To the Graduate Council:

I am submitting herewith a thesis written by Timothy W. Sherrill entitled "Biodegradation of Phenanthrene in Freshwater Environments." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Gary S. Sayler, Major Professor

We have read this thesis
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BIODEGRADATION OF PHENANTHRENE IN FRESHWATER ENVIRONMENTS

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ABSTRACT

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

A rapid Tenax-GC extraction technique was evaluated for use in conjunction with aqueous biodegradation assays for PAH. The method was quantitatively efficient and reproducible for phenanthrene. Aqueous sample volumes and varying concentrations of organic matter influenced the efficiency of PAH extraction. Phenanthrene recovery was decreased by soil extract but unaffected by spent bacteriological culture medium. PAH biodegradation assays, performed with reservoir samples, supported the laboratory evaluation. The study

demonstrated the utility of the Tenax-GC extraction technique for analysis of phenanthrene in the measurement of biodegradation.

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PART I: TENAX-GC EXTRACTION TECHNIQUE

I. INTRODUCTION

Chemical carcinogens in air and water are estimated to cause four of five occurrences of cancer in humans (20). Many polycyclic aromatic hydrocarbons (PAH) are implicated as carcinogenic agents and because of their serious adverse health effects, their widespread contamination of the environment is a source of concern (44). A committee from the National Science Foundation placed PAH on a priority list of hazardous environmental contaminants (37). The National Academy of Sciences has investigated the occurrence of PAH in the atmosphere (35) and the World Health Organization Expert Committee on the Prevention of Cancer has noted the potential hazards to man by carcinogenic compounds such as PAH in water supplies (50). Microbial transformation of PAH in the environment may lead to degradation to innocuous compounds, activation to mutagenic or toxic intermediates, or bioaccumulation of PAH and mobilization into food webs. Development of analytical methods and biodegradation assays which would allow investigation of the environmental fate of PAH are needed.

In studies on the environmental biodegradation of PAH in aquatic habitats, it became obvious that a rapid, relatively inexpensive, yet efficient extraction procedure would greatly facilitate the processing of large numbers of biodegradation samples. Recently, Tenax-GC, a polymer of

diphenylphenylene oxide, was demonstrated effective in the extraction of PAH from aqueous samples, although some variables could adversely affect the routine efficiency and use of Tenax-GC extraction of test contaminants in biodegraded samples (1, 46). Of particular importance in these studies are the interaction of the elution solvent with extractant and the resulting contamination of the extracted test contaminant. In addition, the presence of particulate matter and dissolved organic compounds, indigenous to natural water samples or from bacterial metabolism, could significantly reduce the extraction efficiency and recovery of the test contaminant. Such effects have previously been encountered by other investigators using a variety of other extraction techniques (1, 40, 46). Consequently, studies were undertaken to assess the use of Tenax-GC as an extraction polymer for biodegradation samples and to determine factors affecting the efficient recovery of PAH.

II. LITERATURE REVIEW

Numerous analysis of water and sediment samples show the distribution of PAH is almost universal in the aquatic environment (3, 18, 20, 23, 25, 44). PAH have been found in groundwater at 0.045-0.51 $\mu g\ l^{-1}$ and treated river water at 0.025-0.234 $\mu g\ l^{-1}$, while untreated river water may contain levels ten-fold greater (19). The suspended solids content of a badly polluted river can reach 50-100 $mg\ l^{-1}$ and may greatly enhance the PAH levels, since studies have shown PAH levels up to 56 $mg\ kg^{-1}$ of dried material (19). Comprehensive reviews of marine studies along the French, Italian, and Greenland coasts have found benzpyrene, a carcinogenic PAH, in almost all plankton, algae, fish, shellfish, and sediment (4, 19).

Quantitatively, the most significant origins of PAH are the man-controlled combustion processes with temperatures above 700°C. Effluents from industrial plants associated with high-temperature pyrolytic processes including production of refined petroleum, steel and iron, coal tar, carbon black, and asphalt, as well as open burning, refuse incineration, power generation and internal combustion engines are sources of PAH released into the environment (4, 19, 44). The biosynthesis of PAH by bacteria, algae and higher plants (4, 19) provides a natural background concentration in the environment. In addition, PAH formed

during forest fires and released in volcanic activity are additional sources which contribute to the environmental load. The PAH formed during high temperature pyrolysis and released into the atmosphere are eventually returned to earth by fall-out or precipitation (6, 16). Run-off water can transport the PAH from areas contaminated with atmospheric fall-out into sewer systems, natural surface waters or groundwater (33).

Polycyclic aromatic hydrocarbons are fused benzene ring compounds. Due to their high molecular weight and low polarity, the solubilities of PAH in water are very low. For example, solubility of phenanthrene in water at 25°C is 1.6 mg l^{-1} (30). Studies have shown that the introduction of other organic chemicals will increase PAH solubility in water (30, 48). The presence of organic solvents in polluted waters could possibly enhance PAH concentrations. Although the addition of detergents increases the solubilization of PAH by the formation of micelles (12), the amount of detergents found in industrial wastes and sewage would not be sufficient to cause micelle formation.

Efficient PAH extraction and separation procedures for aqueous sample analysis are necessary because of the low concentration at which they occur in water and the presence of other organic materials. The considerable time involved, toxic solvents used and large volumes of water required for liquid-liquid extraction methods pose difficulties to PAH

analysis of aqueous environmental samples. Tenax-GC, a porous polymer based on 2,6-diphenyl-p-phenylene, has been developed to extract and concentrate PAH in water samples (31). Chromatographic techniques, including thin-layer, gas-liquid and high pressure liquid chromatography are widely employed in the identification and quantification of PAH following extraction (11, 24, 25).

A primary objective of this investigation was the development of an efficient extraction procedure which could be used to assess residual PAH in biodegradation assays of aquatic samples.

III. MATERIALS AND METHODS

Chemicals

Reagent grade phenanthrene (Eastman Kodak Co., Rochester, NY) was used as a model compound for examining the efficiency of extraction of low molecular weight PAH with Tenax-GC. Pesticide grade hexane and acetone (Fisher Scientific Co.) were employed in all PAH extraction studies and preparation of glassware. Phenanthrene (9-^{14}C), 13 mCi/mmole (New England Nuclear, Boston, Mass.), was used in extraction efficiency studies.

Preparation of Phenanthrene Saturated Aqueous Solution

A saturated aqueous solution of phenanthrene was prepared in the following manner: acetone washed 4.5 liters glass carboys were filled with 3 liters of distilled water. To each carboy containing a Teflon stir bar, 1.0 g of phenanthrene was added. The carboys were sealed with an aluminum foil-covered rubber stopper and autoclaved for one hour at 120°C. Each carboy was then placed on a magnetic stir plate and stirred for a period of two months at room temperature. The carboys were then allowed to equilibrate unstirred for an additional two months at which time saturation was assumed complete. Care was taken to avoid exposure of the carboys to sunlight or intense fluorescent light.

Preparation of Organic Matter Solution

To study the effects of naturally occurring dissolved organic matter on the efficiency of Tenax-GC extraction, two representative sources of dissolved organic matter were prepared. An extract of soil organic matter was prepared by adding 10% (wt/vol) soil to 1 liter of distilled water. The mixture was heated to 80-90°C for one hour with moderate stirring and residual soil was removed by clarification through Whatman #1 filter paper and passed through a 0.45 µm Gelman cellulose membrane filter (Gelman Instrument Co., Ann Arbor, Mich.). The resulting extract was sterile and dark golden in color.

A second source of dissolved organic matter was prepared from a spent bacteriological culture medium. Five hundred ml of yeast extract peptone glucose broth (YEPG) (41) were inoculated with 10 ml of raw lake water. This mixed bacterial culture was incubated for 48 hours at 20°C with constant agitation. The bacterial suspension was removed by centrifugation at 3000 x g for 10 minutes and the spent culture supernatant was filtered through a 0.2 µm Nucleopore membrane filter (Nucleopore Corp., Pleasanton, Calif.). The dissolved organic carbon (DOC) concentrations were determined by wet persulfate combustion (17) and found to be 390 mg C l^{-1} and 970 mg C l^{-1} for the soil extract and the culture medium extract, respectively.

Tenax-GC Extraction Column

A 60/80 mesh Tenax-GC (Applied Sciences Laboratories, Inc., State College, Penn.) extraction column was prepared by packing a long stem (15 cm) glass filtration funnel with 10 cm of Tenax-GC (Figure 1). The extraction polymer was held in place with pyrex glass wool plugs at either end. The final dimensions of the column were 10 cm x 0.4 cm. The column was inserted into a nylon mini-adapter (Ace Glass, Inc., Vineland, NJ) and fitted with a Teflon O-ring seal and vacuum adapter (threaded female port with a 24/40 ground glass male port). A 15 ml concentration tube (Kontes, Vineland, NJ) with a 24/40 ground glass neck was used as a receiving vessel for the column eluate. A negative pressure of 5 psi was applied to the column to facilitate the extraction procedure.

Gas Chromatography

Quantification of PAH in the hexane elutions was performed with a Perkin-Elmer 3920 gas chromatograph (GC) equipped with a flame ionization detector. The GC column was packed with 3% SE-52 on Chromosorb WAW DAMCS 80/100 mesh in a 9 ft x 1/4 in O.D. x 2 mm I.D. column. Nitrogen was employed as the carrier gas at a flow rate of 50 ml/minute with the following temperature parameters: Injector, 300°C; column, 180°C to 240°C at 8°C/minute; and detector, 310°C. Phenanthrene was quantified by comparison to a standard peak with the aid of a Perkin-Elmer M-2 integrating calculator.

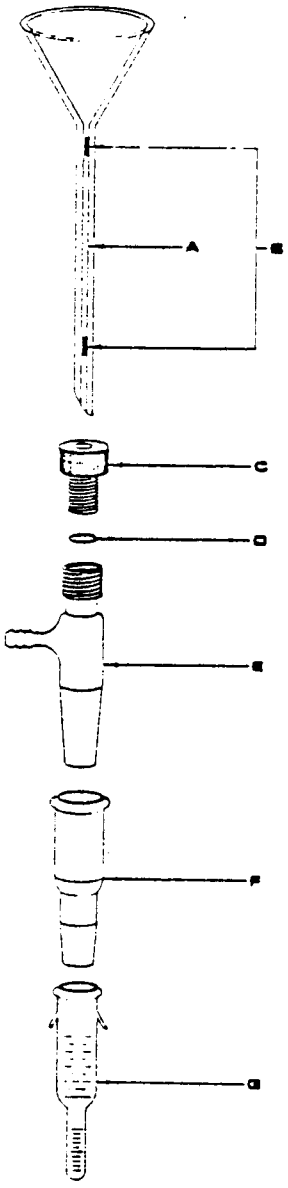


Figure 1. Tenax extraction apparatus. Components: A. 10cm x 4mm Tenax-GC column; B. pyrex glass wool plugs; C-E. nylon fitting, teflon O-ring, and vacuum adapter (mini-adapter, Ace Glass Co.); F. 24/40-19/22, ground glass adapter; and G. 15 ml concentrator tube (Kontes).

Tenax-GC Extraction Efficiency and Substrate Recovery

Radiolabeled phenanthrene was employed to determine the net recovery and extraction efficiency of the Tenax-GC technique. In phenanthrene extraction efficiency studies, 50 to 100 µg of (9-^{14}C) phenanthrene, in 10 µl acetone, were added to 100 ml volumes of filter sterilized lake water in 250 ml screw cap Erlenmeyer flasks. Following mixing and equilibration, a 1 ml aliquot was removed and placed in 15 ml of a 0.8% Omnifluor (New England Nuclear, Boston, Mass.) in dioxane liquid scintillation cocktail and counted on a Tracor 6892 liquid scintillation counter using a preset ^{14}C window. The remaining sample was extracted via the Tenax-GC column and the extracted aqueous sample was again subsampled and counted. The Tenax-GC column was eluted with two 5 ml volumes of hexane and an aliquot was removed from each eluate and counted prior to and after evaporative concentration to 1 ml.

Additional studies were performed for phenanthrene extraction efficiency and recovery in which gas-liquid chromatography (GLC) was employed to determine residual phenanthrene in various sample fractions. These studies, which simulated degradation studies, used 100 ml of filter sterilized lake water to which 10 mg Celite and 100 µg phenanthrene were added. The samples were vacuum filtered through Whatman #1 filter circles and the Tenax-GC column and eluted with two 5 ml volumes of hexane. The filters

were returned to their respective flasks and extracted with 20 ml hexane in the presence of 10 g Na_2SO_4. The filter extracts and the column eluates were subjected to GLC analysis for residual phenanthrene prior to and after evaporative concentration to a 1 ml final extract volume.

Effects of Aqueous Sample Loading and Dissolved Organic Matter on Tenax-GC Extraction

A series of experiments was performed to determine the effects of aqueous sample size and dissolved organic matter on the recovery of phenanthrene by Tenax-GC extraction. Three volumes, 50, 100, and 200 ml, of phenanthrene-saturated water were extracted using the prescribed technique. Each eluate of triplicate samples for each volume extracted was concentrated to 1.0 ml and was analyzed by GLC for quantificating substrate recovery. For assessing the influence of dissolved organic matter (DOM) on Tenax-GC extraction efficiency, 0.01%, 0.1%, and 5.0% (v/v) solutions of either soil extract or culture medium extract were prepared in 50 ml of phenanthrene saturated water. Extraction, concentration, and quantification techniques employed were identical to those described previously.

Data Analysis

Significance between the controls and organic matter treatments was tested by analysis of variance (ANOVA).

The Student-Neuman-Keules multiple range test was used to test for significant differences among the different organic matter treatments.

IV. RESULTS

Tenax-GC Extraction Efficiency and Substrate Recovery

Studies on the recovery of (9-^{14}C) phenanthrene during the analysis of Tenax-GC extraction efficiency indicated that there was no significant volatilization of phenanthrene from the biodegradation reaction vessels (Table 1). Approximately 99% of the total phenanthrene added to the reaction vessels was recovered prior to the actual Tenax-GC extraction of aqueous samples. Following the Tenax-GC extraction and the elution of the phenanthrene from the column, it was observed that virtually all the phenanthrene was recovered in the elution solvent (97.7% and 117.2%, respectively for the 500 and 100 $\mu g\ l^{-1}$ phenanthrene concentrations). However, concentrating the 5.0 ml eluate to 1.0 ml resulted in a significant loss of about 45% of the total phenanthrene recovered in the initial column elution.

The results were substantiated by similar experiments employing gas liquid chromatographic analysis of phenanthrene recovery in an experimental design which duplicated that used for the biodegradation assays (Table 1). Prior to concentrating the individual aqueous and particulate extracts, 96% of the total added phenanthrene was recovered. However, a 45% loss in recoverable phenanthrene resulted from concentrating the individual extracts to a

Table 1. Comparative analytical recovery of phenanthrene, following Tenax-GC extraction.

Method	Phenanthrene Concentration µg/100 ml	Phenanthrene Recovered µg	Phenanthrene Recovered Percent
^{14}C-Phenanthrene Analysis[a]			
Aqueous phase (prior to extraction)	50	49.6 ± 0.7	99.1 ± 1.4
	100	98.4 ± 1.1	98.4 ± 1.1
Column Eluate Prior to Concentration	50	49.4 ± 10.5	98.7 ± 21.0
	100	117.2 ± 11.2	117.2 ± 11.2
Column Eluate After Concentration	50	25.7 ± 4.0	51.4 ± 8.0
	100	56.3 ± 0.8	56.3 ± 0.8
Gas-liquid Chromatography[b]			
Column Eluate Prior to Concentration	100	64.9 ± 1.8	64.9 ± 1.8
Filter Extract Prior to Concentration	100	31.3 ± 4.2	31.3 ± 4.2
Total Phenanthrene Prior to Concentration	100	96.2 ± 1.1	96.2 ± 1.1
Column Eluate After Concentration	100	31.0 ± 3.3	31.0 ± 3.3
Filter Extract After Concentration	100	10.5 ± 1.4	10.5 ± 1.4
Total Phenanthrene After Concentration	100	41.5 ± 3.5	41.5 ± 3.5

[a]Phenanthrene recovery quantificated using liquid scintillation counting.

[b]Phenanthrene recovery quantificated by gas-liquid chromatography.

1.0 ml volume prior to GLC analysis (relative to the total recovered prior to the concentrating step). These combined results indicated the extraction procedures were no less than 96% efficient in terms of phenanthrene extraction and that a predictable 45% loss in phenanthrene could be attributed to the final extract evaporation prior to GLC analysis.

Effects of Aqueous Sample Size and Organic Matter on Tenax-GC Extraction

A phenanthrene saturated solution (50 ml, 100 ml or 200 ml) was extracted via Tenax-GC extraction and eluted with 5.0 ml hexane. Each eluate was concentrated to 1.0 ml for phenanthrene analysis, and quantified by GLC. The recovery of phenanthrene was found to be variable (Table 2). Net recovery from 50 ml and 100 ml volume of phenanthrene saturated water was linear; upon increasing the sample volume two fold (100 ml to 200 ml) only a 20% greater level of phenanthrene was recovered.

All concentrations of soil extract caused a significant ($p > .01$) reduction, about 12%, in the relative extraction efficiency of phenanthrene in aqueous solution (Table 3). There was no significant dose effect upon increasing the concentration of soil organic matter in the aqueous solution. These results would indicate that the threshold effect of soil extract upon extraction efficiency lies below the 0.01% concentration. The equivalent

Table 2. Effect of aqueous sample size on Tenax-GC extraction efficiency of phenanthrene saturated solutions.

Substrate[a]	Recovery[b] µg	C.V.	Concentration µg l^{-1}
50 ml	49.1	4.0	983.2
100 ml	98.7	2.1	987.4
200 ml	117.8	18.6	589.0

[a]Aqueous samples taken from 3.0 liter stock solution of phenanthrene saturated distilled water.

[b]Mean recovery and coefficient of variation (C.V.).

[c]Column eluates concentrated to 1.0 ml.

Table 3. Effect of dissolved organic matter on the Tenax-GC extraction efficiency of phenanthrene.

Organic Matter	Phenanthrene Recovered µg	Relative Recovery Efficiency
Soil Extract		
0.01%	38.8 ± 2.6	79.0%
0.1%	43.6 ± 0.5	88.9%
1.0%	43.7 ± 0.2	89.0%
5.0%	45.3 ± 4.0	92.3%
	Mean Efficiency	87.3 ± 5.6
Culture Medium		
0.1%	48.0 ± 3.9	97.8%
0.1%	50.4 ± 0.2	102.6%
1.0%	52.9 ± 5.2	107.7%
5.0%	50.3 ± 0.7	102.4%
	Mean Efficiency	102.6 ± 4.0

dissolved organic carbon (DOC) concentration at this concentration of soil extract in water is 39 μg C l^{-1}; which is below the DOC levels encountered in natural surface waters.

There was no significant effect of culture medium extract, organic matter on the relative extraction efficiency of phenanthrene in aqueous solutions (Table 3). The relative extraction efficiency for all concentrations of culture medium extract organic matter was 102.6 ± 4.0% as compared to untreated, phenanthrene saturated, aqueous solutions.

V. DISCUSSION

Tenax-GC extraction efficiencies ranging from 98.5% to 99.6% have been demonstrated for high molecular weight PAH at concentrations as low as 0.08 g l^{-1} (31). Comparable results for Tenax-GC-phenanthrene analytical extraction efficiency (i.e., mean recovery using either GC or scintillation quantitation, 106%) were obtained in the present study for higher substrate concentrations employed in biodegradation assessment.

An apparent loss of substrate by volatilization of about 2% (Table 1, page 15) was observed in efficiency studies using membrane filtered lake water. Herbes has previously shown that up to 15% of a radiolabeled anthracene can be lost in short term incubations (21). However, in the presence of particulate matter, yeast cells, it was demonstrated that the surface adsorption-desorption phenomena occur kinetically much faster than the rate of PAH volatilization (21). If these results can be applied to the present study, decreased volatilization in the presence of detritus and microbial cells in whole lake water would render the initial PAH substrate more susceptible to microbial attack. A practical modification in the procedure described here would be the elimination of the negative pressure extraction step and the use of a moderate positive pressure to facilitate the column flow

rate and reduce potential volatilization.

Significant, reproducible analytical losses upon concentrating the column eluate were observed (Table 1, page 15). However, by concentrating the elution volume to 2.0 ml rather than 1.0 ml the loss of the extracted substrate is reduced to insignificant levels.

An estimate of phenanthrene solubility from the linear values reported in aqueous saturation studies (Table 2, page 17) is 1.79 mg l^{-1} when normalized to the 45% loss on concentration. This value compares with a solubility of 1.6 mg l^{-1} reported by Wodzinski and Johnson (49). It is apparent that phenanthrene recovery decreased significantly upon increasing the total phenanthrene column load from 100 µg to 200 µg.

These studies on the effects of soluble organic matter on substrate recovery by Tenax-GC extraction demonstrate the potential for effective recovery of phenanthrene in biodegradation assays. In addition, replicate variability was low which contributed to the sensitivity of the biodegradation assay technique. The results of these studies indicate the Tenax-GC extraction is a valuable tool for the analysis of residual phenanthrene substrate, following biodegradation, and suggests its potential use for other PAH substrates.

PART II: PHENANTHRENE BIODEGRADATION

I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are distributed throughout the environment, predominantly as a result of high-temperature pyrolytic processes (25, 44). Many of these aromatic compounds eventually enter aquatic systems in storm-water runoff from metropolitan areas as well as wastewater effluents from industrial processes utilizing fossil fuels (3, 33). Several studies have demonstrated the carcinogenic properties of PAH (10, 26) which may be considered a potential health risk to man upon long-term, chronic exposure. The transport and bioaccumulation of PAH in aquatic food webs (27, 36) may increase the potential hazard to humans.

The enzymatic pathways for microbial PAH degradation have been elucidated (7, 43) and several studies have demonstrated the ability of various microorganisms to mediate the decomposition of these compounds (9, 14). The primary objective of this investigation was the evaluation of the potential for significant PAH degradation by the indigenous microbial populations in freshwater environments.

II. LITERATURE REVIEW

Several investigators have demonstrated the microbial degradation of PAH (5, 9, 13, 14, 28, 29, 49). The enzymatic mechanisms of PAH degradation have been characterized in reviews (7, 43). The Beta-ketoadipate pathway, the common route of bacterial degradation of single-ring aromatic hydrocarbons, appears to be the major pathway of PAH degradation (43). Numerous bacterial isolates have inducible enzymatic systems capable of degrading smaller unsubstituted PAH. The initial enzymatic step in degradation of the aromatic nuclei is a substrate specific oxygenase linked to a reduced nicotinamide adenine dinucleotide (NADH) coenzyme system (14). The requirement of free oxygen by the oxygenase system indicates a limitation of PAH degradation in anaerobic environments. The substrate specificity of the oxygenases may allow the degradation of one of many possible PAH pollutants. Substitution on the ring structure may cause shifts in the enzymatic degradative processes (9, 15). The transformation of PAH by microorganisms may yield intermediates which are more toxic than the original pollutant.

Early investigations demonstrated bacteria with PAH degradation potential were present in various soils and sediments (43, 46). Several studies followed which investigated the biochemistry of PAH degradation by soil microorganisms (8, 13, 39). Other researchers used activated

sludge (34), sediment (22), and river water (32) samples instead of individual bacterial isolates to monitor biodegradation of lower molecular weight hydrocarbons. The results of these investigations indicate that larger PAH, structures with more than 3-rings, are more resistant to microbial degradation than the smaller structures such as naphthalene, anthracene and phenanthrene. Much of the biodegradation work has been directed at the study of enzymatic pathways by which chemically pure PAH are metabolized by pure cultures in a well defined cultural medium. The lack of standardization in assessment and the laboratory conditions employed in the biodegradation assays make the estimation of potential microbial degradation in the environment difficult. The development of an assay which would evaluate the PAH degradation potential of microorganisms in freshwater environment was a main objective of this investigation.

III. MATERIALS AND METHODS

Chemicals

Certified reagent grade phenanthrene and pyrene (Eastman Kodak Co., Rochester, NY) were employed as the model low molecular weight PAH for the biodegradation studies. Pesticide grade solvents, acetone, hexane, and petroleum ether (Fisher Scientific Co., Pittsburg, Penn.) 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 (Figure 2) which represented a relatively pristine aquatic system with no prior or apparent contamination by chronic sources of PAH input. A 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.

During 1977, samples from Center Hill Reservoir were taken from a depth of one meter using a Niskin sterile bag sampler (General Oceanics, Miami, Fla.) and maintained at ambient water temperature while being transported to Tech Aqua Biology Field Station where processing began within

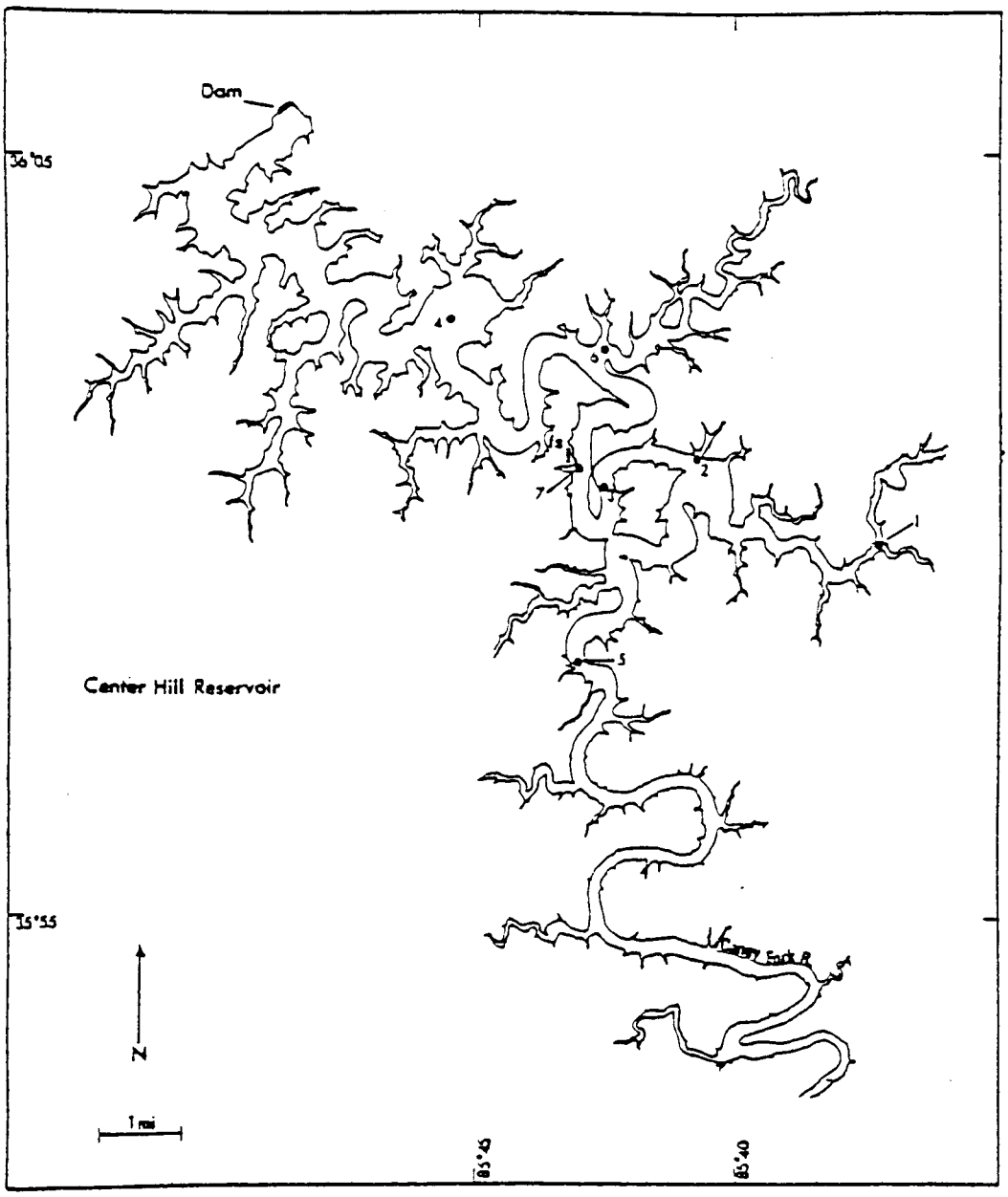


Figure 2. Field sampling site locations on Center Hill Reservoir, Cookeville, Tennessee. F.S. = field station.

one hour of sample collection. In subsequent studies in 1978, samples were collected by immersion of a 20 liter Nalgene container to 0.5 meters.

Field sampling variables assessed during the investigation included: dissolved oxygen (YSI DO meter, Model 51B, Yellow Springs Instrument Co., Yellow Springs, Ohio); water temperature (mercury filled glass thermometer); dissolved organic carbon (Beckman #215A TOC Analyzer, Beckman Instruments, Inc., Irvine, Calif.); nitrate (specific ion electrode, Orion); and orthophosphate was determined by the Vanadomolybdophosphoric acid method (2).

Total viable heterotrophic bacteria (TVC) and PAH-resistant and/or degrading microorganisms were determined by spread plating appropriate dilutions of each sample in autoclaved lake water onto yeast extract peptone glucose (YEPG) agar and phenanthrene (PHE) agar, respectively (41). Colony forming units were counted after two and four weeks' incubation at 25°C.

Measurement of PAH Biodegradation

One hundred ml water samples 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 psi for 20 minutes and checked for sterility periodically. 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 four weeks.

At the end of the incubation period, the contents of the batch culture flasks were filter extracted through Whatman #1 filter circles and a Tenax-GC, 2,6-diphenyl-p-phenylene oxide (Applied Science Labs, Inc., State College, Penn.) column (Figure 1, page 10). The Tenax-GC column was eluted with two 5.0 ml volumes of hexane and a 5.0 ml petroleum ether rinse between each sample extraction. After evaporative concentration of the column eluates and eluates of extracted filterable particulates to a 1.0 ml final volume, they were quantitified by flame ionization gas chromatographic analysis (GC-FID) and comparison to PAH standards. Biodegradation was determined by comparison of total PAH substrate recovery in the degradation flasks relative to the sterile control flasks.

Effect of Incubation Time, Temperature and PAH Concentration on Phenanthrene Biodegradation

Time course studies were conducted to determine the rate of phenanthrene biodegradation as well as the effect of incubation temperature and PAH substrate concentration on the rate and extent of phenanthrene biodegradation. In these studies the standard biodegradation assay was employed while the incubation times, temperatures or

phenanthrene concentrations were varied independently. Incubation times varied over a range of one to five days and thereafter weekly for one month. Incubation temperatures were varied over a range of 5°C to 45°C. In addition, the rate of phenanthrene degradation at 100 μg l^{-1}, and 1000 μg l^{-1} phenanthrene was examined.

Temporal and Spatial Biodegradation

Phenanthrene biodegradation assays were performed at Center Hill Reservoir sites during February, March, April, June, August, September, and December of 1977. These studies were performed to determine the variability and seasonal potential for PAH biodegradation among related samples. Physical, chemical, and microbiological characteristics of the sites were determined concurrently during sampling to determine those factors which accounted for the greatest variation in phenanthrene biodegradation.

Differences in the variation of phenanthrene degradation among sampling sites and sampling dates were examined using analysis of variance (ANOVA) and the contribution of physical, chemical and biological variables to phenanthrene degradation was determined by multiple regression analysis. Analyses were performed using SPSS (38) and 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 phenanthrene in freshwater sample of diverse types, a

study was undertaken to compare phenanthrene biodegradative capacities among Center Hill, Melton Hill, and Fort Loudoun Reservoirs. Within a period of one week in October, 1978, 20 liter grab samples were collected from sites on each reservoir and assayed for phenanthrene load (via, Tenax-GC extraction of five liters of sample and GC-FID quantification) and phenanthrene biodegradation. Triplicate control and biodegradation flasks for each site were subjected to short-term biodegradation assessment (five day) using a phenanthrene concentration of 1000 $\mu g\ l^{-1}$. Biodegradation was determined as previously described.

A comparative study was performed to measure the potential for pyrene biodegradation in Center Hill Reservoir. This study was performed to determine whether or not phenanthrene degradation was an isolated phenomenon and not representative of other PAH. Pyrene biodegradation studies were performed identically to those of phenanthrene biodegradation.

IV. RESULTS

Temporal and Spatial Biodegradation

Significant phenanthrene biodegradation was observed in all water samples collected from Center Hill Reservoir in 1977 (Table 4). The average temporal range in phenanthrene degradation was from 54.2 ± 6.6% to 93.4 ± 1.7%, August and December, respectively; while the average 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; while slightly lower levels of degradation were seen in the late summer months. The overall mean degradation was approximately 80% during a four-week incubation. This value is normalized relative to sterile control samples which exhibited a 45% loss of added phenanthrene. This loss is attributed to the analytical loss, also 45%, due to the concentrating of sample extracts prior to GLC analysis (Table 1, page 15).

In order to determine the significance and predictability of the inherent temporal and spatial variability of the relative phenanthrene biodegradation, it was necessary to statistically analyze the components contributing to variation. Analysis of the individual sites, sampling dates and rate of phenanthrene biodegradation suggested that there is no significant variation among

Table 4. Temporal and spatial variation in phenanthrene biodegradation[a] among Center Hill Reservoir Samples, 1977.

Site	Relative Degradation (%)[b]						
	Feb.	Mar.	Apr.	June	Aug.	Sept.	Dec.
Site #1	N.D.	65.86 ±13.65	91.75 ±0.80	85.51 ±3.75	N.D.	N.D.	N.D.
Site #2	100.00 ±0.00	90.25 ±9.47	80.39 ±4.44	79.76 ±9.78	N.D.	N.D.	N.D.
Site #3	N.D.	78.71 ±7.87	86.33 ±3.89	82.03 ±4.13	N.D.	N.D.	N.D.
Site #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
Site #5	55.52 ±17.38	79.45 ±1.59	85.20 ±4.58	84.61 ±5.74	N.D.	N.D.	N.D.
Site #6	N.D.	82.71 ±0.97	80.70 ±5.19	82.73 ±5.45	N.D.	N.D.	N.D.
Site #7	N.D.	N.D.	N.D.	N.D.	58.84 ±8.50	78.46 ±1.00	94.60 ±0.84

[a]Relative to sterile controls.

[b]Mean of three observations ± standard deviation.

N.D. = not determined.

sampling sites or dates at the 95% probability level (Table 5).

A multiple regression analysis was performed in which degradation was regressed on the physical, chemical, and biological variables for each sample examined (Table 6). The multiple regression analysis indicated that 46% of the total variation in phenanthrene degradation could be attributed to the individual site characteristics (Table 7). 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 was found to be insignificant at the 95% level. The regression coefficient for TVC 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 the regression of degradation on the independent variables was not significant at the 95% level. The regression analysis indicated approximately 25% of the total variation can be attributed to the two biological parameters, phenanthrene 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

Table 5. Analysis of variance in phenanthrene biodegradation by sample site[a] and date.

Source of Variation	Sum of Squares	d.f.	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.

Table 6. Center Hill Reservoir field site characteristics used in the multiple regression analysis of phenanthrene biodegradation.

Site	Date	PHE Degradation[a] (%)	TVC[b] (ml^{-1})	PHE Degrading[c] Bacteria (ml^{-1})	TEMP (C°)	DOC ($mg\ l^{-1}$)	PO$_4$ ($mg\ l^{-1}$)	NO$_3$ ($mg\ l^{-1}$)	SSED ($mg\ l^{-1}$)	DO ($mg\ l^{-1}$)	COND ($mg\ l^{-1}$)	TRNS (m)	PH
1	3-25-77	65.9	3.85	3.56	15.0	10	.05	.9	27.8	9.5	130	1.0	8.6
	4-22-77	91.8	5.43	3.96	19.3	-	.09	1.18	4.2	10.0	138	1.2	9.5
	6-9-77	85.5	4.57	2.90	27.5	-	0	.49	108.3	2.2	-	1.3	9.6
2	2-12-77	100	3.58	2.95	3.5	21	.02	.78	0.8	13.8	90	2.5	7.3
	3-25-77	90.3	4.26	3.20	15.0	11	.018	.9	24.6	10.3	105	1.0	8.3
	4-22-77	80.4	4.95	3.43	20.0	-	.027	2.5	9.1	9.4	90	.5	8.5
	6-9-77	79.8	4.52	2.73	27.0	-	.01	.62	112.3	-	-	1.8	9.4
3	3-25-77	78.7	4.15	2.60	16.0	8.0	-	.9	28.3	10.5	100	.8	8.6
	4-22-77	86.3	4.88	3.79	18.0	-	.055	2.66	3.6	9.4	90	1.3	8.8
	6-9-77	82.0	4.54	2.11	28.0	-	-	.72	37.7	-	-	1.7	9.2
4	2-12-77	93.7	3.53	2.11	3.9	50	.018	.92	1.3	13.0	90	4.0	7.8
	3-25-77	77.3	4.62	2.69	11.5	7.0	.015	.9	24.4	10.5	95	1.5	8.3
	4-22-77	82.8	-	-	-	-	.028	4.25	6.5	-	-	-	-
	6-9-77	85.8	4.79	2.30	25.5	-	.040	.80	105.3	-	115	2.0	8.2
	8-19-77	49.6	3.54	2.15	30.0	-	.094	-	3.8	7.2	131	3.0	7.7
	9-15-77	67.6	2.48	1.00	28.5	-	-	-	-	7.8	160	3.4	6.4
	12-19-77	92.3	3.36	2.60	13.0	-	-	-	-	13.6	80	0.6	-
5	2-12-77	55.5	3.58	2.92	4.5	32	.02	.70	1.4	13.2	80	3.2	7.1
	3-25-77	79.5	4.23	3.15	12.0	8.0	.02	.9	37.3	10.4	80	0.5	8.0
	4-22-77	85.2	4.95	3.43	20.0	-	.05	2.42	.47	9.4	115	1.3	-
	6-9-77	84.6	4.79	2.30	26.5	-	-	-	-	-	160	-	-
6	3-25-77	82.7	4.15	3.53	14.5	8.0	.048	.9	34.0	11.0	103	1.3	8.4
	4-22-77	80.7	-	-	-	-	.043	2.49	13.7	11.0	-	-	-
	6-9-77	82.7	4.57	2.91	26.5	-	.030	.95	124.7	-	128	2.0	9.3
7	6-9-77	58.8	-	-	29.0	-	-	-	-	-	140	-	-
	8-19-77	78.5	3.38	3.59	29.0	-	0	-	2.9	7.6	131	2.5	8.4
	9-15-77	94.6	2.54	1.90	27.5	-	-	-	-	8.2	290	1.9	-
	12-9-77	77.3	3.77	2.88	11.5	-	-	-	-	10.5	100	-	-

[a]Relative to sterile control sample.

[b]Log 10 total viable heterotroph count.

[c]Log 10 total phenanthrene resistant or degrading bacteria.

Table 7. Multiple regression analysis of phenanthrene biodegradation[a] on physical, chemical and microbiological variables characteristic of Center Hill Reservoir samples.

Independent Variable	Multiple Coeff. of Determination (r^2)	Change in (r^2)	Simple Correlation Coefficient (r)	Regression Coefficient (b)	Fb
pH (X_1)	.0007	.0007	-.0272	-.89	.784
TRAN (X_2)	.0153	.0146	-.1148	-.45	.031
COND (X_3)	.0195	.0042	-.0484	-.35	.502
DO (X_4)	.0765	.0569	-.0233	-.67	0.000
SSED (X_5)	.1224	.0549	.0681	.52	.429
NO_3 (X_6)	.1789	.0566	.1597	.15	.961
PO_4 (X_7)	.1870	.0081	.2332	-.14	.641
DOC (X_8)	.1936	.0065	.0611	-.21	.004
TEMP (X_9)	.2076	.0140	-.2090	-.63	1.535
PHE-Deg (X_{10})	.3435	.1358	.2292	-.42	.508
TVC (X_{11})	.4614	.1178	.2555	.81	3.505
Y constant	--	--	--	.780	

[a]Biodegradation relative to sterile control samples, triplicate analysis for each sample collected.

[b]Critical F statistic at 95% confidence level = 4.54.

influence on degradation of suspended sediment loads ($r = 0.06$) as compared to phenanthrene resistant organisms ($r = 0.23$) and TVC ($r = 0.26$). This information would indicate that the degradation responses are biological and not merely an irreversible partitioning or binding of phenanthrene to particulate matter.

Center Hill Reservoir samples in June, July, and September of 1977 were assayed for pyrene as well as phenanthrene degradation (Table 8). In all cases the relative pyrene degradation was significantly less than that observed with phenanthrene. The mean pyrene biodegradation was 16.7% as compared to 75% mean phenanthrene degradation in the same samples.

Effect of Incubation Time, Temperature and Phenanthrene Concentration on Phenanthrene Biodegradation

It was apparent that individual sites' characteristics were partially responsible for variability among rates of phenanthrene biodegradation. It was felt that the arbitrary condition 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 in order to more fully understand seasonal and temporal phenanthrene biodegradation.

Table 8. Comparative phenanthrene and pyrene batch culture biodegradation[a] in Center Hill Reservoir samples, 1977.

Date	Site	Phenanthrene Degradation[b]	Pyrene Degradation[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; phenanthrene and pyrene concentrations 1000 µg l^{-1}.

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

A time course estimation of phenanthrene biodegradation in samples collected from Center Hill Reservoir indicated the potential differences in phenanthrene biodegradation rates were masked by prolonged incubation (Tables 9 and 10). Biodegradation responses for both sites 4 and 7 had reached their maximum, ca. 90%, within the first week of incubation (Table 10). The rate of phenanthrene biodegradation at site 7 progressively increased to a relative 60.8% and 46.1% degradation within five days at 100 µg l^{-1} and 1000 µg l^{-1} phenanthrene, respectively (Table 9).

The effect of incubation temperature was even more pronounced than the effect of incubation time as indicated by the temperature dependent biodegradation response for site 7 samples dosed with either 100 µg l^{-1} or 1000 µg l^{-1} phenanthrene and incubated at different temperatures for five days (Table 11). Phenanthrene biodegradation was virtually not detected at either end of the temperature extremes (5°C and 45°C) after the incubation period for either substrate concentration. Biodegradation was, however, directly related to incubation temperature between 15°C and 37°C with maximum rates of biodegradation approaching 90% at 37°C (at 1000 µg l^{-1} phenanthrene).

Five day phenanthrene biodegradation assays at 25°C employing concentrations of 100 µg l^{-1} and 1000 µg l^{-1} suggest that degradative rates are themselves dependent

Table 9. Comparative time courses of phenanthrene biodegradation[a] in site 4, Center Hill Reservoir samples.

Time of Incubation	Percent Degradation[b]	
	100 $\mu g l^{-1}$	1000 $\mu g l^{-1}$
1 Day	0	2.2 ± 5.2
2 Days	N.D.	13.5 ± 3.7
3 Days	66.4 ± 5.9	39.6 ± 9.6
4 Days	N.D.	33.8 ± 1.0
5 Days	60.8 ± 3.7	46.1 ± 6.5
6 Days	N.D.	N.D.
7 Days	43.9 ± 18.7	N.D.

[a]Relative to sterile controls.

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

N.D. = not determined.

Table 10. Effect of incubation time on phenanthrene biodegradation[a] in Center Hill Reservoir samples.

Time of Incubation	Percent Degradation[b]	
	Site #4	Site #7
1 Week	90.12 ± 2.01	86.70 ± 4.11
2 Weeks	90.36 ± 4.63	95.04 ± 0.72
3 Weeks	94.02 ± 2.91	93.55 ± 0.81
4 Weeks	94.81 ± 1.64	90.84 ± 1.79

[a]Relative to sterile controls; phenanthrene concentration, 1000 $\mu g l^{-1}$.

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

Table 11. Effect of incubation temperature on the rate of phenanthrene biodegradation[a] in site 4, Center Hill Reservoir samples.

Incubation Temperature	Percent Degradation[b]	
	100 $\mu g l^{-1}$	1000 $\mu g l^{-1}$
5°C	0	0
15°C	N.D.	10.0 ± 6.3
20°C	N.D.	37.8 ± 7.1
25°C	60.8 ± 3.7	47.1 ± 11.6
37°C	N.D.	89.0 ± 7.8
45°C	0	0

[a]Relative to sterile controls.

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

N.D. = not determined.

upon phenanthrene concentrations. Upon increasing the concentration ten fold (100 µg l^{-1} to 1000 µg l^{-1}), a three fold greater increase in the relative rate of phenanthrene biodegradation was observed (12.1 ± 1.2 µg l^{-1} day^{-1} and 34.4 ± 8.3 µg l^{-1} day^{-1}, respectively; these data are normalized to 100% analytical recovery).

In October, 1978 the biodegradation potentials of samples from Center Hill, Melton Hill and Fort Loudoun Reservoirs were compared during a five-day incubation (Table 12). Significantly greater biodegradation was observed in the Melton Hill and Fort Loudoun Reservoir samples as compared to the Center Hill samples which indicated that those samples with a history of higher levels of enrichment and/or pollution exhibited a greater capacity for PAH biodegradation. More extensive comparative assessments of biodegradation are necessary to establish the validity of this trend. Five liters of water from each of the three sites were extracted via a 10 cm x 1 cm Tenax-GC column and analyzed for background phenanthrene levels. None of the three samples contained phenanthrene residues within the limits of detection (1.0 µg l^{-1}) of the gas chromatographic method employed for analysis.

Table 12. Comparative phenanthrene biodegradation[a] among Tennessee reservoirs.

Site	Percent Degradation[b]
Center Hill Reservoir	17.2 ± 18.4
Melton Hill Reservoir	60.8 ± 17.0
Ft. Loudon Reservoir	83.9 ± 13.7

[a]Relative to sterile controls for each site.

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

V. 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 (13, 28). This conclusion is based on the observed loss of phenanthrene, relative to sterile controls, in biodegradation assays. The microbial nature of the phenanthrene 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 phenanthrene degrading and/or resistant populations. 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 phenanthrene biodegradation capacity was observed in all samples from three varied freshwater systems. In addition, the temperature response profile of the biodegradation assay clearly indicated phenanthrene loss was a result of 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 measurement at 25°C supports this since samples collected during winter months exhibited phenanthrene degradation potentials as high as those collected during warmer weather.

Additional insights into PAH decomposition resulted from the biodegradation assays of this investigation. Increased capacity for phenanthrene biodegradation was observed in Melton Hill and Fort Loudoun Reservoirs compared to Center Hill Reservoir which indicated that samples with a historical background of enrichment and/or pollution to have an increased capacity for PAH biodegradation. Although data for PAH concentrations and the numbers of PAH degrading microorganisms in all three reservoirs are unavailable, 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 (47).

Comparison of phenanthrene and pyrene biodegradation in Center Hill Reservoir water samples indicated phenanthrene was more readily subject to microbial decomposition. These results agree with a recent study

which suggested that PAH are more resistant to microbial transformation as the number of rings in the compound is increased (22).

These studies indicate that lower molecular weight PAH such as phenanthrene are subject to degradation in surface waters of aquatic environments. Furthermore, some aquatic systems exhibit a greater potential for accommodation of PAH contamination which may be useful information in environmental impact analysis.

CONCLUSIONS

This investigation demonstrates the potential for significant degradation of phenanthrene in natural aquatic environments. The data indicate the lower molecular weight PAH such as phenanthrene, a three-ring structure, are more readily degraded than larger PAH such as pyrene, a four-ring compound. A history of pollution in an aquatic system appears to enhance the capacity for PAH degradation which might be important in predicting the environmental fate of PAH contaminants.

This investigation also demonstrates the efficiency of the Tenax-GC extraction technique for phenanthrene analysis in biodegradation measurement and may be applicable to other PAH pollutants. The biodegradation assay described in this study may prove to be an appropriate method for evaluation of the potential for microbial degradation of other organic pollutants of aquatic systems.

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