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Phenanthrene Biodegradation in Freshwater Environments

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Phenanthrene, a low-molecular-weight polycyclic aromatic hydrocarbon, was incubated with water samples from various reservoir systems in Tennessee to evaluate the potential for significant polycyclic aromatic hydrocarbon degradation by the indigenous microbial populations. Biodegradation was assessed by comparison of total polycyclic aromatic hydrocarbon substrate recovery in degradation flasks relative to sterile control flasks. During 1977 field studies, the mean phenanthrene biodegradation was approximately 80% after a 4-week incubation. Within a given habitat, 45% of the total variability in phenanthrene biodegradation was attributable to the physical, chemical, and microbiological site characteristics examined. Polycyclic aromatic hydrocarbon degradation was directly related to the historical environmental pollution of the sampling sites examined, the length of biodegradation assessment, temperature, and the molecular size of the polycyclic aromatic hydrocarbon substrate.

Polycyclic aromatic hydrocarbons (PAH) are distributed throughout the environment, predominantly as a result of high-temperature pyrolytic processes (9, 19). Many of these aromatic compounds eventually enter aquatic systems in stormwater runoff from metropolitan areas as well as wastewater effluents from industrial processes utilizing fossil fuels (2, 13). Several PAH have demonstrated carcinogenic properties (5, 10) and may be considered a potential health risk to humans upon long-term, chronic exposure. The transport and bioaccumulation of PAH in aquatic food webs (11, 14) may increase the potential hazard to humans.

The enzymatic pathways for microbial PAH degradation have been elucidated (3, 18), and several studies have demonstrated the ability of various microorganisms to mediate the decomposition of these compounds (4, 7). The primary objective of this investigation was the evaluation of the potential for significant PAH degradation by the indigenous microbial populations in freshwater environments.

MATERIALS AND METHODS

Chemicals. Certified reagent-grade phenanthrene (PHE) and pyrene (Eastman Kodak Co, Rochester, N.Y.) were employed as the model low-molecular-weight PAH for the biodegradation studies. Pesticide-grade solvents, acetone, hexane, and petroleum ether (Fisher Scientific Co., Pittsburgh, Pa.) were used in PAH extraction and preparation of the glassware. Celite (Fisher) was used as an inert carrier for the PAH substrate.

Field sampling and site characteristics. The majority of the studies were performed at seven sites on Center Hill Reservoir which represented a relatively pristine aquatic system with no prior or apparent contamation by chronic sources of PAH input (16). A later comparative study also involved two East Tennessee reservoirs, Fort Loudoun and Melton Hill Reservoirs, which receive industrial and domestic wastes from the surrounding watershed and urban regions.

Center Hill Reservoir samples during 1977 were taken from a depth of 1 m, using a Niskin sterile bag sampler (General Oceanics, Miami, Fla.), and were maintained at ambient water temperature while transported to Tech Aqua Biology Field Station, where processing was initiated within 1 h of sample collection. In subsequent 1978 studies, grab samples were collected by immersion of a 20-liter Nalgene container, 0.5 m below the surface.

Field sampling variables assessed during the investigation included: dissolved oxygen (YSI DO meter, model 51B, Yellow Springs Instrument Co., Yellow Springs, Ohio); water temperature and conductivity (YSI conductivity meter, model 33); transparency (Secchi disk, Wildlife Supply Co., Saginaw, Mich.); pH (Orion model 407A pH-specific ion meter, Orion Research Inc., Cambridge, Mass.); and air temperature (mercury-filled glass thermometer). Dissolved organic carbon was determined by dry combustion infrared analysis (Beckman no. 215A TOC Analyzer, Beckman Instruments, Inc., Irvine, Calif.). NO_3 was determined by a specific ion electrode (Orion). Orthophosphate was determined by the Vanadomolybdophosphoric acid method (1).

Total viable heterotrophic bacteria and PAH-resistant or -degrading microorganisms were determined by spread plating appropriate dilutions of each sample in autoclaved lake water onto yeast extract peptone glucose agar and PHE agar, respectively (16). Colony-forming units were counted after 2 and 4 weeks of incubation at 25°C. Basal salts media, without added PHE, supported 1 to 10% of the total viable cell counts obtained from duplicate inoculation of PHE agar.

PAH biodegradation assessment. Water samples (100 ml each) were placed in sterile, Teflon-lined, screw-capped, 250-ml Erlenmeyer flasks containing 10 mg of Celite (Fisher) per flask to serve as an inert carrier of the PAH. Control flasks were autoclaved at 121°C, 15 lb/in^2 for 20 min. Degradation flasks and sterile controls, each in triplicate, were supplemented with 100 μg of the PAH substrate in 100 μl of acetone and incubated without agitation at 25°C in the dark for 4 weeks.

Upon termination of the incubation period, the contents of the batch culture flasks were filter-extracted through Whatman no. 1 filter circles and a Tenax-GC, 2,6-diphenyl-*p*-phenylene oxide (Applied Science Laboratories Inc., State College, Pa.) column (17). The Tenax column was eluted with 5.0 ml of petroleum ether between each sample extraction. The column eluates and eluates of extracted filterable particulates were condensed by evaporation in a Kuderna Danish evaporator (Kontes) and quantitated by flame ionization gas-chromatographic analysis and comparison to PAH standards (17). Biodegradation was assessed by comparison of total PAH substrate recovery in the degradation flasks relative to the sterile control flasks. Sterility of control flask was determined by spread plate inoculation of yeast extract peptone glucose agar before and after incubation.

Effect of incubation time and temperature and PAH concentration on PHE biodegradation. Studies were conducted to assess the time course rate of PHE biodegradation as well as the effect of incubation temperature and PAH substrate concentration on the rate and extent of PHE biodegradation. In these studies, the standard biodegradation assay was employed while the incubation times, temperatures, or PHE concentrations were varied independently. Incubation times were varied over a range of 1 to 5 days and weekly thereafter for 1 month. Incubation temperatures were varied over a range of 5 to 45°C. In addition, the rates of PHE degradation at 100 and 1,000 μg of PHE liter^{-1} were examined.

Temporal and spatial biodegradation assessment. PHE biodegradation assessment was performed at Center Hill Reservoir sites during February, March, April, June, August, September, and December of 1977. These studies were performed to assess the variability and seasonal potential for PAH biodegradation among related samples in a relatively pristine environment. Physical, chemical, and microbiological site characteristics were determined concurrently during sampling to determine those variables which accounted for the greatest variation in PHE biodegradation.

Significant differences in the variation of PHE degradation among sampling sites and sampling dates was examined, using computerized analysis of variance. The variance component contribution of physical, chemical, and biological, independent variables on PHE degradation, the dependent variable, was assessed by multiple regression analysis. Computer program subroutines for analysis of variance and multiple regression analysis were derived from *Statistical Package for the Social Sciences* (15). Statistical analysis employed a DEC 10, IBM 360/5-370/148 computer. Confidence limits were set at $\alpha = 0.05$.

In an effort to determine the biodegradative fate of PHE in diverse freshwater sample types, a study was undertaken to compare PHE biodegradative capacities among Center Hill, Melton Hill, and Fort Loudoun reservoirs. Within a period of 1 week in October 1978, 20-liter grab samples were collected from sites on each reservoir and assessed for PHE load (via Tenax extraction of 5 liters of sample and GC-FID quantitation) and PHE biodegradation. Triplicate control and biodegradation flasks for each site were subjected to short-term biodegradation assessment (5 days), using a PHE concentration of 1,000 μg liter^{-1}. Biodegradation assessment as previously described was employed.

A comparative study was performed to assess the potential for pyrene biodegradation in Center Hill Reservoir. This study was performed to determine whether or not PHE degradation was an isolated phenomenon and not representative of other PAH. Pyrene biodegradation was performed in a manner identical to that for PHE biodegradation.

RESULTS

Temporal and spatial biodegradation assessments. Significant PHE biodegradation was observed in all water samples collected from Center Hill Reservoir in 1977 (Table 1). The average temporal range in PHE degradation was from 54.2 ± 6.6% to 93.4 ± 1.7% for August and December, respectively, whereas the average

TABLE 1. *Temporal and spatial variation in PHE biodegradation among Center Hill Reservoir samples, 1977*

Site	Relative degradation (%)[a]						
	Feb	Mar	Apr	June	Aug	Sept	Dec
1	ND	65.86 ± 13.65	91.75 ± 0.80	85.51 ± 3.75	ND	ND	ND
2	100.00 ± 0.00	90.25 ± 9.47	80.39 ± 4.44	79.76 ± 9.78	ND	ND	ND
3	ND	78.71 ± 7.87	86.33 ± 3.89	82.03 ± 4.13	ND	ND	ND
4	93.69 ± 4.91	77.33 ± 2.64	82.79 ± 2.31	85.76 ± 3.30	49.58 ± 6.55	67.61 ± 14.03	92.26 ± 1.32
5	55.52 ± 17.38	79.45 ± 1.59	85.20 ± 4.58	84.61 ± 5.74	ND	ND	ND
6	ND	82.71 ± 0.97	80.70 ± 5.19	82.73 ± 5.45	ND	ND	ND
7	ND	ND	ND	ND	58.84 ± 8.50	78.46 ± 1.00	94.60 ± 0.84

[a] Each value represents the mean of three determinations (±standard deviation) relative to sterile controls. ND, Not determined.

spatial degradation ranged from 76.2 ± 14% at site 5 to 87.0 ± 9.6% at site 2. Although few general trends are apparent, the highest levels of degradation were observed during the winter months, whereas slightly lower levels of degradation were seen in the late summer months. The overall mean degradation, temporally and spatially, was approximately 80% during a 4-week incubation. This value is normalized relative to sterile control samples which exhibited a 45% loss of added PHE. This loss is attributed to the analytical loss, also 45%, due to the concentrating of sample extracts before gas-liquid chromatographic analysis (17).

To determine the significance and predictability of the inherent temporal and spatial variability of the relative PHE biodegradation, it was necessary to statistically analyze the components contributing to variation. Analysis of the individual sites, sampling dates and rate of PHE biodegradation using *Statistical Package for the Social Sciences* (15) analysis of variance subroutine suggested that there is no significant variation among sampling sites or dates at the 95% probability level (Table 2).

A multiple regression analysis was performed in which the dependent variable, degradation, was regressed on the individual physical, chemical, and biological variables for each sample examined (Table 3). The multiple regression analysis indicated that approximately 46% of the total variation in PHE degradation could be attributed to the individual site characteristics (Table 4). This value was estimated from the multiple r^2 value (0.461) obtained upon the inclusion of the final independent variable in the multiple regression equation. The individual regression coefficients and the regression were found to be insignificant at the 95% level. The regression coefficient for total viable heterophobic bacteria was significant at the 90% level, and the overall regression line was significant at the 75% level. A larger sample size would be necessary before one could conclude that the regression of degradation on the independent variables was not significant at the 95% level; although, it appears that many independent variables could be eliminated as factors affecting biodegradation. The regression analysis indicated that approximately 25% of the total variation can be attributed to the two biological parameters, PHE-resistant bacteria and total viable cell counts (13.5 and 11.8%, respectively). Dissolved oxygen, suspended sediments, and nitrate-nitrogen levels each accounted for approximately 5% of the total variation. The simple correlation coefficients (r) are informative in that they demonstrate a less significant influence on degradation of suspended sediment loads ($r = 0.06$) as compared with PHE-resistant organisms ($r = 0.23$) and total viable heterotroph count ($r = 0.26$). This information would indicate that the degradation responses are biological and not merely an irreversible partitioning or binding of PHE to particulate matter.

TABLE 2. *Analysis of variance in PHE biodegradation by sample site[a] and date*

Source of variation	Sum of squares	Degrees of freedom	Mean square	F^b
Sample site	280.91	6	46.82	0.26
Sampling date	627.36	6	104.56	0.58
Residual	2723.12	15	181.54	
Total	3631.39	27	134.50	

[a] Center Hill Reservoir samples collected in 1977.
[b] Site and date variations were not significant at the 95% confidence level. F, F statistic.

Center Hill Reservoir samples in June, July, and September of 1977 were assessed for pyrene as well as PHE degradation (Table 5). In all cases, the relative pyrene degradation was significantly less than that observed with PHE. The mean pyrene biodegradation was 16.7% as compared with 75.0% mean PHE degradation in the same samples.

Effect of incubation time, temperature, and PHE concentration on PHE biodegradation. It was apparent that individual sites characteristics were in part responsible for variability among rates of PHE biodegradation. It was felt that the arbitrary conditions of incubation during biodegradation imposed an artificial bias that was reflected in relative uniform biodegradation rates among the samples examined. Consequently, both the length of incubation time and the temperature of incubation were varied independently to more fully understand seasonal and temporal PHE biodegradation.

A time course estimation of PHE biodegradation in samples collected from Center Hill Reservoir indicated that the potential differences in PHE biodegradation rates were masked by prolonged incubation (Table 6). Biodegradation responses for both sites 4 and 7 had reached their maximum, ca. 90%, within the first week of incubation (Table 6). The rate of PHE biodegradation at site 7 progressively increased to a relative 60.8 and 46.1% degradation within 5 days at 100 and 1,000 μg of PHE liter^{-1}, respectively (Fig. 1).

The effect of incubation temperature was even more pronounced than the effects of incubation time, as indicated by the temperature-dependent biodegradation response histograms for site 7 samples dosed with either 100 or 1,000 μg of PHE liter^{-1} and incubated at varied tempera-

TABLE 3. *Center Hill Reservoir field site characteristics used in the multiple regression analysis of PHE biodegradation*[a]

Site	Date (1977)	PHE degradation (%)[b]	TVC (ml^{-1})[c]	PHE degrading bacteria (ml^{-1})[d]	Temp (°C)	DOC (mg $liter^{-1}$)	PO_4 (mg $liter^{-1}$)	NO_3 (mg $liter^{-1}$)	SSED (mg $liter^{-1}$)	DO (mg $liter^{-1}$)	COND (mg $liter^{-1}$)	TRNS (m)	pH
1	3-25	65.9	3.85	3.56	15.0	10	0.05	0.9	27.8	9.5	130	1.0	8.6
	4-22	91.8	5.43	3.96	19.3	—	0.09	1.18	4.2	10.0	138	1.2	9.5
	6-9	85.5	4.57	2.90	27.5	—	0	0.49	108.3	2.2	—	1.3	9.6
2	2-12	100	3.58	2.95	3.5	21	0.02	0.78	0.8	13.8	90	2.5	7.3
	3-25	90.3	4.26	3.20	15.0	11	0.018	0.9	24.6	10.3	105	1.0	8.3
	4-22	80.4	4.95	3.43	20.0	—	0.027	2.5	9.1	9.4	90	0.5	8.5
	6-9	79.8	4.52	2.73	27.0	—	0.01	0.62	112.3	—	—	1.8	9.4
3	3-25	78.7	4.15	2.60	16.0	8	—	0.9	28.3	10.5	100	0.8	8.6
	4-22	86.3	4.88	3.79	18.0	—	0.055	2.66	3.6	9.4	90	1.3	8.8
	6-9	82.0	4.54	2.11	28.0	—	—	0.72	37.7	—	—	1.7	9.2
4	2-12	93.7	3.53	2.11	3.9	50	0.018	0.92	1.3	13.0	90	4.0	7.8
	3-25	77.3	4.62	2.69	11.5	7	0.015	0.9	24.4	10.5	95	1.5	8.3
	4-22	82.8	—	—	—	—	0.028	4.25	6.5	—	—	—	—
	6-9	85.8	4.79	2.30	25.5	—	0.040	0.80	105.3	—	115	2.0	8.2
	8-19	49.6	3.54	2.15	30.0	—	0.094	—	3.8	7.2	131	3.0	7.7
	9-15	67.6	2.48	1.00	28.5	—	—	—	—	7.8	160	3.4	6.4
	12-19	92.3	3.36	2.60	13.0	—	—	—	—	13.6	80	0.6	—
5	2-12	55.5	3.58	2.92	4.5	32	0.02	0.70	1.4	13.2	80	3.2	7.1
	3-25	79.5	4.23	3.15	12.0	8	0.02	0.9	37.3	10.4	80	0.5	8.0
	4-22	85.2	4.95	3.43	20.0	—	0.05	2.42	0.47	9.4	115	1.3	—
	6-9	84.6	4.79	2.30	26.5	—	—	—	—	—	160	—	—
6	3-25	82.7	4.15	3.53	14.5	8	0.048	0.9	34	11.0	103	1.3	8.4
	4-22	80.7	—	—	—	—	0.043	2.49	13.7	11.0	—	—	—
	6-9	82.7	4.57	2.91	26.5	—	0.030	0.95	124.7	—	128	2.0	9.3
7	6-9	58.8	—	—	29.0	—	—	—	—	—	140	—	—
	8-19	78.5	3.38	3.59	29.0	—	0	—	2.9	7.6	131	2.5	8.4
	9-15	94.6	2.54	1.90	27.5	—	—	—	—	8.2	290	1.9	—
	12-9	77.3	3.77	2.88	11.5	—	—	—	—	10.5	100	—	—

[a] DOC, Dissolved organic carbon; SSED, suspended sediment; DO, dissolved oxygen; COND, conductivity; TRNS, transparency.
[b] Relative to sterile control sample.
[c] Log 10 total viable heterotroph count.
[d] Log 10 total PHE-resistant or -degrading bacteria.

TABLE 4. *Multiple regression analysis of PHE biodegradation on physical, chemical, and microbiological variables characteristic of Center Hill Reservoir samples*[a]

Independent variable[b]	r^{2c}	Change in r^2	r^d	Regression coefficient	
				b	F^e
pH (X_1)	0.0007	0.0007	−0.0272	−0.89	0.784
TRNS (X_2)	0.0153	0.0146	−0.1148	−0.45	0.031
COND (X_3)	0.0195	0.0042	−0.0484	−0.35	0.502
DO (X_4)	0.0765	0.0569	−0.0233	−0.67	0.0
SSED (X_5)	0.1224	0.0549	0.0681	0.52	0.429
NO_3 (X_6)	0.1789	0.0566	0.1597	0.15	0.961
PO_4 (X_7)	0.1870	0.0081	0.2332	−0.14	0.641
DOC (X_8)	0.1936	0.0065	0.0611	−0.21	0.004
Temp (X_9)	0.2076	0.0140	−0.2090	−0.63	1.535
PHE-degrading bacteria (X_{10})	0.3435	0.1358	0.2292	−0.42	0.508
TVC (X_{11})	0.4614	0.1178	0.2555	0.81	3.505

[a] Biodegradation relative to sterile control samples, triplicate analysis for each sample collected.
[b] See footnote *a* to Table 3. *Y* constant was 0.780.
[c] r^2, Multiple coefficient of determination.
[d] r, Simple correlation coefficient.
[e] Critical F statistic at 95% confidence level = 4.54.

TABLE 5. *Comparative PHE and pyrene batch culture biodegradation in Center Hill Reservoir samples, 1977*[a]

Date	Site	PHE degradation (5)[b]	Pyrene degradation (5)[b]
6-77	1	85.5 ± 3.7	14.6 ± 7.5
	2	79.7 ± 9.7	0.0
	3	82.0 ± 14.1	0.0
	4	85.7 ± 3.3	0.0
	5	84.6 ± 5.7	13.4 ± 26.4
	6	82.7 ± 5.4	57.1 ± 17.9
8-77	4	49.5 ± 6.5	3.8 ± 19.4
	7	58.5 ± 8.5	4.0 ± 27.0
9-77	4	67.6 ± 14.0	0.0
	7	78.5 ± 1.1	54.1 ± 11.9

[a] Relative to sterile control samples; PHE and pyrene concentrations were 1,000 μg liter^{-1}.

[b] Mean of three observations ± 1 standard deviation.

TABLE 6. *Effect of incubation time on PHE biodegradation in Center Hill Reservoir samples*[a]

Time of incubation (week)	% Degradation relative to controls	
	Site 4	Site 7
1	90.12 ± 2.01	86.70 ± 4.11
2	90.36 ± 4.63	95.04 ± 0.72
3	94.02 ± 2.91	93.55 ± 0.81
4	94.81 ± 1.64	90.84 ± 1.79

[a] PHE concentration, 1,000 μg liter^{-1}.

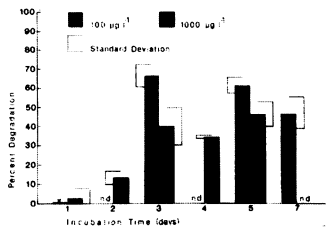

FIG. 1. *Comparative time courses of PHE biodegradation at 100 and 1,000 μg liter^{-1}. Percent degradation is relative to sterile controls, site 4. ND, Not determined.*

tures for 5 days (Fig. 2). PHE biodegradation was virtually not detected at either end of the temperature extremes (5 and 45°C) after the incubation period for either substrate concentration. Biodegradation was, however, directly related to incubation temperature between 15 and 37°C, with maximum rates of biodegradation approaching 90% at 37°C (at 1,000 μg of PHE liter^{-1}).

Five-day PHE biodegradation assays at 25°C employing concentrations of 100 and 1,000 μg liter^{-1} (Fig. 2) suggest that degradative rates are themselves dependent upon PHE concentrations. Upon increasing the concentration 10-fold (100 to 1,000 μg liter^{-1}), a 3-fold greater increase in the relative rate of PHE biodegradation was observed (12.1 ± 1.2 μg liter^{-1} day^{-1} and 34.4 ± 8.3 μg liter^{-1} day^{-1}, respectively; these data are normalized to 100% analytical recovery).

In October 1978 the biodegradation potential of water samples from Center Hill, Melton Hill, and Fort Loudoun reservoirs was compared during a 5-day incubation (Fig. 3). Significantly greater biodegradation was observed in the Melton Hill and Fort Loudoun Reservoir samples as compared with the Center Hill samples, which indicated that those samples with a history of higher levels of enrichment or pollution, or both, exhibited a greater capacity for PAH biodegradation. More extensive comparative assessments

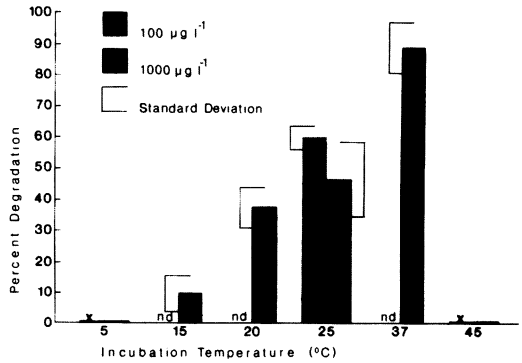

FIG. 2. *Effect of incubation temperature on the rate of PHE biodegradation in site 4, Center Hill Reservoir samples. Percentage degradation is relative to sterile control samples. ND, Not determined.*

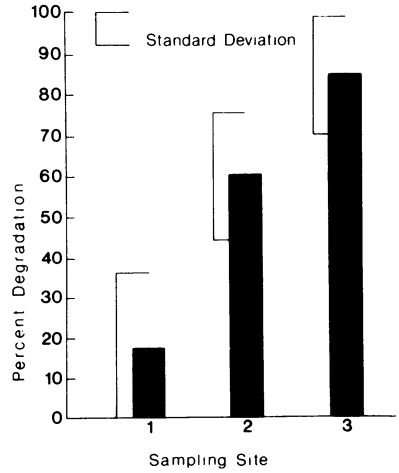

FIG. 3. *Comparative PHE biodegradation among Center Hill Reservoir (site 1), Melton Hill Reservoir (site 2), and Ft. Loudoun Reservoir (site 3). Percent degradation is relative to sterile control samples for each site.*

of biodegradation are necessary to establish the stability of this trend. Five liters of water from each of the three sites was extracted via a Tenax column (10 by 1 cm) and analyzed for background PHE levels. None of the three samples contained PHE residues within the limits of detection (1 μg of PHE liter^{-1}) of the gas-chromatographic method employed for analysis.

DISCUSSION

This investigation demonstrated significant PAH-degradative potential of aquatic microorganisms in studies which employed natural mixed microbial populations in contrast to earlier reports of microbial PAH degradation which used pure cultures of isolates grown under optimal laboratory conditions (6, 12). This conclusion is based on the observed loss of PHE, relative to sterile controls, in biodegradation assays. The microbial nature of the PHE degradation is supported by the statistical analysis in which a large proportion of the total variation in phenanthrene degradation was correlated with densities of both total viable heterotrophic bacteria and microbial populations that are PHE degrading, PHE resistant, or both. The apparent lack of correlation between suspended sediment loads and degradation in the analysis also suggests that particulate partitioning does not contribute significantly to the observed biodegradation in these assays. Furthermore, substantial PHE biodegradation capacity was observed in all samples from three varied freshwater systems. In addition, the temperature response profile of the biodegradation assay clearly indicated that PHE loss was a result of a microbial decomposition processes and not due to volatilization of the PAH. Although biodegradation was directly related to incubation temperatures, it was not correlated with ambient site temperatures. These data would suggest that microbial populations, capable of degradation, are present during winter months in Center Hill Reservoir, but their degradative capacity is suppressed at lower temperatures. Biodegradation assessment at 25°C supports this point, since samples collected during the winter months exhibited PHE degradation potentials as high as those of samples collected during warmer weather.

Additional insights into PAH decomposition resulted from the biodegradation assays of this investigation. The increased PHE biodegradation capacity observed in Melton Hill and Fort Loudoun Reservoirs compared to Center Hill Reservoir indicated a trend of samples with historical background of greater levels of enrichment or pollution, or both, to exhibit an increased capacity for PAH biodegradation. Although data for PAH concentrations and the numbers of PAH-degrading microorganisms in all three reservoirs are not available, the observed trend is consistent with an investigation which showed a correlation between the concentration of oil in water and sediment and the number of petroleum-degrading bacteria (20).

Comparison of PHE and pyrene biodegradation in Center Hill Reservoir water samples indicated that PHE was more readily subject to microbial decomposition. These results agree with a recent study indicating that PAH become more resistant to microbial transformation as the number of rings in the compound is increased (8).

Although the mixed-culture, metabolic fate of PHE was not examined, these studies indicate that lower-molecular-weight PAH, such as PHE, are subject to environmental removal by microorganisms in surface waters of aquatic environments. Furthermore, some aquatic systems exhibit a greater potential for accommodating PAH contamination which may be useful information in impact assessment and environmental risk analysis.

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