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ABSTRACT

Title of Thesis: Determination of Benzo(a)-pyrene Metabolites in the Bile of Winter Flounder from the New York Bight

Xingmin Liu, Master of Science in Chemistry, 1989

Thesis directed by: Dr. Arthur Greenberg, Professor of Department of Chemical Engineering, Chemistry and Environmental Science

A method for detecting the concentration of BaP Metabolites in fish tissues has been developed. The analytical procedure includes extraction of PAH and BaP Metabolites from fish bile, liver extract and muscle extract; reduction of metabolites to BaP using hydriodic acid; and subsequent analysis of the BaP by thin layer chromatography with fluorescence detection. The method is applied to tissues from fish collected from polluted as well as clean reference waters and experimental tanks with dilutions of treated industrial wastewater. Experimental results on one kind of bottom-dwelling fish, winter flounder, which comes from

the Hudson-Raritan Estuary, NY, where the water and sediment contain toxic chemicals as BaP and PAH, show fish do uptake BaP from the environment. The bigger the fish, the more BaP they uptake. The concentration of BaP metabolites in fish liver is higher than in bile and muscle. Generally, during the summer, fish uptake more BaP than in the winter.

DETERMINATION OF BENZO(A)PYRENE METABOLITES IN THE BILE OF WINTER FLOUNDER FROM THE NEW YORK BIGHT

by
Xingmin Liu

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
1989

APPROVAL SHEET

Title of Thesis: Determination of Benzo(a)pyrene Metabolites in the Bile of Winter Flounder from the New York Bight

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I. Introduction

A. Background

Polycyclic aromatic hydrocarbons (PAH) can be formed by thermal decomposition of any organic material containing carbon and hydrogen. Its formation is based on two major mechanisms:

1. Pyrolysis or incomplete combustion
2. Carbonization processes

The main man-made sources of PAH, are divided into two categories: stationary and mobile sources. The stationary sources include industrial sources, power and heat generation, residential heating, incineration, and open fires. The second category is that of the mobile sources, which include gasoline-engine automobiles, diesel-engine automobiles, trucks, airplanes, and sea traffic.

Benzo(a)pyrene (BaP), one of the PAH compounds, has for different reasons has been measured more frequently than other PAHs. BaP can be an indicator of total PAH emission by comparing it to values of BaP emission from other sources. However, BaP is only a minor component, usually less than 5% of the total amount of PAH.

PAH compounds and BaP are widespread environmental

contaminants which are present in air, water, sediment, dry soil, and foods (1,2). Certain PAHs, such as BaP, have been shown to be carcinogenic in various organs of mammals (3). BaP and PAH compounds enter water, soil and marine biota by fallout from the atmosphere, in rainwater and runoff from road surfaces as well as from direct discharges in industrial and domestic effluents. PAHs are readily taken up by marine organisms. In some species such as vertebrate fish, PAHs are rapidly metabolized and excreted and steady-state levels of PAH in tissues are low or undetectable. However, some organisms, notably shellfish, lack any appreciable capacity to metabolize PAH and therefore accumulate them in their tissues. Such bioaccumulation may pose a risk to human health if carcinogen-contaminated seafoods are harvested and consumed. Table 1 shows some data for BaP levels in biota.

At polluted locations, BaP concentrations in biota depend on the particular species - those with high lipid content usually accumulate more BaP (10). BaP accumulation has been shown to be highest in migratory fish compared to that found in sea, salt-water, semimigratory or freshwater fish. Semimigratory fish have the lower levels of BaP than nonfatty ones (11).

Table 1. BaP Concentrations in Freshwater Biota

Organism	Location	Concentration of BaP (ug/kg or ppb)	Ref.
phytoplankton	Lake Constance	2.0	4
Algae:		475	5
Algae spec.	River above city (USSR)	0.6-6.0	5
Algae spec.	River below city (USSR)	75	5
Cladophora	Reservoir (USSR)	0.16-1.7	5
Spirulina	Lake Texcoco (Mexico)	2.6-3.8	6
Mollusks:			
Uniopictorum	Moscow River (USSR)	0.03-1.3	5
Uniopictorum	Reservoir (USSR)	10.0-12.0	5
Cipangopaludina	Pounds (Hamilton, Ca.)	2.5	7
Fish:			
Lake trout	Lake Maskinonge (Ca.)	<1.0	8
5 species	River prior to sewage discharge (USSR)		
body		1.06-7.89	5
gills		6.1-9.1	5
4 species	River after sewage discharge (USSR)		
body		2.4-66.8	5
gills (sterlet only)		62-93	5
prdatory and nonpredatory fish	water bodies in Estonia	0.11-5.96	9

Malins et al (2), reported a 4-year, multidisciplinary study examining the relationships between pollutants and diseases of fish in Puget Sound, WA. They found over 900 individual organic compounds in sediment from one urban bay (Commencement Bay), and evidence was obtained for the presence of numerous additional compounds. Many of the chemicals accumulated in bottom-dwelling fish, and high levels of certain toxic chemicals in the urban bays were linked to serious diseases (e.g. liver carcinomas) of English sole (Parophrys vetulus) and other demersal fish species. Estuaries and other coastal waters receive many of the approximately 70000 synthetic chemicals in commercial use (12). These chemicals enter coastal waters from a number of sources, including spills, dumping operations, urban runoff, and municipal and industrial waste discharges. Numerous studies have shown that sediments are major reservoirs for pollutants. For example, surface sediment from the Hudson-Raritan Estuary near Newton Creek in New York was found to contained 180 ppm of aromatic hydrocarbons (AHs), including 5.6 and 1.3 ppm of the carcinogens benz(a)anthracene and benzo(a)pyrene (13). Higher concentrations of these carcinogens, 11000 and 9000 ppm respectively, were found in surface sediments from the estuary of the Elizabeth River, VA (14). Fish accumulate many of the chemicals from polluted aquatic environments (14). A number of pathological conditions have been

observed in marine fish taken from polluted coastal waters and estuaries. Fin erosion was found in a variety of species taken from the Southern California Bight, the New York Bight, and the Duwamish Waterway in Seattle (15-17). In most cases, the highest prevalences of this disease were in fish from areas with highly contaminated bottom sediments. Liver neoplasms have been reported in fish from areas with highly contaminated sediments: Atlantic hagfish (yxine glutinasa) from a fjord in Sweden (18); in Atlantic tomcod (Microgadus tomcod) from the Hudson River estuary (19), and in two species of bottom fish (English sole, Parophrys vetulus, and starry flounder, Platichthys stellatus) from Puget Sound, WA (20, 21). Liver neoplasms have also been reported in a freshwater bottom-dwelling fish species, the brown bullhead (Ictalurus nebulosus) from polluted sites in a number of rivers associated with the Great Lakes (22-24).

Compounds such as aromatic hydrocarbons (AHs) primarily exert their toxic effects after metabolic activation. AH metabolites have recently been detected in the bile of fish taken from contaminated areas, showing that AHs are taken up by fish from their environment (25,26). Usha Varanasi et al (27) found that BaP was extensively metabolized by shrimp and fish (Table 2).

B. Objective

In their research proposal, Weis et al (28) noted that the Hudson-Raritan Estuary is a typical urban estuary in that a once-productive area received a tremendous burden of toxic pollutants from many point and non-point sources. The pollutants mostly come from municipal discharges (29). The toxic pollutants are organics and heavy metals (30).

Sediments serve as a sink for both organics and heavy metals. Considerable levels of both categories of pollutants can accumulated in fish. Despite a century of such abuse, there are still sufficient numbers of finfish and shellfish to support both recreational and commerical fisheries in several areas.

The present study is concerned with analysis of benzo(a)pyrene metabolite content and originally existing benzo(a)pyrene content in bile and liver of fish (winter flounder) from a polluted site such as Hudson-Raritan Estuary using a published HI reduction method and TLC of the BaP produced by this "reverse metabolism" reaction.

Table 2. Benzo(a)pyrene and Metabolites in Benthic Organisms Exposed to Duwamish River Delta Sediment.[a] (27)

hydrocarbon & metabolites	length of exposure weeks	shrimp (*P.platyceros*)	fish (*P. vetulus*)		
		body	body	liver	bile
p mol of BaP-equiv/g of tissue (wet weight)	1	13±4	36±6	890±170	14000±7900
	4	41±13	35±5	960±270	14000±13000
% BaP		NA	6±1	1.0±0.3	<1
% BaP metabolites		NA	94±1	99±0.3	>99

a. Estimate of BaP concentration in tissues is based on specific activity of [^{3}H]BaP in sediment after solvent extraction.

NA = not analyzed

II. Sample Collection and Treatment

A. Sample Location

See Figure I: Map of the Sampling Site

Field Site: The R.V. Wilson in the Hudson-Raritan Estuary.

Reference Site: Shinnecock Bay, Long Island, NY.

B. Sample Collection and Treatment

Samples were collected by Professor P. Weis of N.J. Medical School

Winter flounder adult fish (pseudopleuronectes americanus) were considered to be a representative benthic species. Collection was made during all seasons; both sexes were sought. Fish weighing from 150 g to 340 g / per adult winter flounder were either captured by trawler from the Hudson-Raritan Estuary or grown in experimental tanks. After capture, fish were placed in holding tanks containing fresh seawater until they could be necropsied aboard the research vessel. The fish were sacrificed on the vessel by cervical section. Bile was removed from the gall bladder and frozen until analyzed. Liver was excised and treated using a soxhlet extraction, according to a NBS method. (This was done by Professor P. Weis of N.J. Medical School).

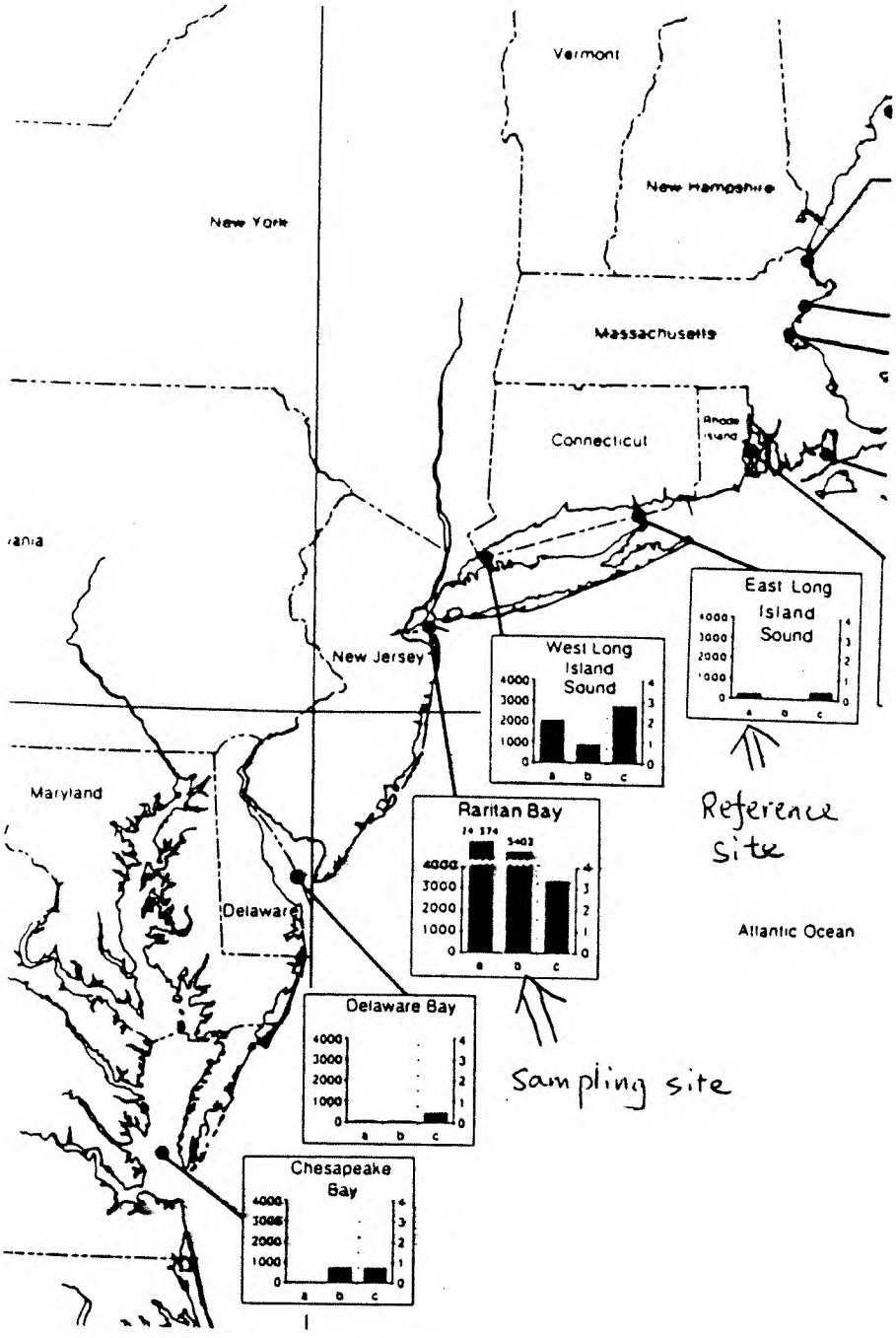


Fig. 1 Map of the Sampling Sites

III. Theory and Method Development

A. TLC Method

Thin Layer Chromatography is a separation technique. A glass plate is coated with a thin layer of suitable adsorbent and the bottom of the plate is spotted with a solution of organic compound (or mixture) to be investigated. Mixtures are separated by the movement of a solvent or solvent mixture, the moving phase, through a thin layer of adsorbent, the stationary phase. Compounds are separated due to their differing affinities for the moving and stationary phases of the TLC system.

Reviews of the use of TLC for the separation of PAH compounds in general and the benzopyrenes in particular have been published by Sawicki et al. (31) and White and Howard (32). The advantages of TLC analytical methods for the separation of PAH compounds include speed, simplicity, low cost, and sensitivity.

BaP is a significant member of the PAH fraction in most media. It is a potent carcinogen in animals and can be separated from other PAH using TLC methods. In 1967, Sawicki and co-workers (33) compared a number of TLC

methods with other methods for the determination of BaP in airborne particulate matter. Most TLC methods for the isolation of BaP employ acetylated cellulose as an adsorbent. Schultz and co-workers (34) used 30% acetylated cellulose / methanol-ether-water (4:4:1) to isolate BaP from the organic fraction of airborne particulate matter. A scanning spectrofluorometer was used to quantitate BaP on the TLC plate at levels as low as 1 ng with a reproducibility of $\pm$10%. Howard and co-workers (35) also used acetylated cellulose / ethanol-toluene-water (17:4:4) to identify BaP in a PAH fraction isolated from smoked food. Recovery of BaP was 73 - 100% at the 2 ppb level for the overall analysis.

B. Metabolism of PAH and BaP Compounds

Cancer induction is dependent on the metabolism of PAH to form electrophilic intermediates which can react with critical cellular constituents such as DNA (36-38). The major pathway for the metabolism of PAH is via the oxidative reaction catalyzed by a cytochrome P-450-dependent-mixed-function oxidase (MFO) system (39,40), although other enzymes are also implicated in PAH metabolism (41, 42). The chief site of metabolism is the liver, and mainly the parenchymal cells there.

The mechanism of BaP oxidation has been reviewed by

Estabrook et al (43). The first stage is the transfer of an oxygen atom from the active form of P-450 to BaP, usually to produce an epoxide. The cytochrome contains an iron atom, which is activated by an 'oxene' intermediate, Fe(III)-O: the reduced P-450 is then re-oxidized by a process involving reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. The whole process may be described by:

$$BaP + O_2 + NADPH + H+ \xrightarrow{\text{oxidase}} BaP.O + NADP+ + H_2O$$

Metabolism of BaP takes place in two phases (44). Phase 1: conversion to oxidized derivatives (epoxides, phenols, diols, tetrols, quinones), is performed by oxidative enzymes and epoxide hydrolases attached to the microsomal membrane. Phase 1 is carried out in liver mainly by a system comprising a monooxygenase and epoxide hydrolase. In phase 2, these moderately polar metabolites are conjugated with sulphate, glucuronic acid or glutathione by enzymes mainly present in the cytosol, to give substances which are sufficiently soluble in water to be exereted. The epoxides are generally unstable and undergo different reactions to form (a) phenols through isomerization of the epoxides, (b) trans-dihydrodiols by the action of epoxide hydrolase (EH), and (c) glutathione (GSH)

conjugates by addition of GSH to one of the electrophilic carbons of the epoxide, a process catalyzed by glutathione s-transferase (GST) (see Figure 2). These primary metabolites can also undergo several other reactions (45). For example, trans-7,8-dihydroxy-7,8-dihydro-BaP (BaP 7,8-dihydrodiol) can be oxidized to form the 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydro-BaP (BPDE), which can undergo nucleophilic addition reactions with such molecules as DNA and GSH (Figure 2). In addition to the above reactions, BaP 7,8-dihydrodiol can also be conjugated with a glucuronic acid or sulfate moiety to form glucuronide or sulfate conjugates (46,47). Both glucuronidation and sulfation are the major pathways of oxidized PAH metabolites in various species (46-48). A recent laboratory study on English sole by Stein and Varanasi and co-workers has shown that BaP metabolites, such as BaP 9,10-dihydrodiol, BaP 7,8-dihydrodiol, 1-hydroxyBaP, and 3-hydroxyBaP, were present in the form of glucuronide and sulfate conjugates in the bile of English sole (49-52). Also recent work done by Nishimoto, M.M. (27) using a low concentration of [^{3}H]-BaP (5 nm) as substrate found that the same BaP metabolites were formed by English sole liver microsomes. Quinones were also found to be present in appreciable amounts, although the identity of their monohydroxy precursor(s) has not been established.

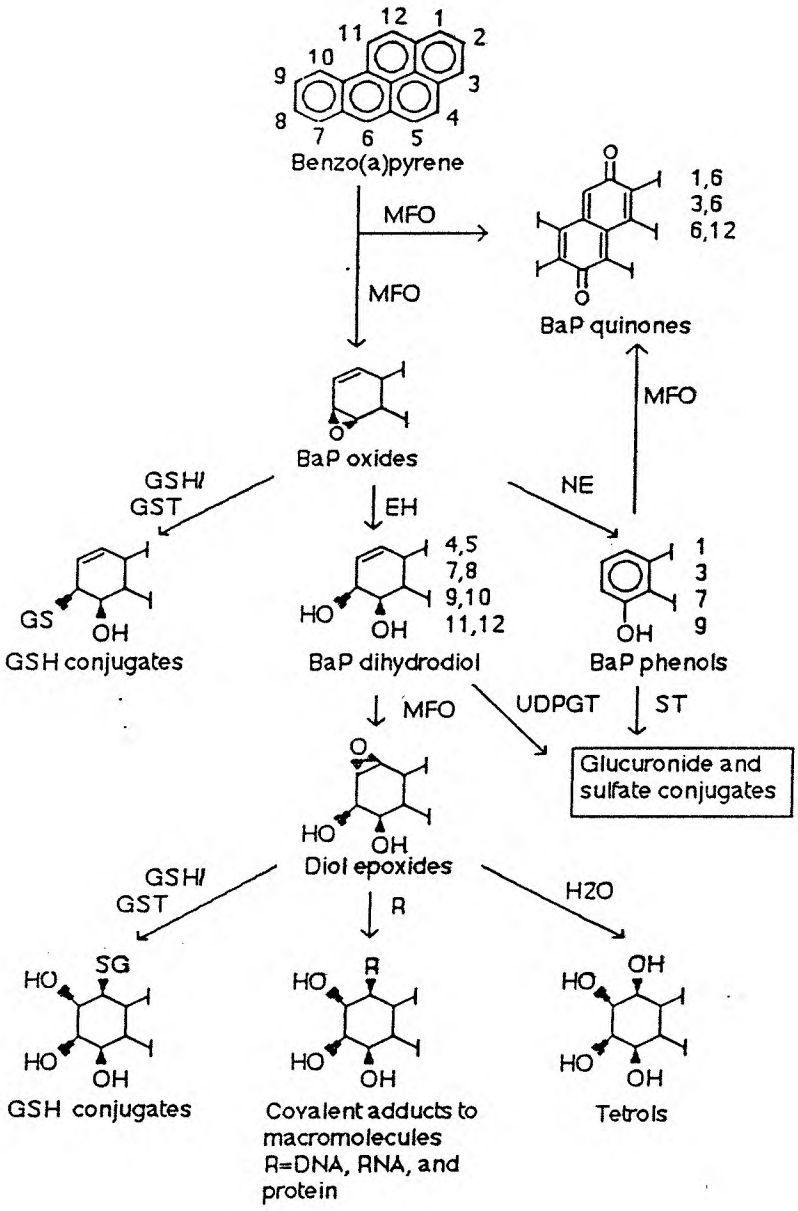


Fig. 2 **Major pathways of the enzymatic oxidation of BaP** observed in several fish. The numbers refer to the position on BaP where the oxides, dihydrodiols, and phenols are formed. The stereochemistry of the hydration products of oxides are generally <u>trans</u> addition of H_2O. Abbreviations: MFO: mixed-function oxidase; GSH: glutanthione; GST: glutathione S-transferase; EH: epoxide hydrolase; NE: non-enzymatic; UDPGT: uridine diphosphoglucuronyl transferase; ST: sulfotransferase. (45)

C. Reduction of Metabolites to PAH

According to Konieczny and Harvey (53) and Hudlicky (54), reduction of several polycyclic quinones using hydriodic acid without or with red phosphorus, offers advantages over other methods; i.e. deoxygenation may be effected directly on the phenol without the necessity for the preparation of a special derivative, and secondary hydrogenation of polycyclic ring systems with HI is poorly competitive in comparison with the alternative methods where this often a serious problem (55,56).

Hydrogen iodide, one of the oldest reducing agents, is available as a gas, but is used mainly in the form of its aqueous solution, hydriodic acid. Hydrogen iodide dissociates at higher temperatures to iodine and hydrogen, which affects hydrogenations. The reaction is reversible. Its equilibrium is shifted in favor of the decomposition by the reaction of hydrogen with organic compounds to be reduced but it can also be affected by removal of iodine. This can be accomplished by allowing iodine to react with phosphorus to form phosphorus triiodide which decomposes, in the presence of water, to phosphorus acid and hydrogen iodide. In this way, by adding phosphorous to the reaction mixture, hydrogen

iodide is recycled and the reducing efficiency of hydriodic acid is enhanced (54). Reduction with hydriodic acid can be accomplished by refluxing organic compounds with the azeotropic acid. Acetic acid is added to increase the miscibility of the acid with the organic compound. Hydriodic acid is a reagent of choice for the reduction of alcohols (58), some phenols (53), some ketones (59,60), quinones (57), and other organic compounds.

The mechanism of how HI works involves successive protonation and hydride transfer from HI with the formation of 1 molar equivalent of I_2 and dehydration at each stage (61). This is shown in Figure 3.

However in Konieczny and Harvey's study they found that HI in acetic acid without phosphorus was found to be a convenient general reagent for the reduction of polycyclic quinones, hydroquinones, and phenols (or their esters or ethers which are readily cleaved under the conditions employed) to fully aromatic hydrocarbons in a single step. In their experience, phosphorus is generally not required and has a deleterious effect through promotion of undesired hydrogenation of the aromatic products.

In the present research, phosphorus was not used for

reaction with fish bile samples, but in fish liver samples, a comparison test was done using red phosphorous along with HI.

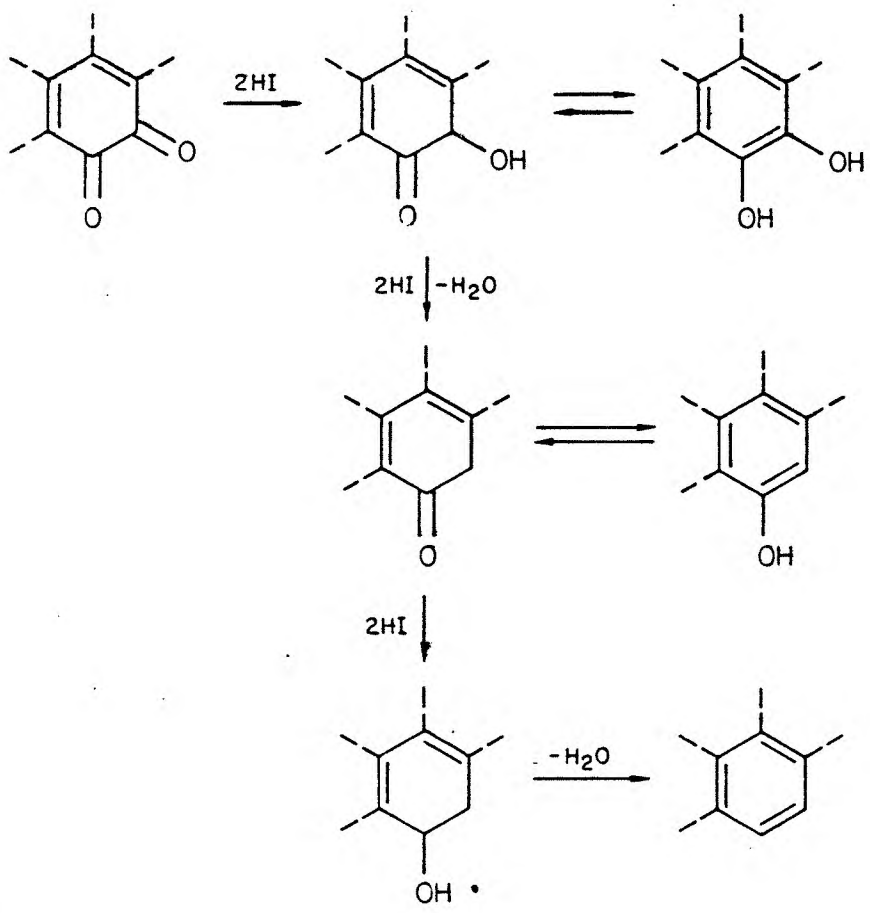


Fig. 3 Protonation and Hydride Transfer from HI with Formation of I_2 and dehydration at Each Stage (61)

D. Determination of BaP in Fish Bile and Liver

1. Chemicals

1) Benzo(a)pyrene from Aldrich Chemical Co.
   A series of working standard solutions were prepared by diluting the stock solution with cyclohexane. They are 1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 40 ng/ml, 70 ng/ml. They are all kept in refrigerator before using.
2) Hydrochloric acid from Mallinckrodt Co. (AR)
3) Diethylether anhydrous 99% from Alfa Products (HPLC)
4) Hydriodic acid 47%-51% from J.T. Baker Chemical Co. (AR)
5) Acetic acid > 99.5% from Fluka Co. (AR)
6) Potassium disulfate from Fluka Co. (AR)
7) Cyclohexane from J.T. Baker Chemical Co. (HPLC)
8) Sodium sulfate anhydrous from Mallinckrodt Co. (AR)
9) Methylene chloride from J.T. Baker Chemical Co. (HPLC)
10) Alcohol anhydrous from J.T. Baker Chemical Co. (HPLC)
11) Phosphorus red Amorphous from Fisher Scientific Company

2. Apparatus

1) TLC plate is coated with 250 microns layer of 20% cellulose-acetate on 20 x 20 cm glass plate, (from Analtech Co.) with 18 channels.
2) Fluorescence spectrophotometer (from Perkin-Elmer, MPF-44B).

emission wavelength: 428.5 nm

excitation wavelength: 387.0 nm

3. Application to Samples for Methods Development

1) Analysis BaP in bile and liver and muscle

Fish bile samples: kept in plastic bottles in frozen state

color: green to dark green

volume: 0.12 - 0.48 ml in liquid state

fish weight: 150 - 340 g

Liver samples: kept in plastic bottles in frozen state

color: yellow to dark brown

fish weight: not available

Muscle samples: kept in plastic bottles in frozen state

color: yellow to brown

fish weight: most not available

a. Acidification

Whole fish bile /liver / muscle was defrosted at room temperature in the containing bottle. 5 ml hydrochloric

acid is added and the pH adjustd to 3 by pH meter. The sample then is moved to water bath and heated to 30 - 40 C to let hydrolyze throughly for 0.5 hour.

b. Extraction

In a separatory funnel, anhydrous (99%) diethyl ether is used as extraction solvent. Mix 15 ml ether with hydrolyzed fish sample and shake 3 minutes, keeping the organic phase (BaP-metabolites contained) for later use and providing the residual aqueous phase to UMDNJ for other uses, notably metals analysis.

c. Reduction of Metabolites

Put the organic extract into a 50 ml round flask bottle, evaporate to dryness by air, then add 2 ml hydriodic acid 47% - 51%, and 15 ml acetic acid > 99.5%, reflux for 15 - 18 hours, at 130 C in oil bath.

* When a comparison test was done on some of fish liver samples, red phosphrous was added to reflux with HI and HAc.

The hot solution is poured into a separatory funnel, 80 ml 1% potassium disulfate solution added, the resulting solution extracted twice with 40 ml and 20 ml cyclohexane solution (rinse the condensor to avoid unnecessary loss). The organic phase is washed with 100

ml distilled water. A small amount of anhydrous crystalline sodium sulfate is added to absorb water.

The extract is blown down to 0.5 ml under air in a 8 ml brown bottle.

* The procedure is done under conditions as dark as possible to avoid BaP photodecomposition.

d. Thin Layer Chromatography method

A series of 100 ul BaP standards and 50 - 250 ul samples are injected onto TLC plate using a multispotter. After sample spotting, the plate is developed with 150 ml solvent (methylene chloride : anhydrous ethanol = 1 : 2) for about 80 minutes, then dried in the air. All the procedures should be done in the dark.

Using a plate-scanning fluorescence spectrophotometer, BaP is identified by comparison with retention times of BaP standards and quantitation is done by comparing the sample area with a standard peak area which is similar in magnitude to the sample.

2) Recovery Test

a) BaP standard

Add 100 ul 70 ng/ml BaP standard into 50 ml round flask, evaporate to dryness by air, then perform the same

procedure as employed for fish samples.

b) BaP metabolites

Since we know there are quinones, phenols, diols and other PAH derivatives in the fish sample as BaP metabolites, some of these can be reduced to BaP.

BaP-metabolite-conjugates --hydrolysis--> BaP-metabolites (quinones, phenols, diols)

BaP-metabolites --reduction--> BaP

The following standards of BaP metabolites have been prepared: BaP 7,8-dione, concentration 3.3 mg/50 ml in acetonitrile; 3-OH-BaP, concentration 1 mg/100ml in DCM; BaP 3,6-dione, concentration 3.9 mg / 50 ml in acetonitrile; BaP 4,5-dione, concentration 3.7 mg/50 ml in acetonitrile; BaP 6,12-dione, concentration 5 mg / 50 ml in acetonitrile. 100 ul aliquots have been examined for identification and calibration.

3) Test of detection limit

Take 1 ng/ml BaP standard, use 100 ul multispotters to inject 5 ul, 10 ul, 20 ul, 30 ul, 40 ul, 40 ul, 50 ul, 60 ul, 70 ul, 80 ul, 90 ul on TLC plate, then develop and run on fluorescence spectrophotometer.

4. Experimental Results (Table 3 to Table 14)

For all the bile samples, no red phosphorus has been added when refluxing with HI and HAc.

Table 3. Detection limit of BaP analysis using fluorescence spectrophotometer (Perkin-Elmer MPF-44B)

A linear regression graph is attached. (Figure 4)

The result: detection limit is 0.010 ng or 10 pg.

Table 4. Dosage testing on flounders in experimental tanks in January 1988.

It gives the BaP metabolite concentration in fish which exposed to treated municipal wastewater, (0% TMW to 8% TMW). The result shows: the higher percentage of TMW, the more BaP fish take.

Table 5. BaP metabolites in fish bile from New York Bight and the reference site - Long Island in September 1987

Table 6. BaP metabolites in fish bile from New York Bight on November 19, 1987

Table 7. BaP metabolites in feral fish bile from Raritan Bay in February 1988

Bile sample numbers from 49 to 60.

Table 8. BaP metabolites in fish bile from New York Bight in July 1987

Table 3. Detection Limit of BaP Analysis Using Fluorescence Spectrophotometer

1 ng/ml BaP standard inject: 5 ul -----> 90 ul

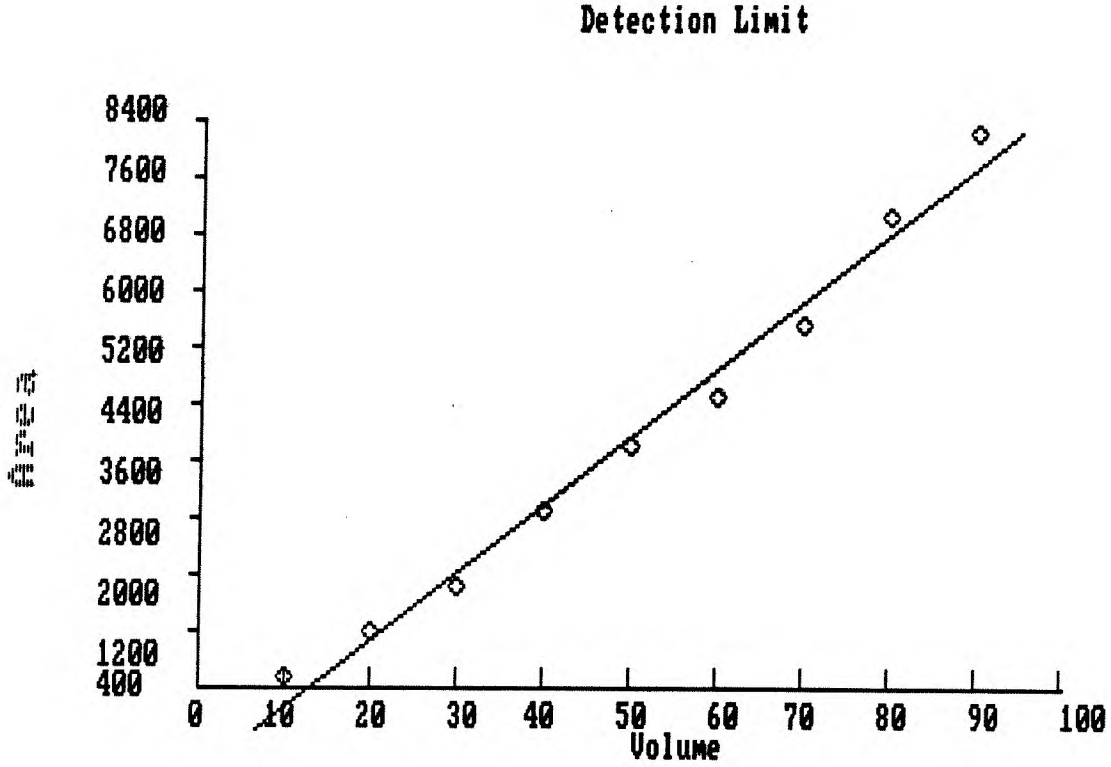
Volume (ul)	RT (second)	Area
5	ND	ND
10	59	576
20	59	1178
30	57	1860
40	56	2895
50	56	3792
60	57	4489
70	57	5538
80	57	7041
90	59	8222

Detection limit: 10 ul BaP = 1 ng/ml x 10 ul x 10^{-3} ml/ul

= 0.010 ng

* Set computer "MA" (smallest area can be detected) = 400

ND = undetectable



Detection Limit

POINT	X	Y
1	10	576
2	20	1178
3	30	1860
4	40	2895
5	50	3792
6	60	4489
7	70	5538
8	80	7041
9	90	8222

Slope = 95.205 +/- 4.381585
Intercept = -805.6945 +/- 339.3961
Correlation = .9926681
Calculated on points 1 TO 9

Fig. 4 Detection Limit of BaP Analysis

Table 4 Dosage Testing on Flounders in Experimental Tanks in 1/1988

Flounder (Juvenile) in Experimental tanks in January 1988, temperature maintain 8 C, 23 days total exposure time. Treated municipal wastewater (TMW) concentration in experimental tanks from 0% to 8%.

C = control dose (0% TMW)

M = medium dose (4% TMW)

H = high dose (8% TMW)

Bile #	Volume (ml)	BaP metabolite concentration (ng/bile)	ppb[a]
C_2 (pooled 6 samples)	0.20	ND[b]	ND
M_2 (pooled 5 samples)	0.22	0.09804	0.4456
H_2 (pooled 4 samples)	0.16	0.1157	0.7237

a. Concentrations of ppb are based upon assumption that density of bile is approximately 1.0 g/ml.

b. ND = undetectable (0.010 ng or lower)

Table 5 BaP Metabolites in Fish Bile from New York Bight and the Reference Site - Long Island in 9/1987

Bile #	BaP metabolite concentration (ng/bile)
1	ND[a]
2	ND
3	ND
4	ND
5	ND
6	ND
7	ND
8	ND
9	ND
10	ND

a. ND = undetectable (0.010 ng or lower)
bile # 1, 2, 3 come from reference site (Long Island)
bile # 4 to 10 come from New York Bight
fish weight and bile volume are not available

Table 6 BaP Metabolites in Fish Bile from New York Bight on 11/19/1987

Winter flounder 11/19/87 newly arrived from New York Bight, food still in gall, and poor bile collection.

Bile #	Fish wt. (g)	Bile vol. (ml)	BaP metabolite conc. (ng/bile)	ppb[a]
11/87-1B (37)	215	0.12	0.1125	0.9375
11/87-2 (38)	150	0.14	ND[b]	ND
11/87-3 (39)	205	0.20	0.0697	0.3485
11/87-9 (45)	186	0.03	ND	ND
11/87-10 (46)	160	0.08	ND	ND
11/87-11 (47)	192	0.12	ND	ND
11/87-12 (48)	189	0.18	0.2871	1.595

a. Calculations of ppb are based upon assumption that the density of bile is approximately 1.0 g/ml.

b. ND = undetectable (0.010 ng or lower)

Table 7 BaP metabolites in Feral Fish Bile from Raritan Bay in 2/1988

Bile #	BaP concentration (ng/bile)
49	ND[a]
50	ND
51	ND
52	ND
53	ND
54	ND
55	ND
56	ND
57	ND
58	0.08575
59	ND
60	0.0465

a. ND = undetectable (0.010 ng or lower)
fish weight and bile volume are not available

Table 8 BaP Metabolites in Fish Bile from New York Bight in 7/1987

Bile #	Fish wt. (g)	Bile vol. (ml)	BaP metabolite conc. (ng/bile)
7/1/87 (33)	337	0.23	0.1496
7/1/87 (34)	275	NA[a]	0.0622
7/1/87 (36)	175	NA	0.0926

a. NA = not available

Table 9 BaP Metabolites in Fish Bile from Raritan Bay in 5/1988

Bile #	Bile vol. (ml)	BaP metabolite concentration (ng/bile)[b]	ppb[a]
61	0.30	ND	ND
62	0.35	ND	ND
63	0.68	0.02856	0.042
64	0.55	ND	ND
65	0.32	ND	ND
66	0.73	0.05210	0.071
67	0.35	ND	ND
68	0.70	ND	ND
69	0.38	0.03995	0.105
70	0.28	ND	ND
71	1.02	ND	ND
72	0.50	0.03266	0.065

a. Calculations of ppb are based upon assumption that density of bile is approximately 1.0 g/ml.

b. ND = undetectable (0.010 ng or lower)

fish weight is not available

Table 10 BaP Metabolites in Fish Biles: Comparison the Results of Fish Captured from Reference Site and Polluted Site

Bile #	BaP metabolite concentration (ng/bile)
S2	0.1488
B2	0.3187
S8	0.2780
B8	0.2932
S3	ND[a]
B11	0.0520

a. ND = undetectable (0.010 ng or lower)

bile # S2, S8, S3 come from reference site (Long Island)

bile # B2, B8, B11 come from New York Bight

Table 11 BaP Metabolites in Fish Livers: Comparison of Results when Reflux with or without Red Phosphorus

Fish were captured from Raritan Bay in 5/1988

Liver #	BaP metabolite concentration (ng/liver)	
	reflux without red phosphorus	reflux with 0.2 g red phosphorus
61	ND[a]	ND
63	ND	6.490
64	2.442	2.769
65	1.300	2.483
66	ND	1.340
68	ND	ND
69	ND	ND
70	ND	ND
71	ND	1.118
72	ND	ND

a. ND = undetectable (0.010 ng or lower)

fish weight is not available

Table 12 BaP Metabolites in Fish Muscle

Muscle #	Fish wt. (g)	Muscle wt. (g)	BaP metabolite conc. (ng/fish)
46M	160	4.93	0.135
C_2 M	NA[a]	NA	0.114
H_1 M	NA	NA	0.0610
H_2 M	NA	NA	0.0705

a. NA = not available

muscle # 46M comes from New York Bight in 11/1987

C_2 M comes from experimental tank (0% TMW)

H_1 M comes from experimental tank (8% TMW)

H_2 M comes from experimental tank (8% TMW)

Table 13 Comparison of BaP Metabolite Concentrations in Fish Bile, Liver and Muscle

	volume or weight (ml or g)	BaP metabolite conc. (ng/fish)
46 Bile	0.08 ml	ND[a]
46 Liver	1.44 g (organic extract)	0.132
46 Muscle	4.93 g (organic extract)	0.135

a. ND = undetectable (0.010 ng or lower)

* No red phosphorus added when reflux for bile and muscle samples.

Table 14 Reduction of BaP Metabolite standard and the Recovery of BaP standard

BaP metabolites	Recovery	
	without phosphorus	with phosphorus
3-OH-BaP	2.0%	2.0%
BaP 3,6-dione	--	0.2%[a]
BaP 4,5-dione	--	0.1%[a]
BaP 7,8-dione	0.1%	0.1%
BaP 6,12-dione	--	ND[a, b]
BaP standard		
BaP	--	37.3% ± 13.5% (n=3)

a. data from Ouyang Zheng (62).

b. ND = undetectable (0.010 ng or lower)

IV. Discussion

The fish we analyzed came from a polluted site. The environment in which they lived, either the water or the sediment, is polluted. The species, winter flounder, is a bottom-dewelling, non-fatty fish. Because it lives near the sediment where many chemicals are accumulated, they may digest more PAH compounds than surface-dewelling fish, but accumulated less PAHs than fatty fish (11).

The results of any study of PAHs in the marine environment are of necessity highly dependent on the analytical methodolgy employed, and the analysis of PAH in marine samples represents one of the more difficult tasks in the field of PAH determination. Concentrations of individual compound are frequently small and amounts of sample (i.e. fish bile, liver) are very limited. In organisms like fish, PAHs are associated with a complex mixture of natural and biogenic hydrocarbons, so sample manipulation and purification can lead to low recoveries.

Because of the complexity of metabolites in fish samples, we can not tell exactly what kinds of PAH metabolite compounds are found in fish bile and liver. Nishimoto, M.M., University of Washington (26), by

using HPLC and [^{3}H]-BaP and [^{14}C]-BaP method on English sole and starry flounder (a species similar to winter flounder) found that the major metabolites produced by both species were the BaP 9,10-dihydrodiol, BaP 7,8-dihydrodiol, 1-hydroxyBaP and 3-hydroxyBaP. He also reported that fish do not produce significant amounts of BaP quinones.

In my research work, the fish samples were found to contain BaP-metabolite-conjugates and BaP. When the hydrolysis step was done, both BaP metabolites and BaP were detected. Using hydriodic acid as a reductor to reduce BaP- diol, hydroxy and quinone were all reduced to BaP. In comparing results of fish liver reflux with or without red phosphorus, I found that phosphrous did help the hydriodic acid to do a slightly better job, as shown in Figure 4.

The results of BaP concentration in fish bile show that, in general, the bigger the fish, the more BaP they uptake. But in fish liver analysis, fish and liver weight are not available and we can not compare the results. For the same individual fish, when there are BaP metabolites in bile, there are usually BaP metabolites present in liver and muscle. The concentration in the liver is the highest probably

because the chemicals which enter body are concentrated and metabolized first in the liver and then pass into the bile.

Fish captured from the reference site were assumed to be clean. However, low levels of BaP metabolites in fishes biles have been found as shown in Figure 5.

Analysis the fish bile sample data for seasonal effects, yields the following results: in February, 16.7% fish (12 samples, fish weight is not available) uptake BaP, the average concentration is 0.06613 ng/bile; in May, 33.3% fish (12 samples, fish weight is not available) uptake BaP, the average concentration is 0.03832 ng/bile; in July, 100% fish (3 samples, average weight is 262 g) uptake BaP; the average concentration is 0.1015 ng/bile; in September, 0% fish (7 samples, fish weight is not available) uptake BaP; in November, 42.9% fish (7 samples, average weight is 203 g) uptake BaP; the average concentration is 0.1564 ng/bile. This monthly variation is shown in Figue 6. According to these data, we find that, during the summer (July), fish uptake more BaP than during the winter. This is probably because during the summer, fish are more active; they eat more, and their metabolism is faster. The September data seems not to be consistant with the

above conclusion. This may be because the fish samples of that month are smaller and smaller fish uptake less BaP than bigger ones.

Generally, the whole bile sample weight is about 0.1 g to 0.3 g. Such a small amount of sample was divided into two parts: one part was used for BaP metabolite analysis; the other for PCBs analysis.

The recovery procedure for BaP standard was 37.3% $\pm$ 13.5%. The recovery of refluxing with HI and HAc is about 30%; recovery of extraction using cyclohexane twice (40 ml and 20 ml) is about 75%; recovery of blow down to 1 ml using air is about 90% (62). The most important step that greatly affects the recovery is the refluxing step. Even though the recovery of BaP standard is not high, it does not necessarily mean that the recovery of the real samples is that low. Because the BaP metabolites are not as light sensitive as BaP itself, so the photodecomposition of BaP metabolites is much less than that of BaP. The same results are obtained when a similar procedure is used to analyze for BaP in human urine.

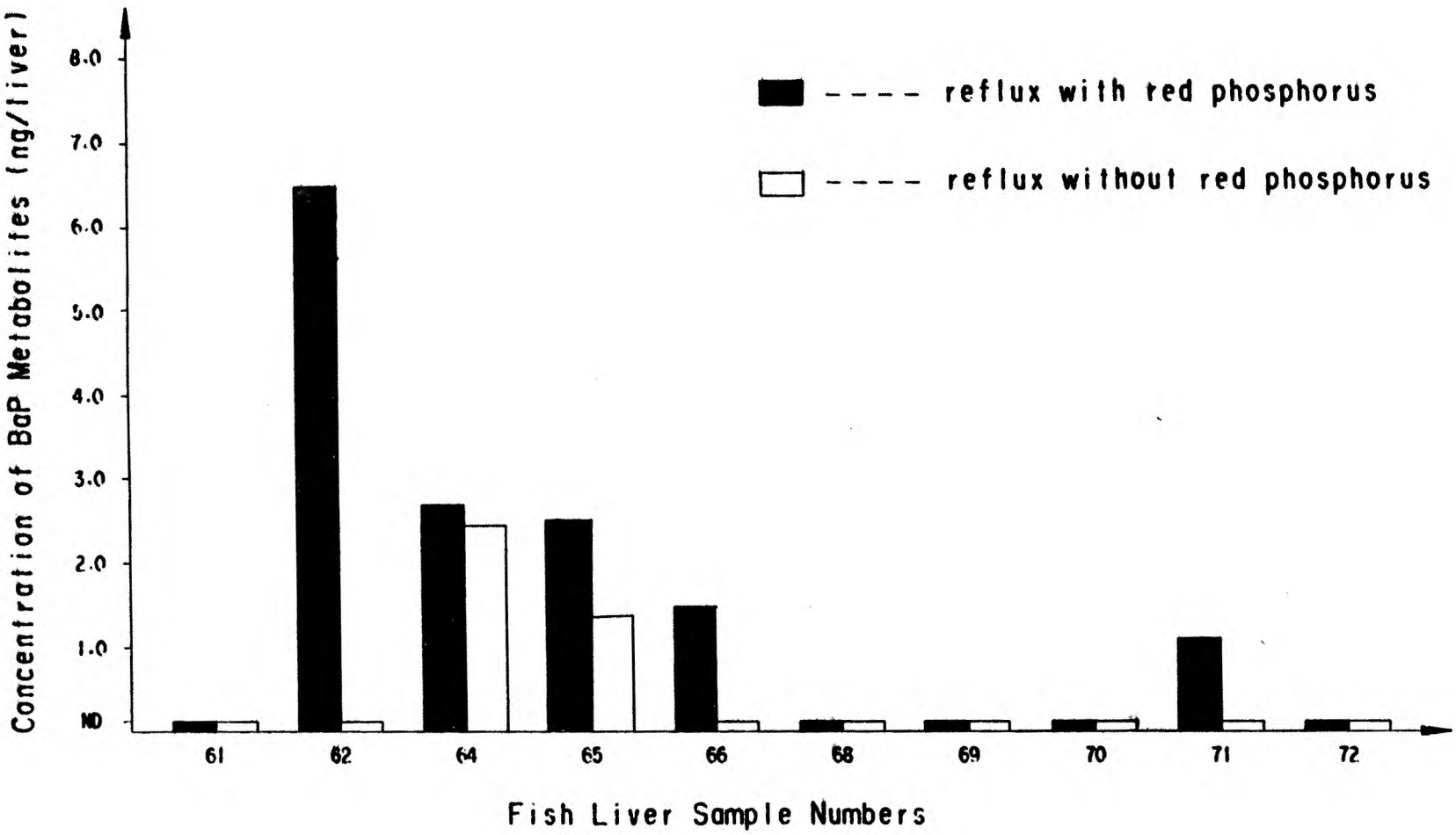


Fig. 5 Comparison the Results of BaP Metabolite Concentration in Fish Livers Refluxing with or without Red Phosphorus

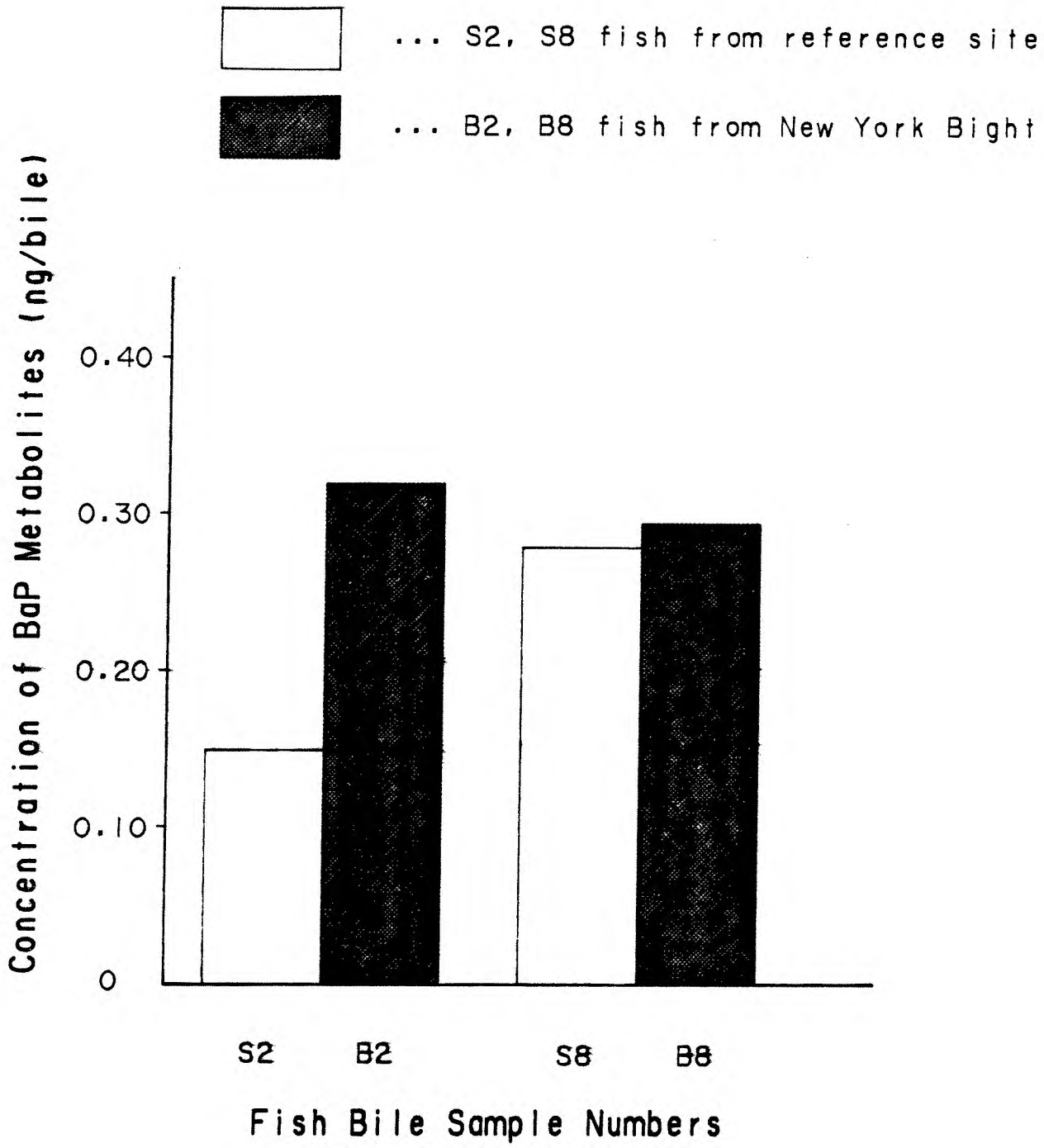


Fig. 6 Comparision the results of BaP metabolite concentration in fish bile from reference site and polluted site

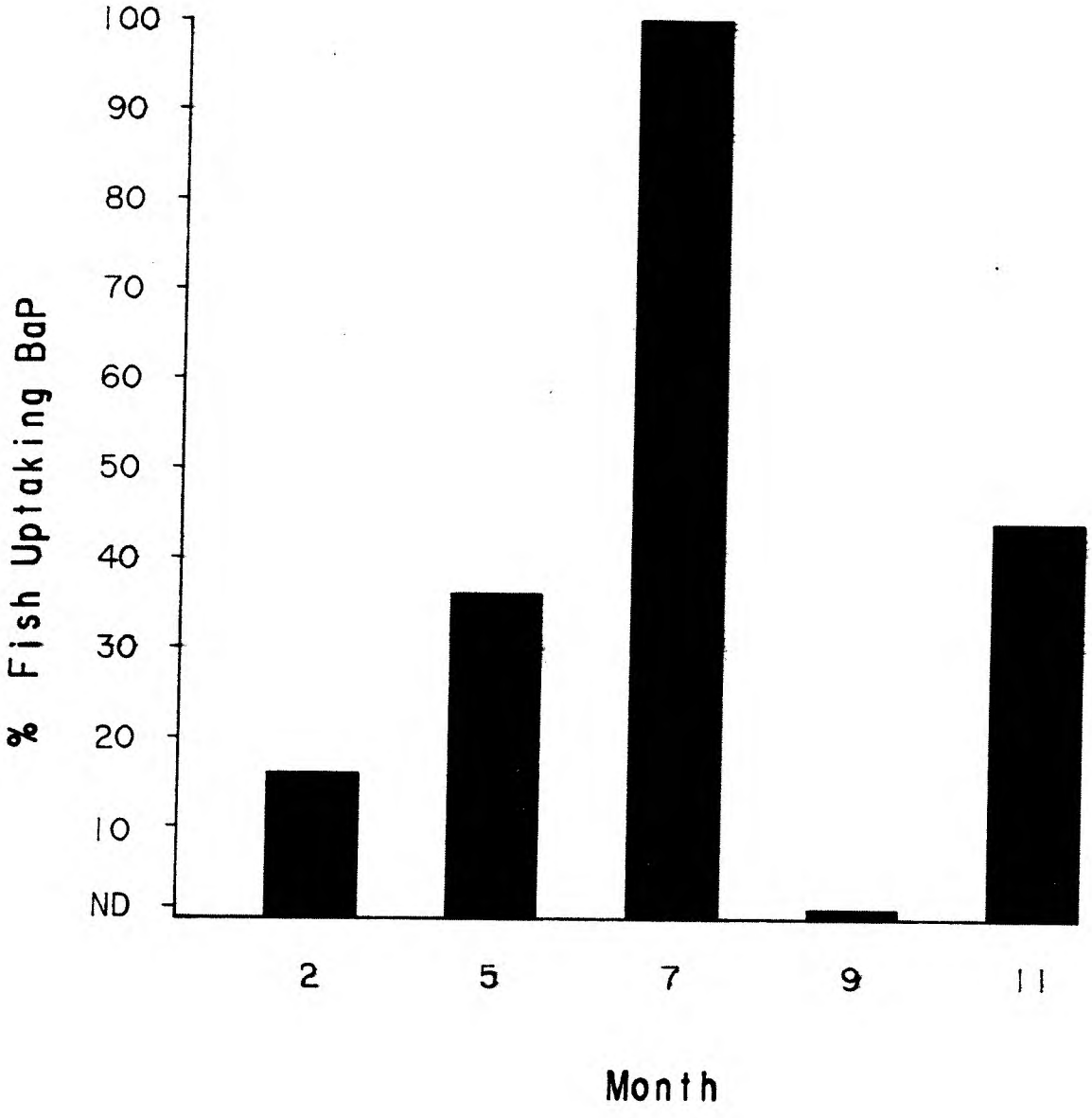


Fig. 7 Seasons Effect on Fish Uptaking BaP

V. Conclusion

The study of BaP concentration in fish (winter flounder) taken from a polluted bay, using a TLC-fluorescence method is a simple and easy analytical procedure for determination of BaP uptake in fish bile, liver and muscle. This is one of the methods to detect BaP metabolites in marine organisms. In this method, hydriodic acid reflux with BaP metabolites with or without red phosphrous is a very important step.

The winter flounder is a bottom-dwelling fish which was captured from New York Bay where water and sediment accumulate a lot of toxic chemicals such as PAHs and BaP. An analysis of the results show that fish do take up PAHs or BaP from the environment in which they live; the bigger the fish, the more BaP they uptake. Fish sex seems to have no effect on the fish taking up BaP, but seasons do. Generally, during the summer, fish take up more BaP than the winter. In the same fish, if BaP metabolites can be found in liver, there might be BaP metabolites in bile and muscle, but the concentration of BaP metabolites in fish liver is the highest.

VI. References

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