	19.1*	18.1*	8.3*
Pyrene ^d						0.3	17.5	8.3	7.0	5.6
Chrysene ^d						0.8	13.3	9.6	6.5	5.2
Benzo[a]pyrene ^d						0.5	0.6	1.0	0.5	0.2

^a Control reactors were treated with sodium azide and mercuric chloride. Active reactors were supplemented with N and P (1 M) and sparged with breathing air. Values represent cumulative $^{14}\text{CO}_2$ represented as a percentage of the total ^{14}C -labeled substrate added. *, Significantly less than the corresponding control value ($P = 0.05$).

^b Two reactors, three subsamples per reactor per time point.

^c Three reactors, three subsamples per reactor per time point.

^d Secondary microcosms using sediment from the control bioslurry reactors were not established for these labeled substrates.

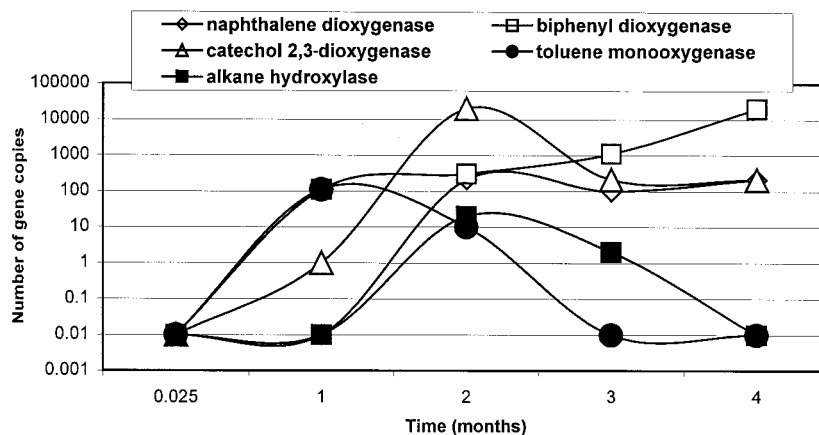


FIG. 3. The total number of gene copies (plotted logarithmically over time) of specific enzymes involved in PAH biodegradation as identified by multiplex PCR analysis of active bioslurry reactor sediments. Two categories of enzymes are represented: (i) class I carboxylated proteins (toluene monooxygenase and alkane hydroxylase) and (ii) intra- and extradiol aromatic cleavage dioxygenases (naphthalene dioxygenase, biphenyl dioxygenase, and catechol 2,3-dioxygenase). The time course spanned a 4-month period with each point (T_1 to T_4) indicating 1 month.

carbon and energy sources liberated from the slurried sediment.

By synthesizing results obtained by independent analytical methods, we were able to show significant relationships among changes in contaminant chemistry, microbial community structure, and microbial function. Throughout the study, rates of phenanthrene mineralization exceeded mineralization rates for pyrene and chrysene (both four-ring PAH moieties). We also observed a substantial number of significant negative correlations between the three-ring PAH moieties (MWs 154 to 178) and specific PLFA (Table 4). PLFA that correlated with the loss in the three-ring PAHs also covaried and defined a unique community type, identified as community 3 (see Fig. 1b). This community increased in relative abundance throughout the 4-month period (Fig. 2). The increase in relative abundance of this community correlated significantly with an increase in gene copy numbers for naphthalene dioxygenase, biphenyl dioxygenase, and catechol 2,3-dioxygenase (Table 3). Thus, the biodegradation of three-ring PAH moieties (the potential for which was confirmed by radiorespirometry) was identified in terms of an affect on in situ microbial community structure, which in turn was directly related to the in situ occurrence of genes coding for catabolic enzymes.

The relationships between PAH and PLFA can be further interpreted. For example, i17:0, a PLFA that correlated signif-

icantly with the loss of fluorene, phenanthrene, and anthracene, is common to species of *Arthrobacter*, *Streptomyces*, and *Rhodococcus*. Species of *Rhodococcus* are also known to synthesize 10me16:0 and 10me18:0, PLFA biomarkers that are most often associated with the actinomycetes (13). Species of *Alcaligenes* and *Pseudomonas* are known to be capable of mineralizing fluoranthene, and both genera show an abundance of cyclopropyl fatty acids in the phospholipid bilayer (18, 20). However, since most PLFA are distributed across multiple genera, overinterpretations can easily lead to ambiguous results. Therefore, based on the PLFA analysis alone, it can only be inferred that a taxon of bacteria known to synthesize a particular PLFA is present in the sample. But when taken in context with the detection of catabolic enzymes and the correlations that exist between PLFA biomarkers and individual PAH moieties, it can be inferred from Table 1 that the three-ring PAHs were being degraded by *Rhodococcus* species and the four-ring PAHs were being degraded by *Alcaligenes* and/or *Pseudomonas* species.

Although it is unlikely that the PAHs served as the sole source of carbon and energy for the extant biota in the slurry reactors, the above discussion indicates that the availability of these compounds had a marked affect on the microbiology of the system. Values related to PAH mineralization, PAH loss, and microbial growth furthers the argument. For example,

TABLE 3. Significant ($P < 0.05$) correlations resulting from the comparison of PLFA group (k-means defined) mole percentages and numbers of gene copies in the active reactors ($n = 3$) across all time points

Group ^a	Spearman rank order score (R) with:				
	Naphthalene dioxygenase	Biphenyl dioxygenase	Catechol 2,3-dioxygenase	Toluene monooxygenase	Alkane hydroxylase
Community 1				0.65	
Community 2	-0.54	-0.65	-0.94		-0.64
Community 3	0.68	0.67	0.90		0.75
Trace compounds			0.63		

^a PLFA in each community are as follows: community 1, 18:1w7c, cy17:0, a15:0, i15:0, and 18:0; community 2, 16:1w7c and 16:0; community 3, 12me 18:0, 10me 18:0, 10me 16:0, br 19:1, cy19:0, br17:1, i15:1w7c, 17:1w8c, 16:1w9c, a17:0, 17:0, i16:0, and 14:0. Trace compounds, PLFA comprising <6% of the total.

TABLE 4. Significant ($P < 0.05$) correlations resulting from the comparison of PLFA and PAH relative percentages taken from active reactors ($n = 3$) over a 4-month incubation period

PLFA	Spearman rank order score (R) with PAH ^a (no. of rings, MW):														
	Naph (2, 128)	AceNaph (3, 152)	Fluor (3, 166)	Phen (3, 178)	Anth (3, 178)	Pyr (4, 202)	Fluoranth (4, 202)	Chry (4, 228)	B(a)A (4, 228)	B(b)F (5, 252)	B(k)F (5, 252)	B(a)P (5, 252)	Indeno (6, 276)	B(g,h,i)P (6, 276)	Dibenzo (5, 278)
n14:0	-0.65														-0.64
n15:0															
n16:0										-0.59					-0.74
n17:0		-0.66	-0.60	-0.55	-0.53										
n18:0		-0.58		-0.51						0.59					
i15:0					-0.74							0.60			
a15:0							0.60	0.62							
i16:0															
i17:0		-0.64	-0.66	-0.61	-0.64						0.62				
a17:0	-0.59						0.63		0.64						
n16:1w9c		-0.58	-0.59	-0.61	-0.66	-0.76				0.71			0.58	0.63	0.58
n16:1w7c		0.90	0.87	0.79	0.82	0.69		0.60				-0.69	-0.71	-0.55	
n16:1w7t		0.63	0.52					0.83					-0.53		
n16:1w5c		-0.65	-0.64	-0.66	-0.65	-0.66							0.68	0.63	
n17:1w8c	0.67														0.55
cy17:0	0.78						-0.68		-0.51						0.62
n18:1w7c		0.56	0.54		0.51				-0.52						
cy19:0	0.66						-0.55			0.68					0.77
i15:1w7c	0.56							-0.54		0.49					0.61
br17:1		-0.82	-0.84	-0.86	-0.84	-0.86				0.73		0.69	0.77	0.75	0.57
i18:1										0.64	-0.58				0.70
br19:1	0.56									0.68					0.75
n10me16:0		-0.87	-0.83	-0.80	-0.76	-0.64						0.55	0.78	0.67	
n10me18:0		-0.60				-0.54		-0.60					0.53		
n12me18:0		-0.70	-0.61	-0.64	-0.70	-0.68	-0.74					0.66	0.60		

^a PAH abbreviations: Naph, naphthalene; AceNaph, acenaphthylene; Fluor, fluorene; Phen, phenanthrene; Anth, anthracene; Pyr, pyrene; Fluoranth, fluoranthrene; Chry, chrysene; B(a)A, benzo[*a*]anthracene; B(k)F, benzo[*k*]fluoranthrene; B(a)P, benzo[*a*]pyrene; Indeno, indeno[1,2,3-*c,d*]pyrene; B(g,h,i)P, benzo[*g,h,i*]perylene; Dibenzo, dibenzo[*a,h*]anthracene.

phenanthrene loss at between $T_{0.025}$ and T_1 was approximately 4 mg/kg. This loss occurred over a 30-day period. At $T_{0.025}$, phenanthrene mineralization was occurring at a rate of 2% day⁻¹, whereas at T_1 the mineralization rate had increased to 5% day⁻¹. Taking an average mineralization rate of 3.5% day⁻¹ and multiplying this value by the total amount of PAH lost over the 30-day incubation period yields a value of 4.2 mg of phenanthrene per kg. Thus, an estimated amount of phenanthrene that the extant microbiota was capable of mineralizing over the 30-day period corresponds with the realized amount of phenanthrene lost in the same time period.

The microbial biomass also increased between $T_{0.025}$ and T_1 . An increase of 2,782 pmol of PLFA g⁻¹ corresponds to approximately 5.6×10^6 cells/g (assuming 1 pmol of PLFA is equivalent to 2×10^4 bacterial cells) (3). Boonchan et al. (4) showed a bacterial consortia isolated from a creosote-contaminated soil to increase (using a most-probable-number enumeration) by 10^4 cells over a 20-day period in which pyrene, at 0.25 mg/ml, was supplied as the sole source of carbon in a basal salts medium. Although the magnitude of the cell number increase was 2 orders of magnitude greater in our bioslurry reactors, it must be noted that the organisms in the bioslurries were exposed to PAH mixtures and not to a single moiety. In their study, Boonchan et al. (4) found only a 10^2 increase in bacterial biomass as a result of benzo[*a*]pyrene exposure, which is 2 orders of magnitude less than that observed with pyrene. It is very likely that carbon sources other than the PAH contamination contributed to microbial growth and metabolism in our active bioslurry reactors. However, the measured affect on

microbial community composition and activity suggest a strong biodegradation component within the system.

The work previously described by Langworthy et al. (14) involved a riverine system, a natural environment quite distinct from the forced environment examined in this study. This difference provides one explanation for the contrast in microeukaryotic input observed between the two studies. The abrasive action of the slurry treatment likely limited the growth of many higher organisms, such as fungi, algae, and protozoa, which were identified as significant members of the microbiota in the riverine system. Also, in contrast to the survey of catabolic genes presented in this study, Langworthy et al. looked for gene abundance via direct probing of environmental DNA. Nevertheless, both systems showed the presence of *nahA* (naphthalene dioxygenase), *nahH* (catechol 2,3-dioxygenase), and *alkB* (alkane hydroxylase). Wikstrom et al. (21) also demonstrated the occurrence of catechol 2,3-dioxygenase in various soil types and that a relationship exists between gene abundance and PAH concentration. The results from all three of these studies further the argument presented by Ghiorse et al. (10) that PAH contamination can, in fact, be a basis for a unique food web. Bacteria enriched by the presence of the contamination can, in turn, enrich protozoa and other higher organisms.

The biodegradation potential of the Milwaukee CDF sediment was determined by correlating the microbial community structure (through PLFA analyses) to gene presence (using DNA analyses) to PAH loss (by chemical analyses). This is the first time the application of in situ biomarkers has been used to

define the capability of PAH degraders in real sediment systems. This approach provides direct nonbiased measurements to define in situ biodegradation potential, which can be related to kinetic rates. Currently, laboratory studies are routinely performed to provide this information. The combination of microbial techniques reported here could minimize the future need for extensive laboratory treatability studies, resulting in more timely and more cost-effective treatment assessment. However, additional work is needed on other sediments and soils before the phenotype and genetic potentials of the extant microbiota can be used as biomarkers to assess the absolute intrinsic biodegradative potential. A more comprehensive assay of biodegradative genes is needed so as not to bias results toward any particular group of microorganisms. Continued research is also needed to expand beyond the assessment of an activity potential (DNA analyses) to a direct measurement of the expressed capability (RNA analyses). Overall, this work provides the framework for developing a new and useful approach with which to assess the potential bioavailability and treatability of PAHs in dredged sediments.

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