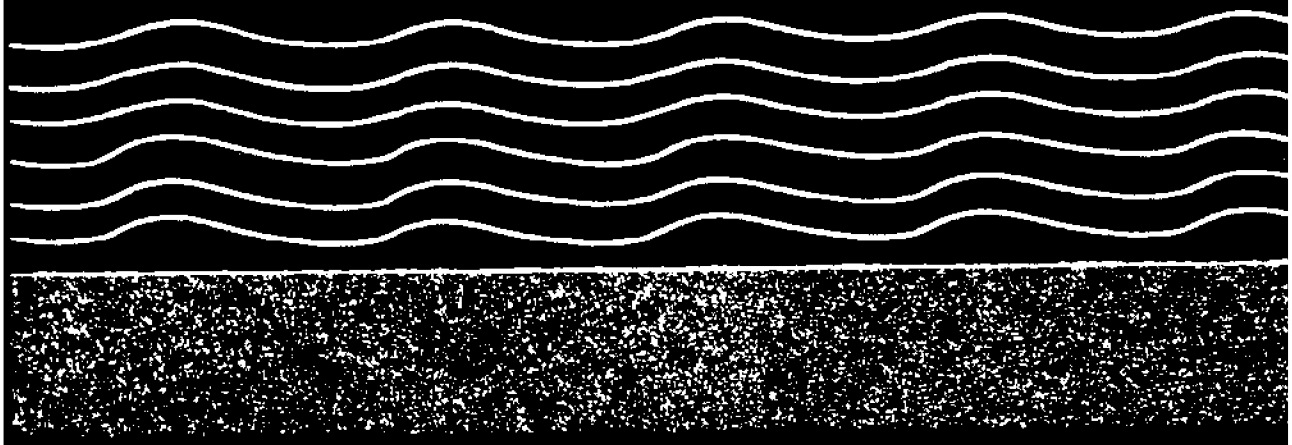
Polycyclic Aromatic Hydrocarbons In Chesapeake Bay: A System for Monitoring Levels In Water, Sediments, and Shellfish And for Estimating Potential Hazards

By Howard DeVoe and Mary Voll



Technical Report
Maryland Sea Grant Program
H.J. Patterson Hall, Room 1224
College Park, Maryland 20742

Publication Number
UM-SG-TS-80-05

POLYCYCLIC AROMATIC HYDROCARBONS IN CHESAPEAKE BAY: A SYSTEM FOR MONITORING LEVELS IN WATER, SEDIMENTS AND SHELLFISH AND FOR ESTIMATING POTENTIAL HAZARDS

by
Howard DeVoe
Department of Chemistry
and
Mary Voll
Department of Microbiology
University of Maryland

Report Date: August, 1980

Copies of this publication are available from:

Sea Grant Program
University of Maryland
1224 H. J. Patterson Hall
College Park, MD 20742

UM-SG-TS-80-05

The publication of this technical report is made possible by grant #NA80AAD-00013, awarded by the National Oceanic and Atmospheric Administration to the University of Maryland Sea Grant Program.

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PREFACE

Polycyclic aromatic hydrocarbons--PAH's--are a recalcitrant class of hydrocarbons not amenable to evaporation or microbial degradation in natural water systems, a residue of the petroleum products spilled into the marine environment. Some compounds of this class have been shown to induce cancer in laboratory animals. Shellfish, already shown to concentrate PAH's taken up in filter feeding from hydrocarbon-polluted waters, could pose a public health hazard as carriers of carcinogenic PAH compounds.

In this project, chemist Howard DeVoe extracted PAH fractions from oyster tissue and from the thick black sediment of the Colgate Creek section of the Baltimore Harbor in Chesapeake Bay, then measured the PAH levels in the samples by high-pressure liquid chromatography. Microbiologist Mary Voll, adapting the standard bacterial assay for detecting carcinogens developed by Ames, measured the ability of the PAH's extracted to induce mutations in bacteria, a characteristic strongly correlated with cancer induction.

DeVoe's procedure for isolating PAH's from sediments and oysters is simpler and faster than others described in the literature. First, he treated wet sediment and oyster samples with anhydrous sodium sulfate to tie up the water, then extracted the resulting dry powder with hexane. But hexane extracts from the sediment samples contained a yellow oil; oyster extracts retained their hexane-extractable lipids. Though

published methods for extracting PAH's from oysters, for example, describe time-consuming saponification of the oyster lipids, Devoe tried passing the hexane extracts of both sediment and oyster samples first through a column of silica gel to adsorb polar pigments and lipids, and then through a gel permeation column to separate the PAH's from nonpolar substances of higher molecular weight. With this procedure he was able to obtain a cleaned-up preparation of PAH's--ready for chromatographic analysis--in just a few hours.

Promutagenic, procarcinogenic PAH compounds pile up in the sediments of harbors and in other waters where hydrocarbon-rich wastes are discharged. Shellfish take them up without apparent harm. Only when they are ingested by mammals and converted by metabolic enzymes to biological derivatives, do they become active. Once activated, they cause frameshift changes in the structure of DNA: mutation.

After activating the PAH fractions with rodent liver homogenate, Voll scored growth colonies of Salmonella typhiminium TA 100 against a spontaneous mutant control assay. At least four of the PAH's identified by chromatogram caused mutations in the bacteria strain. DeVoe tentatively identified one of these as benzo(a)-pyrene, a five-ring PAH compound well known to be carcinogenic.

By spiking oyster flesh with PAH compounds, then extracting and measuring the recovery, DeVoe demonstrated that PAH's can be recovered from contaminated oysters even though large amounts of lipids must be separated out. Next,

he grew oysters on PAH-rich Colgate Creek sediment and on Eastern Bay sediment transported to the laboratory. Oysters were grown on clean sand as a control. No PAH's were found in the laboratory-grown oysters. Perhaps the layer of sand used to keep the sediments in place prevented uptake of the PAH compounds known to be present in the Colgate Creek sediment.

Of the large class of PAH compounds, only some, converted by enzymes, become carcinogenic. By combining the shortened analytical procedure for isolating and measuring PAH compounds with the Ames method for singling out those that are biologically active, DeVoe and Voll have developed a useable system to test for hazardous PAH compounds in natural waters.

The Editors

INTRODUCTION

A number of polycyclic aromatic hydrocarbons (PAH's) are known to be potent carcinogens, as well as to cause mutagenic activity. Some well-known examples are the four-ring compound, benz(a)anthracene, and the five-ring compounds, benzo(a)pyrene and dibenz(a,h)anthracene. In Chesapeake Bay, potential sources of PAH's such as these include industrial discharges, dredge-spoil material taken from Baltimore Harbor, and petroleum products carried by shipping.

That shellfish are able to take up PAH's from the environment is well documented. Cahnmann and Kuratsune (1957) measured a level of 2 - 6 ppb of benzo(a)pyrene in oysters collected from an area that was moderately polluted with petroleum (1 ppb is 1 μg per kg of wet flesh). Benzo(a)pyrene levels of 45 ppb and 26 ppb were found in mussels obtained from contaminated areas by Dunn and Stich (1976) and by Mix and Schaffer (1979) respectively. Lee et al. (1978) reported the extremely high benzo(a)-pyrene level of 360 ppb in oysters which had been suspended for two days in seawater containing a dispersion of benzo(a)pyrene (and other PAH's) dissolved in crude oil.

If shellfish are able to concentrate PAH's from water and from the particulate matter and plankton which they take up as filter feeders, then shellfish collected from polluted areas could pose a public health threat because of the carcinogenic character of some of the PAH's they retain.

In the first phase of the project, a simple analytical procedure for isolating and measuring polycyclic aromatic hydrocarbons in environmental samples was developed and used for preparing PAH fractions from two samples of sediment collected from the highly polluted Colgate Creek area of Baltimore Harbor. The system developed by Ames and his colleagues that uses bacteria as test organisms was employed to assay these fractions for mutagenicity. Mutagenicity is known to be strongly correlated with carcinogenicity. The analytical procedure was used for oyster samples too, and a spiking experiment demonstrated that recovery of PAH's added to a hexane extract of oyster flesh was 25% for fluoranthene, 75% for benz(a)anthracene, 85% for benzo(a)pyrene, and 103% for dibenz(a,h)-anthracene.

Further work attempted to measure PAH's taken up by oysters grown on polluted sediment. The purpose of this work was to demonstrate the utility of the analytical procedure for analyzing PAH's in oysters contaminated by industrial discharges, petroleum spills, and other potential sources of pollution.

MATERIALS AND METHODS

Sample Collection

Sediment samples. Samples of sediment collected in the spring of 1977 from Eastern Bay (an uncontaminated area) and from the Colgate Creek area of Baltimore Harbor (highly polluted) were supplied by the Marine Microbiology Laboratory of the Department of Microbiology,

University of Maryland. Other sediment samples were collected by grab sampler from the research vessel Ridgely Warfield in April, 1978, at the mouth of the Chester River, at Colgate Creek site A (10 1/2 m off the west shore from a bulkhead at the Western Electric Company outdoor cable storage area), and Colgate Creek site B (about 50 m offshore from the same bulkhead). These samples were kept frozen until ready for use.

Oyster samples. Oysters supplied by the Horn Point Environmental Laboratories had been collected on December 6, 1977, from the Green Marsh oyster bar, Choptank River. This bar is located about 1 1/4 mile downstream from the outfall of the Cambridge sewage treatment plant, and is closed to commercial harvesting. The oysters were refrigerated for 7 days before being shucked and frozen.

Analytical Procedure.

The procedure developed to analyze PAH's in sediments and oysters is simpler and more rapid to carry out than any others described in the literature. For oysters, published methods involve time-consuming saponification of the oyster lipids.

High pressure liquid chromatography on reversed phase columns best separates the PAH's from one another. In this method, a solution of the PAH mixture in methanol or acetonitrile is pumped through a column containing extremely small (10 micrometers) silica particles to which oil-like molecules have been bonded. Because the PAH molecules are much more soluble in the

oil-like material than in the solution, they are retained by the column for varying lengths of time, depending on their molecular structure. In this way a separation of the different PAH's is made.

Obtaining the PAH solution for use in the high pressure liquid chromatography presents two basic problems: first, the PAH's must be extracted from the sediment or oyster flesh--they must be moved from a water-like environment into a nonaqueous, nonpolar solvent; second, other nonpolar substances such as lipids must be separated from the PAH's to obtain a homogenous solution in methanol or acetonitrile. The procedure developed handles the first problem by treating the wet sample (sediment or oyster flesh) with anhydrous sodium sulfate to tie up the water as a solid hydrate. A dry granular powder results which can easily be extracted with hexane. The second problem is overcome by passing the extract first through a column of silica gel to absorb polar pigments and partially-polar lipids and then through a gel permeation column that separates the PAH's from nonpolar substances of higher molecular weight. By this procedure, a cleaned-up preparation of PAH's dissolved in methanol, ready for the final high pressure liquid chromatography analysis, can be obtained in several hours.

Extraction and cleanup. A portion (40 - 60 g) of each of the wet sediments collected was mixed with an equal mass of anhydrous Na_2SO_4 to dessicate the sample. The dessicated sample was pulverized in a glass mortar, placed in a glass column, and extracted with hexane (usually about

160 ml) to produce a yellow extract which fluoresced under ultraviolet light. The extract was evaporated and the resulting residue treated with 1 ml acetonitrile to dissolve nonpolar constituents. The acetonitrile solution was analyzed by high pressure liquid chromatography as described below.

Because the residue from the hexane extract included a yellow oil which did not dissolve appreciably in the final acetonitrile solution, it is possible that PAH's were not completely transferred into the acetonitrile phase. Two avoid this problem, a simple cleanup procedure was devised to separate the PAH's from other hexane-extractable substances. The same cleanup procedure was used for hexane extracts of the oyster samples, in which the hexane-extractable lipids would otherwise cause the same problem.

For a sediment sample, the wet sediment was mixed with an equal mass or more of anhydrous Na_2SO_4 to desiccate the sample. The mixture was cooled in a freezer and periodically cut into chunks as the solid decahydrate formed at the peritectic temperature. The resulting granular material was pulerized in a glass mortar and swirled with several portions of hexane. The extract was filtered and concentrated to a volume of about 20 ml. Any crystals which formed were filtered off.

For an oyster sample, the drained flesh was mixed with an equal mass or more of anhydrous Na_2SO_4 and cooled, and the mixture macerated as the decahydrate formed. This resulted in a loose granular material resembling light brown sugar in appearance. The material was ground in

a mortar to a fine powder and shreds of desiccated flesh, and extracted with several portions of hexane.

The hexane extracted from either a sediment sample or an oyster sample was placed on a silica gel column for separation on the basis of polarity. The column contained 15 g of silicia gel deactivated with 5% water by weight, washed into a 15-mm ID glass column with hexane, with a layer of Na_2SO_4 on top. Elution was begun with hexane, and continued with benzene after 60 ml of effluent had come through. The effluent at the interface between the two solvents was collected and evaporated in a rotary evaporator, with care being taken to avoid heating the flask at the end of the evaporation.

The residue was dissolved with gentle heating in 2 ml of a benzene-methanol mixture (equal parts by volume) and placed on a gel filtration column for separation on the basis of molecular size. This column contained Sephadex LH-20 beads, 25 - 100 μm size (Sigma), swollen in the benzene-methanol mixture and packed to a height of 23 cm in a 15-mm ID glass column. Elution was carried out with the benzene-methanol mixture. The first 30 ml of effluent, containing high molecular weight material, was discarded and the next 21 ml was collected. (Elution was then continued to remove remaining material from the column so that it could be reused.) The collected effluent was evaporated to dryness in a rotary evaporator, with no heating at the end of the evaporation, and the residue was dissolved in 1 ml of methanol or acetonitrile. This solution was filtered with a polycarbonate membrane filter (Nuclepore, 0.6 μm pores) held in

a Swinney adapter and injected into the sample loop of the high pressure liquid chromatograph for analysis.

High pressure liquid chromatography. The high pressure liquid chromatograph was assembled from components purchased from various manufacturers. The apparatus consisted of two high pressure pumps (Milton-Roy) with inlet filters, a 3000-psi sample valve (Valco) to which was attached either a 20- µl sample loop (nominal volume) used for analytical runs or a 130 µl sample loop used when fractions were collected, an analytical column packed with a 10- µm reverse-phase packing, an ultraviolet absorbance detector (Waters model 440) with 1-cm pathlength cells detecting simultaneously at wavelengths of 254 nm and 280 nm, and two 10-mv chart recorders (Houston). The analytical column was initially a Waters µBondapak-C18 column; this was later replaced with a Whatman Partisil-10 ODS-2 column.

The solvent for elution was a mixture of methanol and water. The methanol was analytical reagent grade, redistilled in glass. The water was distilled in glass, then pumped through a reverse-phase packing (Waters µBondapak-C18) in order to remove trace organic substances. The flow rate during a run was 2 ml/min. The initial solvent composition was 70% methanol (by volume) for runs using the Waters column and 80% methanol for runs with the Whatman column. The solvent composition was changed exponentially with time by pumping the solvent from an 80-ml volume contained in a stirred reservoir into which methanol was pumped at the same flow rate (2 ml/min). A complete run took about 45 min to

perform. At the end of each run, methanol was pumped through the analytical column in order to elute any tightly bound substances.

Fractions for the biological assays were collected in screw-cap conical tubes and evaporated in a bath of boiling water and ethylene glycol, using Teflon boiling stones (Chemplast, previously rinsed in methanol) to prevent bumping.

Assay for mutagenicity. Among chemicals there is a strong correlation between carcinogenicity, the ability to induce cancer, and mutagenicity, the ability to cause mutation. An assay system developed by Ames and his colleagues (Ames, McCann and Yamasaki, 1975) that uses bacteria as the test organisms directly assays compounds for mutagenicity and is often used to screen chemicals for potential carcinogenicity. The Ames system is quick, inexpensive, and very sensitive.

Chromatogram peak fractions were assayed for mutation-inducing ability in the Ames bacterial assay system. This system employs strains of Salmonella typhimurium, which are mutant in histidine biosynthesis and have other genetic alterations that enhance their sensitivity to mutagenesis by chemicals. The strains mutate to histidine independence at a low spontaneous rate which can be increased by exposure to mutagenic chemicals. The number of cells mutating to histidine independence is readily scored on solidified growth medium lacking histidine. Mutagenic activity is detected in the system as an increase in the number of such mutants above that which occurs spontaneously.

Biologically active PAH compounds are promutagens (Ames, Durston, Yamasaki and Lee, 1973) and procarcinogens (Heidelberger, 1975). They are not active in themselves but are converted to active derivatives by mammalian enzyme systems. The active derivatives cause mutation in bacteria by addition or deletion of one to several consecutive bases in DNA (frameshift mutations) (Ames, Sims and Grover, 1972). Those PAH compounds which are converted metabolically into carcinogenic derivatives can be detected by their mutagenic activity in the Ames system provided they are exposed to an enzyme preparation derived from rodent liver. Mutagenicity of benzo(a)pyrene in quantities as little as 1 µg can be readily detected using liver enzymes and an especially sensitive strain of bacteria, Salmonella typhimuium TA100 (McCann, Choi, Yamasaki, and Ames, 1975). The strain was obtained directly from Ames and was periodically tested for retention of relevant genetic characteristics as described by Ames, McCann and Yamasaki (1975).

Source animals for liver homogenate S-9 fraction were male Sprague-Dawley rats (Flow Laboratories, Inc.) or male strain C57BL/6J mice (Jackson Laboratories). Rats were injected interperitoneally with Aroclor (a mixture of polycholorinated biphenyls) (0.5 ml of a 200 mg/ml solution per 200 g of body weight) six days prior to sacrifice (Ames, McCann and Yamasaki, 1975). Mice were similarly injected with Aroclor (0.3 ml of a 40 mg/ml solution per 25 g body weight) four days prior to sacrifice. Livers were extracted and S-9 fractions prepared as described by Ames, McCann and Yamasaki (1975). Both the rat and mouse S-9 fractions showed high activating activity for PAH's.

Chromatographic PAH samples, evaporated to dryness, were dissolved in dimethylsulfoxide (DMSO) for assay in the Ames system. The direct incorporation assay of Ames was employed (Ames, McCann and Yamasaki, 1979). TA100 cells (0.10 ml of culture), chromatogram fraction, and 0.50 ml of S-9 mix were added to soft molten agar and overlaid on the surface of a minimal agar plate. S-9 mix consists of S-9 fraction in a buffer solution containing necessary co-factors. Plates were incubated for two days and then scored for colonies.

Spontaneous mutant control assay contained TA100 cells, S-9 mix, and an amount of DMSO approximating that added to the assay with the samples. The S-9 sterility assay contained S-9 mix alone. The sample sterility assay contained chromatogram fraction alone. The known mutagenic PAH, benzo(a)pyrene (Gold Label, Aldrich Chemical Co., Inc.), was run in each assay experiment as a control for the assay system. To test whether the fractions might inhibit the activity of the S-9 liver enzymes, assays containing benzo(a)pyrene, chromatogram fraction, TA100 cells, and S-9 mix were run.

The number of mutants induced by the chromatogram fractions or by benzo(a)pyrene was obtained by subtracting the number of mutants obtained in the spontaneous mutant control assay from the number of mutants obtained in the complete assay.

PAH Standards

Stock solutions of the following PAH's were prepared in hexane: fluoranthene (Aldrich, 98%); benz(a)anthracene (Sigma, grade II); benzo(a)pyrene (Aldrich Gold Label); dibenz-(a,h)anthracene (Aldrich, 97%). These stock solutions were diluted 100-fold with methanol in order to obtain a mixture of the PAH's at concentrations of about 3 μg/ml (except dibenzanthracene which was about 1 μg/ml). The methanol solution of the PAH was injected into the 20 μl sample loop of the chromatograph, with the results shown in Table 1. The retention times and the ratios A_{280}/A_{254} were useful in identifying the possible presence of these PAH's in samples; the concentration factor c/Aw could be used to estimate the concentration of a PAH in a sample from the peak area.

Analysis

Sediment Analysis. Figures 1 and 2 are tracings of chromatograms obtained from two different samples of sediment from Colgate Creek. The conditions differed in two important respects: the first chromatogram (Figure 1) is of a sample which was not subjected to the cleanup procedure but was analyzed using the Waters analytical column, whereas the second chromatogram (Figure 2) is a sample which went through the cleanup procedure and was analyzed using the Whatman analytical column.

The peaks appearing on these chromatograms are labelled by arbitrary letters of the alphabet, as indicated in Figures 1 and 2. (Peaks A and B as originally labelled were later found to

TABLE 1. High pressure liquid chromatography of PAH mixture.

PAH[a]	Retention times (min)		A_{280}/A_{254}[b]	c/Aw(mg/ml min) [c]
	Waters column	Whatman column		
Fl	9.4	11.2	1.40	0.63
BA	13.3	16.7	1.59	0.31
BaP	17.3	25.6	0.77	0.28
diBA	20.4	30.3	5.5	1.4

(a) Abbreviations: Fl, fluoranthene; BA, benz(a)anthracene; BaP, Benzo(a)pyrene; diBA, dibenz(a,h)anthracene.

(b) Ratio of absorbances at peak maxima, measured at wavelengths of 280 nm and 254 nm, Waters column.

(c) c = mass concentration of PAH in mixture; A = absorbance at peak maximum, 254 nm, Waters column; w = width of peak in time units. The width is obtained by extrapolating the sides of the peak at the inflection points to the peak base line. For a Gaussian band shape, w is equal to 4 times the standard deviation of the peak.

TABLE 2. Chromatograms of Colgate Creek sediment; 20 µl sample loop.

chromatogram	Figure 1	Figure 2
source of sediment	Colgate Creek, June, 1977	Colgate Creek, April, 1978, site B
wet sediment mass	60 g	53 g
cleanup procedure	no	yes
analytical column	Waters µBondapak-C18	Whatman Partis 10 ODS-2

peak	PAH	retention time	A_{254} (c)	retention time (min)	A_{254} (c)	A_{28} / A_{25}
b	phenanthrene	8.2	0.03	5.9	0.02	0.1
c	anthracene	8.8	0.05	8.4	0.01	0.2
d		(a)		9.2	0.02	0.3
e	fluoranthene	10.4	0.08	11.4	0.07	1.1
f	pyrene	11.0	0.08	12.6	0.02	0.5
A-1		14.1	0.12 (d)	15.5	0.11	0.2
A-2	benz(a)anthracene	14.5	0.12 (d)	17.1	0.15	0.8
A-3		15.2 (b)	0.07	19.2	0.04	0.3
x		(a)		20.4	0.06	0.2
y		(a)		22.1	0.02	0.4
B-1		17.2	0.27 (d)	23.4	0.10	0.8
B-2	benzo(a)pyrene	18.4 (b)	0.05	26.0	0.05	0.7
C		22.6	0.07	34.4	0.04	0.6
D		26.2	0.02	39.2	0.01	0.7
F		32.2	0.08	(e)		

(a) peak missing

(b) shoulder

(c) absorbance maximum of peak, referred to baseline of peak

(d) overlapping peaks

(e) Chromatogram was not carried out as far as peak F; other chromatograms showed peak F is eliminated by the cleanup procedure.

consist of several components; these components have been labelled A-1, A-2, etc.).

The retention times of the peaks were longer on the Whatman column than on the Waters column. The retention times were therefore correlated with the help of the chromatograms of the PAH mixture (Table 1) in order to be able to use the same label for peaks containing the same substance.

The resolution of the peaks is seen to be better with the Whatman column (Figure 2) than with the Waters column (Figure 1). In Figure 2, bands A-1, A-2 and A-3 are separated from one another, and bands B-1 and B-2 are fully resolved. And two new peaks, labelled x and y, appear in the Whatman column chromatogram. The main result of the cleanup procedure as far as these Colgate Creek sediment samples is concerned was a reduction of peak B-1 and the elimination of peak F (Table 2). The cleanup procedure unfortunately did not prevent the gradual rise seen in the baseline of the chromatograms. This rise became a "hump" in chromatograms obtained with the larger 130 µl sample loop; the baseline "hump" reached a maximum in the vicinity of peak B.

Ultraviolet absorption spectra of collected fractions from several chromatograms were measured with a Cary model 14 spectrophotometer. The wavelengths of absorption maxima in the spectra of fractions from six of the chromatographic peaks of Colgate Creek sediment enabled these peaks to be assigned to particular PAH's, as listed in Table 2. The retention times and A_{280}/A_{254} ratios for PAH's listed in Table 1

were also used in making the assignments. There was no indication of the presence of dibenz-(a,h)anthracene in the Colgate Creek extracts.

The absorption spectra of peaks A-1 through D from Colgate Creek sediment were dominated by a broad absorption maximum at about 258 nm. The spectra of PAH's listed in Table 2 were superimposed on this maximum. The material absorbing at 258 nm may possibly be associated with the "hump" observed in the baseline of chromatograms of Colgate Creek sediment. Unpolluted sediments (Eastern Bay and Chester River) gave chromatograms which lacked the "hump"; the largest peak in these chromatograms was at the position of peak B-1, but the absorption maximum of the collected fraction from this peak (Eastern Bay sediment) was at 264 nm instead of at 258 nm as in the case of Colgate Creek sediment.

The hexane extracts of the various desiccated sediment samples, including those from unpolluted sediments, when evaporated, were found to contain white crystals and yellow oil. The white crytals were sparingly soluble in hexane and easily soluble in benzene. They melted at 120°C, and gave solutions in organic solvents that were nonfluorescent in ultraviolet light and had a single broad absorption maximum at 263 nm, with a shoulder at 277 nm. These properties suggest that the substance is not a PAH. The substance chromatographed as a single peak at position B-1. These reults show that peak B-1 from the unpolluted sediments probably consists of this single crystalline substance.

The peak B-1 fraction from the Eastern Bay sample showed no mutgenic activity.

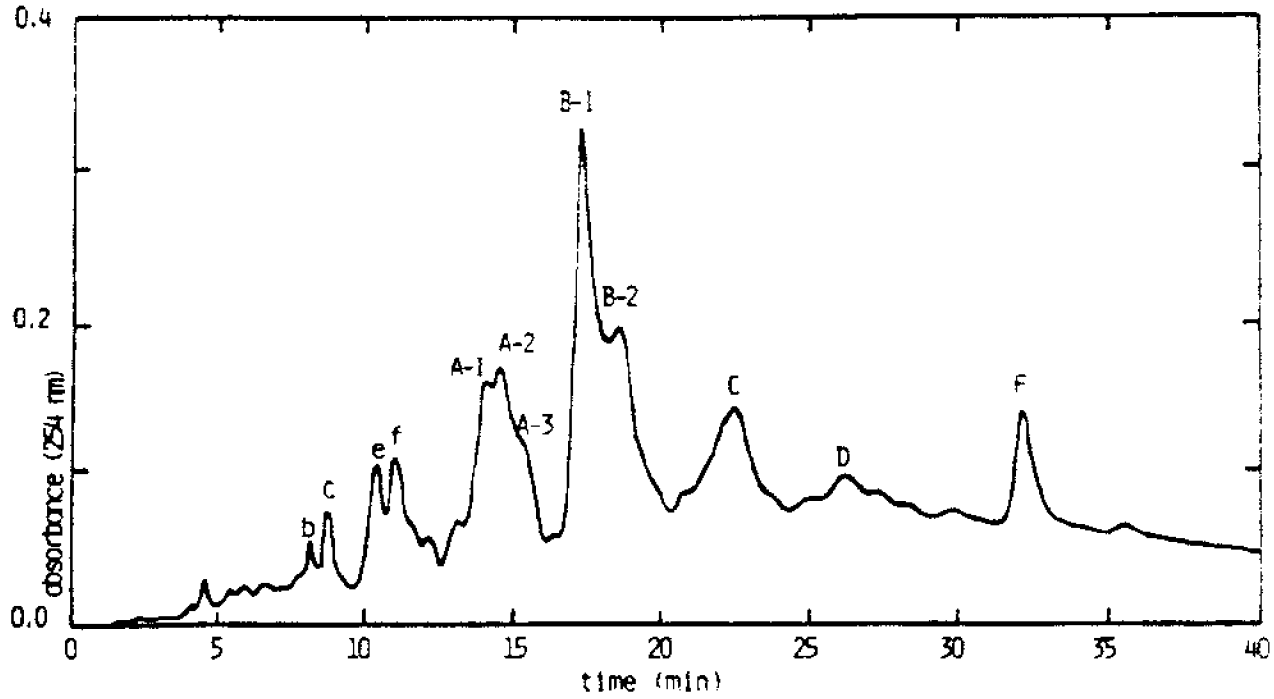

FIGURE 1. Chromatogram of extract from Colgate Creek sediment collected 6/77. 60 g of sediment were dessicated with an equal mass of sodium sulfate and extracted with hexane. The extract was evaporated, dissolved in 1 ml acetonitrile, injected into the 20 µl sample loop and chromatographed with the Waters analytical column. The peaks are labelled by letters.

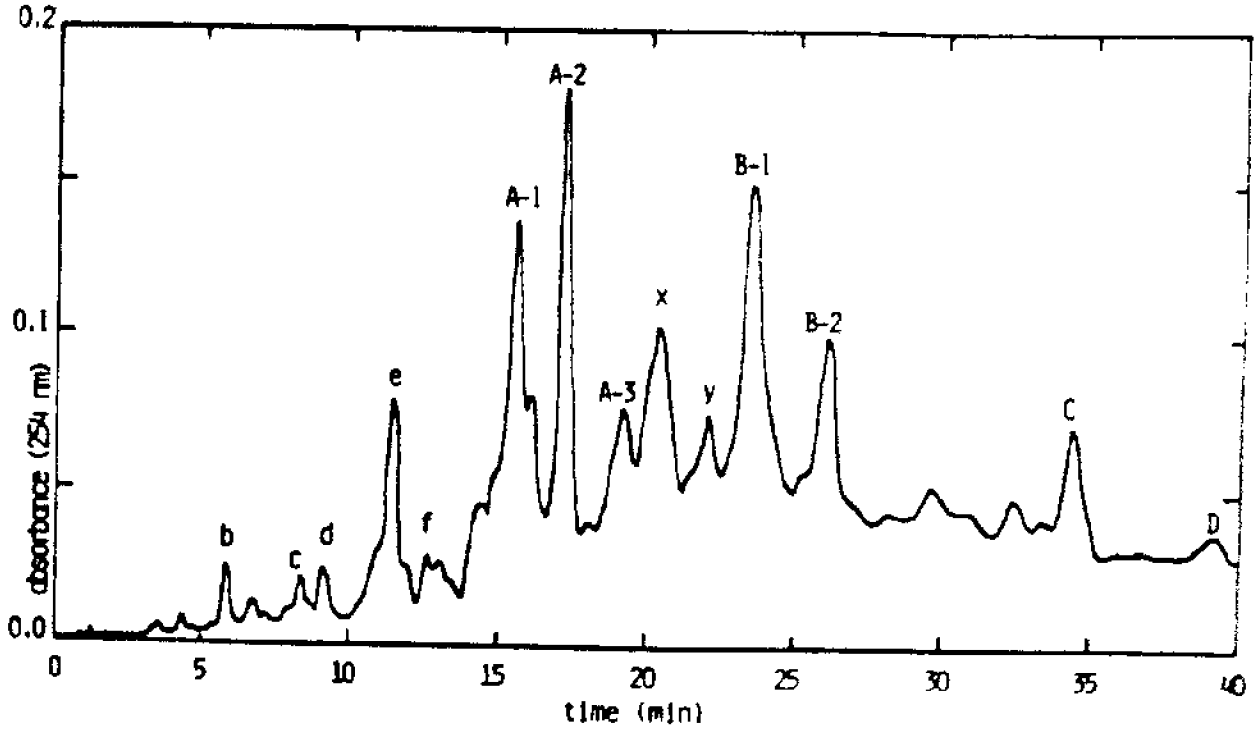

FIGURE 2. Chromatogram of extract from Colgate Creek sediment collected 4/78. 53 g of sediment were dessicated with 100 g sodium sulfate and carried through the extraction and cleanup procedures. The final preparation, dissolved in 1 ml methanol, was injected into the 20 µl sample loop and chromatographed with the Whatman analytical column. The letters label the peaks in order to correlate them with the peaks in Figure 1.

The cleanup procedure eliminates the crystalline substance from the sample because the substance is eluted by hexane from the silica gel column before the PAH's are eluted. Peak B-1 is nevertheless observed in Colgate Creek samples subjected to cleanup, although the cleanup procedure reduces its size (Table 2). Colgate Creek sediment therefore contains the crystalline substance and at least one other substance, with an absorption maximum of about 258 nm, that also chromatographs at position B-1. Mutagenic activity was found in peak B-1 fractions from cleaned-up Colgate Creek sediment samples, but this activity may have been caused by incomplete separation of peak B-2, which is believed to be benzo(a)pyrene.

The cleanup procedure is also able to eliminate peak F from chromatograms. During the cleanup procedure, the material in peak F is eluted from the silica gel column with the PAH's, but is eluted from the Sephadex LH-20 column before the PAH's. The absorption spectrum of a fraction collected from this peak has a broad maximum at 276 nm with a shoulder at 283 nm, and does not resemble the spectrum of a PAH. These results suggest that the substance in peak F is a slightly polar, high molecular weight compound, possibly a constituent of the yellow oil found in the hexane extracts. Peak F was observed in chromatograms of the unpolluted sediments as well as of the Colgate Creek sediment.

The peak F fraction from Colgate Creek sediment, site A, obtained by chromatographing the early-eluting solution from the Sephadex LH-20 column, was found to have essentially no mutagenic activity.

Biological assay. If any fraction proved toxic to the test bacteria or if it inhibited the activity of the liver enzymes, significant numbers of mutants would not be produced. The fractions tested did not exhibit toxicity toward the bacteria, and controls run with each fraction to detect enzyme inhibition were largely negative. Fractions extracted from a sediment sample from an unpolluted area of the Bay have also been tested. None of these fractions was found to have detectable amounts of mutagenic activity.

The same extracts of Colgate Creek sediment samples that gave the chromatograms shown in Figures 1 and 2 using the 20 µl sample loop were chromatographed again using the 130 µl sample loop, and peak fractions were collected. The larger sample loop gave chromatograms of poorer peak resolution, but provided more concentrated fractions for the assay for mutagenicity. The peak fractions were evaporated to dryness and resuspended in a small amount of DMSO.

Results of the assay of peak fractions from Colgate Creek sediment collected 6/77 are given in Table 3. Peaks A-1 and A-2 were combined for testing. All fractions except B-1 and B-2 were dissolved in 0.20 ml DMSO; 0.04 ml was used to assay for fraction sterility, 0.08 ml was assayed for mutagenicity, and the remaining 0.08 ml was used in a liver enzyme inhibition assay. Fractions B-1 and B-2 were dissolved in 0.40 ml and assayed for mutagenicity in duplicate and at two different concentrations. S-9 mix contained S-9 fraction from Aroclor-treated rats.

TABLE 3. Mutagenicity assay of peak fractions from Colgate Creek seditment collected 6/77.

Fraction Peak	ml/plate	TA100 Cells	S-9 Mix[a]	BPb (1 µg/plate)	Colonies per Plate	Induced Mutants per Plate	[c]
None		-	+	-	0		
DMSO	0.08	+	+	-	242,212		
DMSO	0.08	+	+	+	540,580	333	
A-1,A-2	0.04	-	-	-	0		
	0.08	+	+	-	375	148	(370)
	0.08	+	+	+	761		
A-3	0.04	-	-	-	0		
	0.08	+	+	-	219	12	
	0.08	+	+	+	569		
B-1	0.05	-	-	-	0		
	0.05	+	+	-	277,289	56	(448)
	0.10	+	+	-	327,316	95	(380)
	0.05	+	+	+	563		
B-2	0.05	-	-	-	0		
	0.05	+	+	-	354,308	104	(832)
	0.10	+	+	-	458,356	180	(720)
	0.05	+	+	+	594		
C	0.04	-	-	-	0		
	0.08	+	+	-	679	452	(1130)
	0.08	+	+	+	669		
D	0.04	-	-	-	0		
	0.08	+	+	-	552	325	(812)
	0.08	+	+	+	938		
F	0.04	-	-	-	0		
	0.08	+	+	-	386	159	(398)
	0.08	+	+	+	737		

[a] S-9 fraction from Aroclor treated rats was used.

[b] Benzo (a) pyrene.

[c] Number in parentheses is number of induced mutants per total fraction sample.

The average number of spontaneous mutants per assay plate was 227 (second row, Table 3). This number was subtracted from the number of colonies per plate, obtained with benzo(a)pyrene or the peak fractions, to obtain the number of induced mutants per plate (see last column, Table 3). S-9 mix and the peak fractions, when assayed alone, yielded no colonies, indicating absence of bacterial contamination.

All fractions except A-3 gave evidence of mutagenic activity. The number of induced mutants obtained with A-3 was too low to be significant. Fractions C, D, and F, and the combined A-1 and A-2 fractions did, on the basis of a single assay, show significant mutagenic activity, with the number of induced mutants per plate ranging from 148 to 452. Fractions B-1 and B-2, which were more thoroughly assayed, showed definite mutagenic activity as determined by duplicate assays and a dose-proportionate response. With these two fractions, a two-fold increase in concentration of fraction led to an approximate two-fold increase in numbers of mutants induced.

To test whether the peak fractions might inhibit the activating activity of the S-9 liver enzymes, benzo(a)pyrene was assayed in the presence and absence of the peak fractions. If no inhibition occurs, the number of induced mutants obtained when fraction and benzo(a)pyrene are assayed together should approximate the sum of induced mutants obtained when each is assayed separately. In terms of Table 3, since benzo(a)pyrene induced 333 mutants per plate, the colonies per plate (column 5) obtained with the fractions and benzo(a)pyrene should be about 333

greater in number than colonies per plate with fraction alone. This is the case for all fractions except fraction C for which the number of colonies per plate in the inhibition assay was much lower than expected. It is possible that benzo(a)pyrene was inadvertantly ommitted from the inhibition assay, but this could not be retested.

In terms of induced mutants per total fraction sample, peaks B-2, C and D contained the most mutagenic activity.

The results of the assay of peak fractions from Colgate Creek sediment collected 4/78 are given in Table 4. All fractions except B-2 were dissolved in 0.20 ml DMSO; 0.04 ml was used to assay for fraction sterility, and 0.08 ml was assayed for mutagenicity. Fraction B-2 was dissolved in 0.30 ml DMSO and was assayed for mutagenicity at two different concentrations. S-9 mix contained S-9 fraction from Aroclor-treated mice. A liver enzyme inhibition assay was run, but because of an error in delivery of benzo(a)-pyrene, data were not obtained.

S-9 mix assayed alone gave 0 colonies per plate, and fractions assayed alone gave 0-1 colonies per plate. No mutants were induced by fractions e and x. The number of induced mutants obtained with fractions A-1, A-3, y, and B-1 were too low in relationship to the numbers of spontaneous mutants per plate to be considered significant on the basis of a single assay. Fractions A-2 and C gave evidence of significant mutagenicity. Fraction B-2, which gave a dose-proportionate response, was again mutagenic.

TABLE 4. Mutagenicity assay of peak fractions from Colgate Creek sediment collected 4/78.

Fraction Peak	ml/plate	TA100 Cells	S-9 Mix[a]	Colonies per Plate	Induced Mutants per Plate[b]	
Assay 1						
None		-	+	0		
DMSO	0.08	+	+	203,221		
e	0.04	-	-	0		
	0.08	+	+	212	0	
A-1	0.04	-	-	0		
	0.08	+	+	251	39	
A-2	0.04	-	-	0		
	0.08	+	+	379	167	(418)
A-3	0.04	-	-	1		
	0.08	+	+	245	33	
x	0.04	-	-	0		
	0.08	+	+	209	-3	
Assay 2						
None		-	+	0		
DMSO	0.08	+	+	174,175		
y	0.04	-	-	0		
	0.08	+	+	287	113	
B-1	0.04	-	-	0		
	0.08	+	+	258	84	
B-2	0.05	-	-	0		
	0.05	+	+	271	97	(582)
	0.10	+	+	394	220	(660)
C	0.04	-	-	0		
	0.08	+	+	524	350	(875)

[a]S-9 from Aroclor treated mice was used.

[b]Number in parentheses is number of induced mutants per total fraction sample.

In summary, peak B-2, tentatively identified as benzo(a)pyrene, was found to be mutagenic. The mutagenicity found in peak B-1 of sediment collected 6/77 is probably a result of contamination of the B-1 peak with the closely overlapping B-2 peak. The results of the mutagenicity assays also indicated that peaks A-2, C, and D are significantly mutagenic. Mutagenicity was not detected in peak e, tentatively identified as fluoranthene, or in peak x.

Oyster Analysis. Fifteen oysters (Green Marsh oyster bar) having a drained wet weight of 185 g were desiccated and extracted with hexane. A portion of the extract, corresponding to 30 g wet flesh, was subjected to the cleanup procedure. Based on experience with another batch of Green Marsh oysters, this portion of extract contained about 0.3 g of hexane-extractible lipid. The cleanup procedure appeared to remove all of the lipid, and only a few tiny specks of material remained undissolved in the final 1 ml of solution in acetonitrile used for chromatography. The chromatogram had no discernible peaks, and absolutely nothing could be seen in the benzo(a)pyrene region of the chromatogram. The oysters, therefore, contained no measurable amounts of PAH's; an estimated level of 1 ppb of benzo(a)pyrene in the wet oyster flesh would have been detected if PAH's had been present.

A second portion of the hexane extract, corresponding to 90 g wet oyster flesh, was spiked with about 45 μg each of the four PAH's listed in Table 1, carried through the cleanup procedure, and chromatographed. The recoveries of the PAH's by this procedure, as measured by

the product Aw of peak height and peak width, were: fluoranthene, 25%; benz(a)anthracene, 75%; benzo(a)pyrene, 85%; dibenz(a,h)anthracene, 103%. The large loss of fluoranthene probably occurred in the initial hexane elution of the silica gel column. These results show that it is possible to have good recoveries of most PAH's from contaminated oysters despite the large amounts of lipid that must be separated from the PAH's before the PAH's can be analyzed.

PAH Uptake by Oysters

In order to examine the extent to which PAH's are taken up by oysters from polluted sediment, and to demonstrate the utility of the analytical procedure for analyzing PAH's in contaminated oysters, an uptake experiment was conducted at the Horn Point Environmental Laboratory.

Maintenance of Oysters on Sediments. With the cooperation of Dr. George Krantz, 158 cultured oysters were maintained indoors during the summer of 1978 in three 15-gallon aquaria. The bottom of one aquarium contained clean sand as a control; another aquarium contained the Eastern Bay sediment, sandwiched between two layers of sand to prevent dispersion; the third aquarium contained the Colgate Creek sediment (collected 4/78) sandwiched between two layers of sand. The oysters were placed on top of the sediments on June 12, and maintained in a slow flow of water pumped from the Choptank River. Fine solids suspended in the water gradually settled and covered the oysters.

Batches of oysters were removed from the aquaria on the schedule and in the amounts shown in Table 5. The oysters were kept on ice until shucked (on the same or the next day). The wet, drained flesh was weighed (Table 5), placed in clean jars, and frozen until analyzed.

Oyster Sample Preparation. Each sample batch of oyster flesh was placed in a 1-L beaker, and about twice the mass of anhydrous Na_2SO_4 was added to desiccate the sample. The best procedure was to macerate the sample with a metal spatula to chop the flesh and crush the lumps of wet Na_2SO_4, then occasionally to stir and chop the sample as it slowly cooled at room temperature, uncovered, until a dry, fluffy solid resulted. This preparation was ground to a course powder with a glass mortar and pestle.

PAH's were extracted from each desiccated sample with a total of 500 mL of hexane, using a combination of stirring in batches, decantation through filter paper, and rinsing of the solid on the filter paper. The hexane extract was concentrated to several mL in a rotary evaporator.

Removal of polar substances was carried out on a silica gel column. Fifteen g of silica gel (Baker Analyzed 60-200 mesh, "suitable for column chromatography," dried overnight at 130°C and then deactivated with 5% distilled water) was washed into a 15-mm ID glass column with hexane. A short layer of Na_2SO_4 was placed on top. The concentrated hexane extract was run into the column and eluted with hexane until 60 mL or more effluent came out. Benzene was then run through the column, and the effluent in the

TABLE 5. Oyster Samples (summer, 1978).

Date removed	Total exposure to sediment (days)	Controls		Eastern Bay sediment		Colgate Creek sediment	
		number	mass[a]	number	mass[a]	number	mass[a]
June 28	16	17	107	17	110	18	127
July 13	31	15	70	19	108	15	77
August 1	50	16	94	19	114	22	138

[a]Total mass of wet oyster flesh in sample (grams)

interface region, which had a bright blue fluorescence under ultraviolet light, was collected. This procedure left a dark nonfluorescent pigment layer at the top of the silica gel, and a zone of olive-green material with a red fluorescence extending part way down the column.

The collected sample from the silica gel column was evaporated to dryness in a rotary evaporator with a stream of nitrogen, care being taken to avoid heating the last portion of solvent. Droplets of nonvolatile oil with a "fishy" smell remained after this step. The sample was taken up in 2.0 mL of a benzene-methanol mixture (50/50 by volume) and subjected to gel filtration with the same solvent mixture through a Sephadex LH-20 column (Sigma, 25-100 m bead size, packed 23 cm high in a 15-mm ID glass column). The first 30 mL of effluent, containing a band of fluorescent high-molecular-weight material, was discarded; the next 21 mL of effluent, containing a second fluorescent band, was collected and evaporated to dryness as before. This procedure left the LH-20 column free of observable fluorescence so that it could be reused.

The dried sample was taken up in 1 mL of acetonitrile (Fisher HPLC grade), giving a clear solution (except for the sample obtained from 22 oysters; 2mL of acetonitrile was used for this sample). The solution was filtered through a Nucleopore polycarbonate membrane (0.6 μm pore size).

HPLC Analysis. The analytical apparatus and procedure used was high pressure liquid chromatography (HPLC) as described in this

report. The volume of the sample loop was determined to be 22.3 µL by titration of six fillings of the loop with a 1 M solution of HCl. A Waters µBondapak-C18 analytical column was used. An exponential mobile-phase gradient, 70% to 100% methanol in water, was used at a flow rate of 2 mL/min. The methanol and water were HPLC grade (Baker).

RESULTS AND DISCUSSION

While many peaks were observed in the chromatogram from each of the nine oyster samples, the chromatograms were all quite similar in appearance. This finding is illustrated by Figure 3, which compares the chromatograms obtained from oysters maintained for 50 days on Colgate Creek sediment (middle curve) and on Eastern Bay sediment (bottom curve). No extra peaks are seen in the Colgate Creek chromatogram, which could be attributed to PAH's taken up by the oysters from the highly polluted sediment.

By contrast, the upper curve in Figure 3 is the chromatogram of an extract from Colgate Creek sediment showing an entirely different pattern of peaks. The peaks labelled e and f in the chromatogram were previously tentatively identified as the nonmutagens fluoranthene and pyrene, respectively. Peaks A-2 and B-2 were identified as the mutagens benz(a)anthracene and benzo(a)pyrene, respectively. The peaks A-2, B-2, C, and D were all found to be significantly mutagenic.

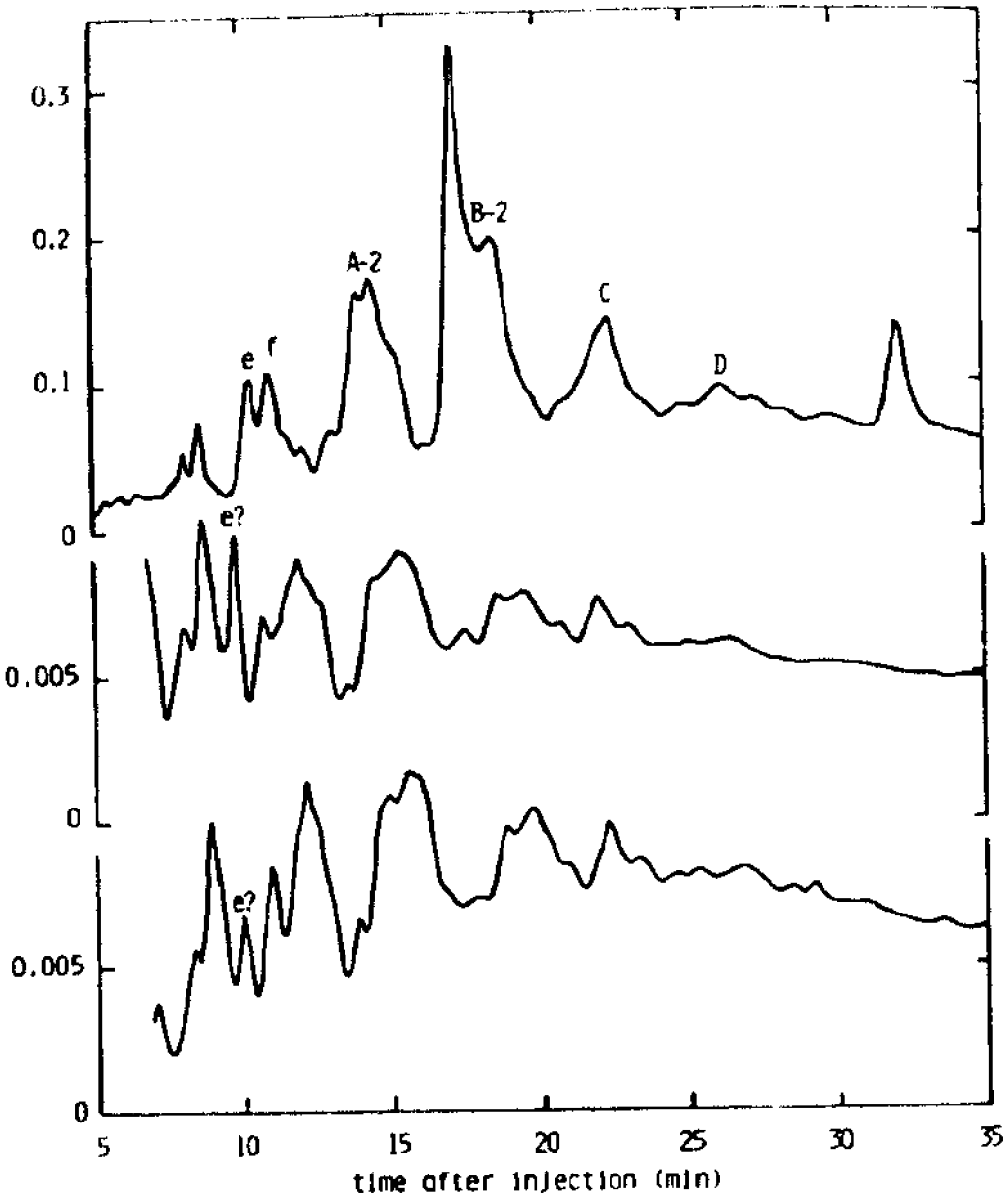

FIGURE 3. Chromatograms of sediment and oyster samples. Top curve: 60 g of sediment from Colgate Creek (collected 7/77) dessicated with an equal mass of Na_2SO_4 and extracted with hexane. Middle curve: oyster maintained for 50 days on Colgate Creek sediment and prepared as described. Bottom curve: oysters maintained for 50 days on Eastern Bay sediment and prepared as described.

The only significant difference that could be seen between the peak patterns of the chromatograms from the different oyster samples was an increase in the height (relative to other peaks) of the peak at 10 min retention time on going from 16 days exposure to 31 days exposure, and from 31 days to 50 days. This peak is labelled "e?" on the middle and bottom curves of Figure 3 because its retention time (10 min) and the value of the absorbance ratio A_{280}/A_{254} (about 2.0) suggest that it is the same as peak e (fluoranthene) in Colgate Creek sediment extracts. The height of this peak was approximately equal for oysters maintained for the same length of time on Colgate Creek sediment, on Eastern Bay sediment, and on sand only; thus the increase in peak height with exposure time may represent the uptake of fluoranthene over time from the sand or water present in all three of the aquaria.

None of the peaks observed in the chromatograms from the oyster samples can be attributed to the expected uptake of PAH's from the polluted Colgate Creek sediment. Instead, all of the peaks (with the exception of the peak at 10 minutes, discussed above) seem to be endogenous to the oysters and may not be PAH's.

The sensitivity of the analytical procedure would easily allow detection of benzo(a)pyrene, for example, at a level of 1 ppb, which is lower than has been measured in mussels and oysters exposed to some polluted environments (see the Introduction). Thus the oysters maintained over the Colgate Creek sediment, covered by sand, apparently were not in contact with a sufficiently high concentration of dissolved or suspended PAH's to take them up to a detectable extent.

This situation, while it thwarts the purpose of the oyster maintenance experiment, does suggest that oysters will not take up PAH's to any appreciable extent or at an appreciable rate unless they are in direct contact with polluted sediment or with dispersed petroleum.

Since PAH's constitute a large class of compounds, only some of which are carcinogenic, the combined use of high pressure liquid chromatography to isolate PAH's and of the Ames system to detect biological activity should be a potent methodology for detecting biologically hazardous PAH compunds in natural water systems.

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