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Comparison of Concentrations and Profiles of Polycyclic Aromatic Hydrocarbon Metabolites in Bile of Fishes from Offshore Oil Platforms and Natural Reefs Along the California Coast

Open-File Report 2012–1248

U.S. Department of the Interior
U.S. Geological Survey

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KEN SALAZAR, Secretary

U.S. Geological Survey
Marcia K. McNutt, Director

U.S. Geological Survey, Reston, Virginia: 2012

Suggested citation:
Gale, R.W., Tanner, M.J., Love, M.S., Nishimoto, M.M., and Schroeder, D.M., 2012, Comparison of concentrations and profiles of polycyclic aromatic hydrocarbon metabolites in bile of fishes from offshore oil platforms and natural reefs along the California coast: U.S. Geological Survey Open-File Report 2012–1248, 27 p. and supplemental tables.

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Conversion Factors

SI to Inch/Pound

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
micrometer (µm)	0.00003937	inch (in.)
nanometer (nm)	0.00000003937	inch (in.)
meter (m)	3.281	foot (ft)
kilometer (km)	0.6214	mile (mi)
	Volume	
liter (L)	61.02	cubic inch (in^3)
milliliter (mL)	1 x 10^{-3} liter	
microliter (µL)	1 x 10^{-6} liter	
	Flow rate	
milliliter per minute (mL/min)	0.06102	cubic inches per minute (in^3/min)
	Mass	
metric ton (t)	1.102	ton, long (2,240 lb)
kilogram (kg)	2.205	pound avoirdupois (lb)
gram (g)	0.03527	ounce, avoirdupois (oz)
milligram (mg)	1 x 10^{-3} gram	
microgram (µg)	1 x 10^{-6} gram	
nanogram (ng)	1 x 10^{-9} gram	
	Concentration	
milligram per milliliter (mg/mL)	=	part per thousand (ppt; 103)
microgram per milliliter (µg/mL)	=	part per million (ppm; 106)
nanogram per milliliter (ng/mL)	=	part per billion (ppb; 109)
nanogram per milligram (ng/mg)	=	part per million (ppm; 106)
	Conductivity	
megaohms per centimeter (MΩ/cm)	=	0.054 micro-siemens per centimeter (µS/m) [this is a reciprocal relationship]

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F=(1.8\times°C)+32$$

Concentrations of chemical constituents in liquid solutions (reagent solution, calibration standards, or bile) are given in nanogram per milliliter (ng/mL), micrograms per milliliter (µg/mL), or milligrams per milliliter (mg/mL).

Concentrations of chemical constituents in solid matrices (protein) are given in nanogram per milligram (ng/mg).

Comparison of Concentrations and Profiles of Polycyclic Aromatic Hydrocarbon Metabolites in Bile of Fishes from Offshore Oil Platforms and Natural Reefs Along the California Coast

Robert W. Gale[1], Michael J. Tanner[1], Milton S. Love[2], Mary M. Nishimoto[2], and Donna M. Schroeder[3]

Abstract

To determine the environmental consequences of decommissioning offshore oil platforms on local and regional fish populations, contaminant loads in reproducing adults were investigated at seven platform sites and adjacent, natural sites. Specimens of three species (Pacific sanddab, *Citharichthys sordidus*; kelp rockfish, *Sebastes atrovirens*; and kelp bass, *Paralabrax clathratus*) residing at platforms and representing the regional background within the Santa Barbara Channel and within the San Pedro Basin were collected. Some of the most important contaminant classes related to oil operations are polycyclic aromatic hydrocarbons (PAHs) because of their potential toxicity and carcinogenicity. However, acute exposure cannot be related directly to PAH tissue concentrations because of rapid metabolism of the parent chemicals in fish; therefore, PAH metabolites in bile were measured, targeting free hydroxylated PAHs (OH-PAHs) liberated by enzymatic hydrolysis of the bound PAH glucuronides and sulfates. An ion-pairing method was developed for confirmatory analysis that targeted PAH glucuronides and sulfates. Concentrations of hydroxylated PAHs in all samples (76 fish from platforms and 64 fish from natural sites) were low, ranging from less than the limits of detection (5 to 120 nanograms per milliliter bile; 0.03 to 42 nanograms per milligram protein) to a maximum of 320 nanograms per milliliter bile (32 nanograms per milligram protein). A previously proposed dosimeter of PAH exposure in fish, 1-hydroxypyrene, was not detected at any platform site. Low concentrations of 1-hydroxypyrene were detected in 3 of 12 kelp rockfish collected from a natural reef site off Santa Barbara. The most prevalent OH-PAH, 2-hydroxyfluorene, was detected at low concentrations in seven fish of various species; of these, four were from two of the seven platform sites. The greatest concentrations of 2-hydroxyfluorene were found in three fish of various species from Platform Holly and were only about threefold above low, yet quantifiable, concentrations found in three fish from Horseshoe Reef, East Anacapa Island, and Coche Point natural sites; the mean concentrations among all sampling sites were not measurably different.

Introduction

The U.S. Bureau of Ocean Energy Management (BOEM) defines decommissioning as the process of ending oil, gas, or sulfur operations and returning the lease or pipeline right-of-way to a condition that meets the requirements of the regulations. The BOEM works to ensure that wells are plugged to prevent pollution, that pipelines are decommissioned and sometimes removed to prevent seepage of hydrocarbons, and to resolve conflicts with other users of the Outer Continental Shelf (Love and others, 2003).

To determine the environmental consequences of decommissioning platforms on local and regional fish populations, the sources of fish recruitment, general health, and contaminant load in reproducing adults must be known. This information will be especially important for platforms harboring large numbers of resident reproducing adults and serve as nursery habitat for juvenile fishes. Juvenile fishes from these platforms potentially could migrate to natural areas and help to replenish populations that are commercial and recreational fishery resources. Decommissioned platforms also provide potential for recruitment and productivity of adults from species that are regionally depleted and are being considered for listing by the Endangered Species Act (U.S. Fish and Wildlife Service, 2012).

The question of contaminant release at platforms continues to be a major concern. The construction and aging of platforms create new reef-like physical environments for fish

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recruitment and productivity. However, platform operation introduces the potential for release of petroleum residues, drilling fluids, and other industrial chemicals related to platform operation and maintenance (Steinberger and others, 2004). Though the question of contamination continues to arise during most discussions of resident fish and shellfish at platforms, very little study has been made of this subject. The most common contaminants discharged at platforms are hydrocarbons and trace metals. There is some evidence from older platforms in state waters that these contaminants accumulate in the shell mounds that build up under platforms (Love and others, 2003). The issue of trace metal contamination was recently reported (Love and others, 2009).

Given the concerns with mercury, organochlorine pesticides, and polychlorinated biphenyls in popular edible fishes, it is timely to identify and quantify contaminants in various resident fish at platforms. Persistent organochlorine pesticides and industrial chlorinated aromatic hydrocarbon chemicals accumulate in lipophilic tissues and residues measured in these tissues reflect contaminant exposure. Some of the most important contaminant classes related to oil operations are polycyclic aromatic hydrocarbons (PAHs) because of their potential toxicity and carcinogenicity (Ariese and others, 1993, 1997, 2005; Escartin and Porte, 1999; Ruddock and others, 2002; St. Ferrari and others, 2002; Jonsson and others, 2003; Fillmann and others, 2004; Hellou and others, 2006; Huggett and others, 2006; Vuorinen and others, 2006; Johnson-Restrepo and others, 2008). However, acute PAH exposure cannot be directly related to tissue concentrations of the parent chemicals in fish. Studies of the fate and dynamics of PAHs in fish indicate that tissue residues of parent PAHs (nonmetabolized polycyclic aromatic hydrocarbons) are rapidly metabolized; forming conjugated hydroxylated PAH glucuronides (or sulfates) in bile (Ariese and others, 1993, 1997, 2005; Johnson-Restrepo and others, 2008). Exposure to PAHs induces *Phase I* and *Phase II* enzymatic detoxification to form hydrophilic metabolites. In *Phase I* reactions, mixed-function oxidases such as cytochrome *P450* monooxygenase, epoxide hydrolase, and others catalyze the addition of one or more hydroxyl groups to the parent PAH. *Phase II* reactions proceed to form polar conjugates of the hydrolyzed PAH with polar moieties, such as glucuronides, sulfates, and glutathiones. The liver is the primary organ for metabolism and the polar metabolites produced are secreted into the bile. Bile is stored in the gall bladder before final excretion. Therefore, exposure to PAHs is better estimated by comparison of concentrations of these PAH metabolites in fish bile and to a lesser extent in the liver (Ariese and others, 1997; Escartin and Porte, 1999; Fillmann and others, 2004; Hellou and others, 2006; Huggett and others, 2006). Chronic bioaccumulation of less metabolized, higher molecular weight PAHs (for example, fluoranthene, pyrene, benzofluoranthenes, benzopyrenes, etc.) also occurs and can be used to assess cumulative exposure over the life history of individual fish (Ariese and others, 1993, 1997, 2005).

Fish collected near natural tar seeps, at nearby natural reefs, and at locations that are from point sources of petroleum previously indicated concentration gradients of PAH metabolites in bile (Ariese and others, 1993; Ruddock and others, 2002; Vuorinen and others, 2006; Johnson-Restrepo and others, 2008). Natural seeps have widely varying hydrocarbon profiles, ranging from very similar to crude oils through tar-like materials depleted in labile, lower molecular weight hydrocarbons. Because of natural regional hydrocarbon sources in the Southern California Bight (Loreson and others, 2009), PAH levels in fish from platform sites must be interpreted within the context of local background levels. To establish the relative importance of any potential platform contamination, given the potential for exposure from natural sources within the region, the exposure of resident fish populations to PAHs at oil platforms and at natural areas adjacent to platforms was investigated for this study.

The variability in levels of PAH metabolites within resident populations of platform and natural seep fishes also is unknown. This variability results from the distribution of exposures of individual fish of differing ages to parent PAH sources from localized water concentrations and individual prey items. Additional variability is introduced by movement of fishes to and from different populations (Straughan and others, 1982; Peterson and others, 1996; Seruto and others, 2005). To determine better the variability in exposure from movement of fish, different exposure scenarios, and rapid metabolism of PAHs, analysis of PAH metabolites in bile was performed on individual fish. Analysis of the individual fish was undertaken to provide critical information about the variance of PAH exposure in fish populations at platforms compared to natural areas, and insight into whether there is any potential risk to nearby areas from movement or recruitment of fish from platforms.

Materials

Water was obtained from a Millipore Synergy UV 18 megaohms per centimeter (MΩ/cm) water-purification system (Millipore Corporation, Billerica, Massachusetts). Methanol (Optima® grade), potassium hydroxide, triolein, and ascorbic acid (American Chemical Society (ACS) grade) were received from Fisher Scientific (Fair Lawn, New Jersey). Tetrabutylammonium phosphate (TBAP; ion-pairing reagent; 96 percent, reagent grade) was received from Acros Organics, Geel, Belgium.

The polycyclic aromatic hydrocarbon (PAH) mixture used for the fish-dosing part of the study was at a concentration of 2,000 micrograms per milliliter (µg/mL) in dichloromethane (Standard Mix #5; Restek Corp., Bellefonte, Pennsylvania). Triphenylamine (98 percent, reagent grade), 1-OH-naphthalene and 2-OH-naphthalene (99 percent, reagent grade), 2-OH-fluorene and 1-OH-pyrene (98 percent, reagent grade), 9-OH-fluorene (96 percent, reagent grade), 9-OH-phenanthrene (90 percent, technical grade), phenolphthalein and phenolphthalein-β-glucuronic acid (ACS grade), bilirubin (mixed isomers), bovine serum albumin Type V

(BSA, referred to as albumin), and sulfatase from *Helix pomatia*, Type H-2 aqueous solution: greater than or equal to 2,000 units per milliliter (units/mL) sulfatase activity and greater than or equal to 100,000 units/mL β-glucuronidase activity (refer to Sigma-Aldrich Inc., St. Louis, Missouri, 2012, for definitions of activity units), were received from Sigma-Aldrich, St. Louis, Missouri (Mo.). 1-OH-chrysene (> 99 percent, reagent grade) was received from Midwest Research Institute – NCI, Kansas City, Mo. 3-OH-benzo(*a*) pyrene (> 99 percent, reagent grade) was received from the European Commission Joint Research Center, Institute for Reference Materials and Measurements, Brussels, Belgium.

Pierce BCA Protein Assay Kit—Reducing Agent Compatible (Thermo Scientific, Rockford, Illinois) was used for bile protein analysis. Spectrophotometric analysis was performed using a Beckman DU640 Spectrophotometer [Beckman Instruments Inc., Fullerton, California (Calif.)]. Luna 3 micrometer (µm) $C_{18(2)}$ 100 Ångström (Å) 150 x 4.60 millimeter (mm) and 150 x 2.00 mm liquid chromatography columns with Security Guard C_{18} 4 x 2.00 mm guard columns were received from Phenomenex Inc., Torrance, Calif. The high-performance liquid chromatographic (HPLC) analysis was performed on an Agilent 1100 Series G1313A Autosampler, G1312A Binary Solvent Delivery Pump, G1316A Column Heater, G1321A Fluorescence Detector, G1314A Variable Wavelength Detector, and ChemStation Revision B.03.02 (341) Software (Agilent Technologies, Santa Clara, Calif.).

A limiting factor to the analysis of PAH metabolites as OH-PAHs in bile is the lack of certified reference materials with biologically incorporated concentrations of PAH-glucuronides. Ariese and others (2005) reported the analysis of two fish-bile reference materials: Bile Certified Reference (BCR) 720 obtained from flounder (*Platichthys flesus*) exposed to PAH-contaminated sediment, and BCR 721 obtained from plaice (*Pleuronectes platessa*) exposed to crude oil. However, at the time of this work these materials were no longer available from the commercial supplier, Sigma-Aldrich. Therefore, bile PAH metabolite (positive control) reference material was prepared by Columbia Environmental Research Center (CERC) for use with this study. All collection, handling, and euthanasia procedures followed animal care and use guidelines (American Fisheries Society and others, 2004).

A PAH dosing solution was prepared by adding 1 mL of a stock solution of 2,000 µg of 16 priority pollutant PAHs in dichloromethane to 2 mL triolein. The carrier solvent was then removed by gentle evaporation under a stream of nitrogen at ambient temperature. The 1,000 µg/mL PAH triolein dosing solution was stored at -20 degrees Celsius (ºC).

Six largemouth bass (*Micropterus salmoides*, LMB) ranging in mass from about 0.5 to 1 kilograms (kg) were caught by hook and line from the catch lagoon at CERC. The fish were tagged, transported to a large indoor enclosure, and allowed to equilibrate for 1 hour. The weight of each fish was recorded and each fish was injected with the 1,000 µg/mL PAH triolein dosing solution at injection volumes corresponding to 200 to 500 µg of each PAH per fish. Using a 1 mL disposable syringe with an 18 gauge (0.838 mm inner diameter x 1.27 mm outer diameter) needle, injections were made directly into the abdominal cavity of each fish. The fish were returned to the enclosure for a 5-day incubation period. Two of the fish died during the incubation period and the gall bladder samples were irretrievable. The remaining fish were euthanized and the gall bladders were excised. The bile from each gall bladder was expelled through the bile duct into individual amber vials and stored at -20 ºC.

Analytical Methods

Both free hydroxylated PAHs and PAH-glucuronide conjugates in fish bile have been studied by a range of methods (Ariese and others, 1993; Ruddock and others, 2002; St. Ferrari and others, 2002; Jonsson and others, 2003; Vuorinen and others, 2006), including: enzyme-linked immunosorbent assays, direct fluorometry, synchronous fluorescence scanning spectrometry (SFS), and HPLC combined with direct fluorescence or SFS detection, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry. To provide the best evidence of relative PAH exposure, while analyzing a large number of fish from platforms and natural areas and maintaining efficient analytical throughput, high-performance liquid chromatography with direct fluorescence detection (HPLC-F) was selected for this study. HPLC-F is a moderately information-rich technique with the well-demonstrated capability to separate and quantify low concentrations of PAH metabolites in the bile of fishes. The HPLC-F technique enabled acquisition of information about both the levels and the profiles of PAH exposure, yielding insights into the similarities and differences among platform sites and natural areas.

Bile fluid volumes are extremely variable and are sensitive to the feeding and fasting status of individual fish; overall bile concentration markedly increases or decreases as water is retained or eliminated. The major bile components bilirubin and total protein, vary in concentration with water retention and thus reflect feeding status. It is generally accepted that the concentrations of PAH metabolites in bile co-vary with these major components (Ariese and others, 1993, 1997, 2005; Escartin and Porte, 1999; Ruddock and others, 2002; St. Ferrari and others, 2002; Jonsson and others, 2003; Vuontisjärvi and others, 2003; Fillmann and others, 2004; Hellou and others, 2006; Huggett and others, 2006; Pikkarainen, 2006; Vuorinen and others, 2003, 2006; Johnson-Restrepo and others, 2008; Goksøyr and others, 2009; Insousti and others, 2009); therefore, bilirubin and protein content may be used to normalize metabolite concentrations between fish of different feeding status. Of the two bile components, bilirubin is more commonly measured (Ariese and others, 1997; Johnson-Restrepo and others, 2008); however, inconsistencies in bilirubin content measurements have been reported (Ariese and others, 1993, 1997, 2005; Huggett and others, 2006). Current research indicates that protein content may be the

more appropriate and consistent measurement of bile content and feeding status, which reflects the condition of the gall bladder (full or empty and with more concentrated or dilute bile) (Ariese and others, 1993, 1997, 2005; St. Ferrari and others, 2002; Huggett and others, 2006; Johnson-Restrepo and others, 2008). In this study bilirubin and total protein content were determined and the utility of each of these measurements for normalizing PAH metabolite concentrations in bile were compared.

Methodologies for analysis of PAH metabolites in bile may target either free OH-PAHs resulting from enzymatic hydrolysis of the bound PAH glucuronides and sulfates, or direct analysis of the PAH glucuronides and sulfates. In this work, the primary method targeted the free OH-PAHs, which required hydrolysis followed by HPLC-F. The direct analysis of the glucuronides and sulfates by ion-pairing HPLC-F was used for determining the completeness of hydrolysis, for confirmation of the OH-PAHs in bile, and for investigating additional bile components.

Total Protein

The analysis of total protein was based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (Smith and others, 1985), and spectrophotometric detection of the copper-bicinchoninic acid (BCA) complex. A solution-compatibility reagent, which modifies disulfide reducing agents such as dithiothreitol, 2-mercaptoethanol, and tris-(2-carboxyethyl) phosphine (TCEP), was used to minimize the effects of these extraneous copper reducing agents (Kessler and Fanestil, 1986). The first reaction step involves the chelation of copper with protein in alkaline sodium potassium tartrate to form a blue complex (the biuret reaction). The number of peptide bonds participating in the reaction is proportional to the intensity of the color. The second color-developing step involves the addition of BCATM reagent to react the cuprous cation formed in the biuret reaction. The resultant purple reaction product exhibits a linear concentration response, which is quantified at an absorbance of 562 nanometers (nm) (Thermo Scientific Inc., Rockford, Illinois, 2012).

Total protein concentrations were determined using a BCA protein assay and calibration with albumin standards. The protein calibration standards are prepared from a 2,000 µg/mL albumin solution. The albumin stock solution was transferred to an amber 1.5 mL vial; additional standard levels were prepared by serial dilution. The calibration standards were stored sealed, at room temperature, when not in use and were prepared each day before each protein analysis. The Compatibility Reagent™ was prepared by adding 500 microliters (µL) of reconstitution buffer to the compatibility reagent tube. Following thorough mixing, 500 µL of water was added and mixed for at least 30 seconds to facilitate dissolution of solids prior to use. The compatibility reagent is stable for as much as 8 hours, if protected from light, and was prepared prior to each set of bile protein analyses. The BCA reagent was prepared by mixing 50 parts BCA reagent A to 1 part BCA reagent B and mixing thoroughly by gentle swirling (both reagents are supplied with the kit). The final volume of BCA reagent prepared was based on the total number of field samples, standards and quality control (QC) samples for analysis (1 mL of BCA reagent per sample).

Protein analysis was performed directly in disposable cuvettes using 25 µL aliquants of the bile sample diluted tenfold with water. Compatibility reagent (25 µL) was added to each cuvette and mixed gently. Each cuvette was capped, the samples were wrapped in aluminum foil, and the samples were incubated at 37 ºC for 15 minutes. Following the initial incubation, 1 mL of BCA reagent was added to each sample, the cuvettes were recapped, and the samples were again gently mixed, rewrapped in aluminum foil and incubated at 37 ºC for an additional 30 minutes. The samples were then allowed to cool to ambient temperature for at least 5 to 10 minutes. Absorbance readings for each sample were recorded at a wavelength of 562 nm. The raw absorbance data were entered into a spreadsheet and corrected for procedural blank (water and compatibility blank solution), and a linear regression curve was constructed. The background corrected protein concentrations were adjusted for the dilution factor, and reported in units of µg-protein (as albumin) per mL. Accuracy and precision were monitored by the replicate analysis of quality control materials and standards. To determine further the reproducibility of the protein analysis, selected field samples were analyzed in replicate.

Bilirubin

To complement the protein normalization method, bilirubin was investigated as another means to normalize PAH metabolite concentrations in bile (Ariese and others, 1997). The solubility of bilirubin in basic aqueous solutions is >10,000 micromolar (µM), and bilirubin is not soluble in acidified aqueous solution, nonpolar solvents, or alcohols, and has only limited solubility in aromatic solvents, ethers, and ketones as reported by Brodersen (1979). The main difficulty with bilirubin monitoring methods is that oxidation and aggregation of bilirubin over time are associated with a concurrent decrease in concentration observed by absorbance in the wavelength range from 448 to 458 nm; this is a result of the conversion of bilirubin to biliverdin (Brodersen, 1979). Under normal conditions, bilirubin is unstable and is continuously being converted to biliverdin; however, Doumas and others (1987) developed a method for direct (total) bilirubin by conversion of bilirubin to biliverdin using bilirubin oxidase and measurement of absorbance at 380 nm with the result being reported as total biliverdin concentration; the Doumas method was used in this study. Normalized concentrations of hydroxylated PAHs in bile to bile bilirubin concentrations and to bile protein concentrations were compared.

PAH-Glucuronide and -Sulfate Hydrolysis

The characterization and quantification of PAH metabolites in the bile of fishes as free OH-PAHs required the complete hydrolysis of PAH-glucuronides and -sulfates. The hydrolysis of phenolphthalein-β-glucuronide to the phenolphthalein and glucuronic acid products was used to monitor the enzymatic hydrolysis efficiency. The reported amounts of combined sulfatase and β-glucuronidase enzymes used for the hydrolysis of PAH metabolites in bile ranged from 30 units/mL (Ariese and others, 1997) to 2,000 units/mL (Escartin and Porte, 1999).

The hydrolysis of phenolphthalein-β-glucuronide was monitored as a function of time at ambient temperature and at 37 ºC for various glucuronide concentrations. Both the phenolphthalein-β-glucuronide and the free phenolphthalein product were monitored by ion-pairing HPLC-F. The optimized conditions for phenolphthalein-β-glucuronide hydrolysis to the phenolphthalein product were verified by analysis of replicate analyses of phenolphthalein-β-glucuronide fortified negative control bile. Phenolphthalein-β-glucuronide hydrolysis samples were included in the overall method validation and separate phenolphthalein-β-glucuronide hydrolysis samples were used to monitor hydrolysis in each analytical set.

The stock *Helix pomatia*, Type H-2 aqueous enzyme solution contained greater than or equal to 2,000 units/mL sulfatase and greater than 100,000 units/mL β-glucuronidase enzyme activity. A working enzyme solution containing greater than or equal to 20 units/mL sulfatase and greater than or equal to 100 units/mL β-glucuronidase was prepared by diluting the aqueous *Helix pomatia*, Type H-2 solution 100-fold with water; the solution was stored at 4 °C when not in use. This working solution was used for the conversion of glucuronides and sulfates to hydroxylated products for HPLC-F analysis. The final sample volume for HPLC-F is typically 1.00 mL in water:methanol [1:1 volume ratio, (v:v)]; therefore, for an aqueous incubation volume of 500 µL, the volume of hydrolysis solution needed was calculated from the volumes of the bile sample aliquant (usually 10 µL), the internal standard (10 µL), and any QC fortification solution added. The reaction was stopped by addition of 500 µL methanol, producing the final water:methanol sample solution.

The bile samples were removed from the freezer and allowed to reach room temperature while wrapped in aluminum foil. The appropriate volumes of hydrolysis solution were added to 15 mL culture tubes and internal standard solution added. The tubes were mixed by vortexing for 10 to 15 seconds and then aliquants of the bile samples were added. After thorough mixing, the samples were wrapped in aluminum foil and incubated at 37 ºC for 3 hours. Following incubation, the samples were removed and allowed to return to ambient temperature. The reaction was halted by addition of 500 µL of methanol and thorough mixing, and allowed to stand for at least 15 minutes. The samples were filtered (Whatman 13 mm GD/X disposable PTFE filters, 0.45 µm) directly into 1.5 mL amber autosampler vials for analysis. The samples were stored at -20 ºC until analysis. All gall bladder contents (bile or gall bladder water homogenates) were recovered, hydrolyzed, and analyzed by HPLC-F within 30 days of receipt.

High-Performance Liquid Chromatography with Ultraviolet-Visible and with Fluorescence Detection

Most HPLC methods reported the use of reverse-phase gradient methods with octadecasiloxane (C_{18}) stationary phases. The mobile phases used were dependent on the analytes quantified: for free OH-PAHs in hydrolyzed bile samples, methanol–water or acetonitrile–water mobile phases were used; for PAH glucuronides and sulfates in non-hydrolyzed bile samples, the simple reversed-phase solvent systems were replaced with aqueous ion-pairing mobile phases such as TBAP-methanol–TBAP-water (Ariese and others, 1997). The C_{18} stationary phase and a reversed-phase methanol–water gradient mobile phase system were selected for analysis of free OH-PAHs as hydrolyzed bile metabolites in this study. The starting fraction of organic modifier and the composition of the gradient were adjusted to produce baseline separation of the nine-component standard. Methods development used ultraviolet (UV) detection at 254 nm for broad-based screening, which was replaced with the more selective and sensitive fluorescence detection in the analytical method, though UV detection continued to be used post-fluorescence detection for additional screening of field samples. The basic parameters for the excitation and emission wavelength pairs for the analytes are replete in the literature and provided a starting point for optimizing the excitation and emission wavelength pairs for the analytes. All detectors have a unique set of operating characteristics (Hewlett-Packard, Inc., Walbronn, Germany, 1999), and spectral information for excitation and emission maxima were optimized for the analytes under the gradient conditions used for their separation. Solutions of individual OH-PAHs were characterized for excitation in the wavelength range from 230 to 380 nm and for emission in the wavelength range from 280 to 450 nm to identify maxima that allowed sufficient sensitivity while minimizing extraneous interferences with detection and confirmation.

The commercial availability of PAH-glucuronide reference materials is limited; however, specific OH-PAHs are available from commercial suppliers. Standard materials were obtained for preparation of a multicomponent calibration solution representative of the different PAHs expected to be present in environmental samples: (2-ring PAHs) 1-OH-naphthalene, 2-OH-naphthalene, 2-OH-fluorene, and 9-OH-fluorene; (3-ring PAHs) 9-OH-phenanthrene; (4-ring PAHs) 1-OH-pyrene and 1-OH-chrysene; and (5-ring PAHs) 3-OH-benzo(*a*)pyrene. Triphenylamine was selected as the procedural internal standard (PIS), which is processed through all preparatory steps to monitor analyte recovery, as reported by Jonsson and others (2003). A working mixture of analytes and internal standard was prepared to have similar fluorescence detection responses for each component.

Quality Control

Exposure to light was avoided or minimized during all sample and standard handling steps to reduce losses from photodegradation of PAH metabolites. Method performance was monitored using several QC measures. The hydrolysis of phenolphthalein-β-glucuronide to phenolphthalein and glucuronic acid was used to assess the efficiency of the enzymatic hydrolysis reaction and infer the completeness of PAH glucuronide and sulfate hydrolysis; this is the first report of monitoring hydrolysis efficiency with this procedure. Negative control materials refer to samples with the same or a similar matrix, but without or with low concentrations of target analytes and is used to represent a real sample without detectable analyte concentrations; negative control materials are also known as matrix blanks. Fortified negative control materials refer to negative control materials fortified with concentrations of the target analytes. Positive control materials refer to real samples with incurred concentrations of analytes. The LMB positive control bile was used as a surrogate for biologically incorporated PAH metabolites (refer to the Control Materials subsection in the Results and Discussion). This positive control bile also was used to monitor hydrolysis reaction performance. Overall method background was monitored using procedural blanks (no bile) and Black Carp (*Mylopharyngodon piceus*, BC) negative control bile processed through all sample preparation steps. Because of the lack of PAH-glucuronide standard materials, method recoveries of the OH-PAH analytes were monitored by fortifying the BC negative control bile with the eight targeted OH-PAHs prior to processing through all sample preparation steps. The procedural internal standard (triphenylamine) was added to all samples at the beginning of the preparatory scheme, prior to the hydrolysis step, to monitor sample handling losses and overall method recoveries for each sample. Method reproducibility was monitored by preparation of replicate (usually triplicate) bile from field samples within each analytical set.

Results and Discussion

A high-performance liquid chromatographic method with fluorescence detection was developed for the analysis of polycyclic aromatic hydrocarbon (PAH) metabolites in bile targeted free hydroxylated PAHs (OH-PAHs) resulting from enzymatic hydrolysis of the bound PAH glucuronides and sulfates and was applied to measurement of PAH metabolites in bile of fishes collected near platforms and nearby natural areas. The enzymatic step was characterized in detail during methods development to define conditions for fast, efficient, and complete hydrolysis. Control samples fortified with phenolphthalein-β-glucuronide or largemouth bass (LMB) PAH metabolites were processed with each set to ensure that conditions for complete hydrolysis were maintained. An ion-pairing method was developed for confirmatory analysis that targeted PAH glucuronides and sulfates. Normalization of OH-PAH concentration by bilirubin and total protein content was compared for both control bile and for field bile samples. After validation, these methods were applied to field samples of fish resident at platforms and natural areas.

Part I—Methods Development

High-Performance Liquid Chromatography with Fluorescence Detection

Standards for the calibration curve were prepared by serial dilutions of a working stock standard solution prepared by combining individual OH-PAH stock solutions at concentrations that provided similar fluorescence responses for each analyte at its excitation and emission wavelength pair. Under similar response conditions, the calibration standard concentration ranges were linear over approximately a 1,000-fold concentration range and are summarized in table 1.

Table 1. Calibration standard parameters for HPLC-F separation and quantification of hydroxylated PAHs.

[HPLC-F, high-performance liquid chromatography-fluorescence detection; hydroxylated-PAHs, hydroxylated-polyaromatic hydrocarbons; min, minutes; excitation, fluorescence excitation wavelength; emission, fluorescence emission wavelength; nm, nanometers; ng/mL, nanograms per milliliter; PIS, Procedural internal standard.]

Calibration parameters	Retention time (min)	Excitation/ emission (nm)	Linear calibration range (similar fluorescence response factors) (ng/mL)
1-hydroxynaphthalene	17.08	290 / 380	4.4 – 5280
2-hydroxynaphthalene	18.38	290 / 380	1.2 – 1450
2-hydroxyfluorene	23.21	270 / 320	0.30 – 360
9-hydroxyfluorene	19.99	270 / 320	1.6 – 1960
9-hydroxyphenanthrene	25.88	256 / 380	0.32 – 390
1-hydroxypyrene	29.56	330 / 380	0.60 – 720
1-hydroxychrysene	32.03	268 / 380	0.60 – 720
3-hydroxybenzo(*a*)pyrene	35.36	380 / 420	1.0 – 1210
triphenylamine (PIS)	38.49	289 / 365	0.67 – 800.

The post-column flow path was routed through the fluorescence detector and then through the variable wavelength ultraviolet-visible (UV-Vis) detector to avoid any potential ultraviolet-induced degradation of the analytes. Ultraviolet detection was used for method development and was monitored during sample analysis to provide additional information about interferences encountered during the analysis of field samples. The enhancements in sensitivity and specificity for fluorescence detection may be seen by comparing the HPLC-F

chromatogram with selected excitation and emission wavelength pair windows, in figure 1*A*, with the HPLC-UV-Vis chromatogram with UV detection at 254 nanometers (nm), in figure 1*B*.

Using fluorescence detection increased sensitivity 10- to 20-fold compared with normal UV detection; selectivity was likewise increased by selection of excitation and emission wavelength pairs that avoided nonspecific fluorophors. However, UV detection was beneficial to determine the retention time windows for the fluorescence excitation and emission wavelength pairs during methods development, and for subsequent quality control (QC). The optimized chromatographic conditions developed for the routine analytical separation of the targeted OH-PAHs are presented in table 2. The retention-time windows for fluorescence detection using the respective excitation and emission wavelength pairs were verified and the window times were adjusted as necessary to include any omitted components prior to calibration and analysis of the analytical sample sets.

Instrumental limits of detection (LOD) and limits of quantitation (LOQ) were determined using solvent blank injections to demonstrate the baseline sensitivity of the instrument under operating conditions. Procedural blanks represent background interferences expected to derive from the method

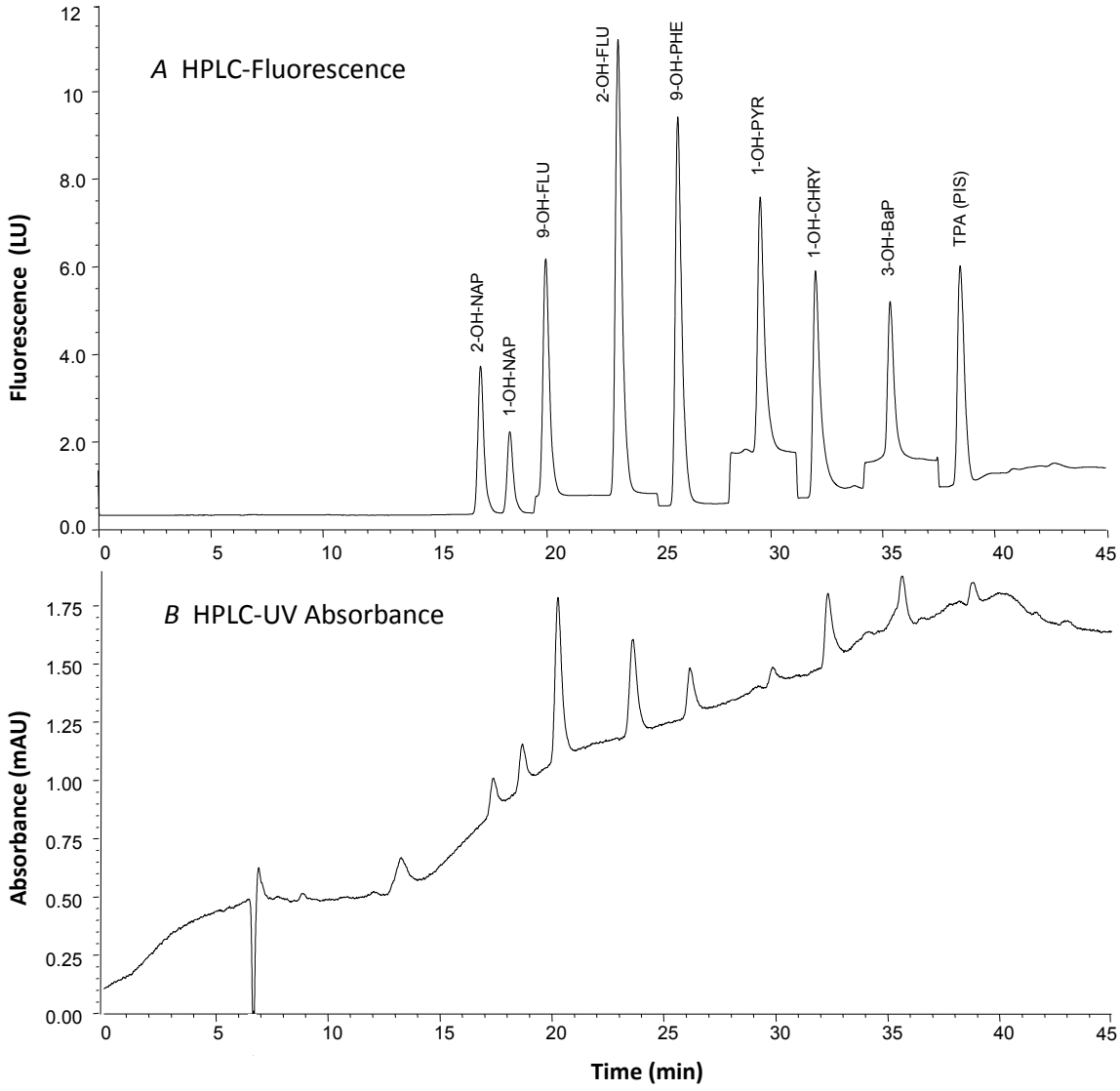

Figure 1. Comparison of sensitivity and selectivity for OH-PAHs by HPLC with *A*, fluorescence detection (LU, luminescence units) and *B*, UV absorbance detection (mAU, milli-absorbance units). OH-PAHs are annotated. 2-OH-NAP, 2-hydroxynaphthalene; 1-OH-NAP, 1-hydroxynaphthalene; 2-OH-FLU, 2-hydroxyfluorene; 9-OH-FLU, 9-hydroxyfluorene; 9-OH-PHE, 9-hydroxyphenanthrene; 1-OH-PYR, 1-hydroxypyrene; 1-OH-CHYR, 1-hydroxychrysene; 3-OH-BAP, 3-hydroxybenzo(*a*)pyrene; TPA, triphenylamine; IS, internal standard.

Table 2. Analytical parameters for HPLC-F separation and quantification of hydroxylated PAHs.

[HPLC-F, high-performance liquid chromatography-fluorescence detection; PAHs, polyaromatic hydrocarbons; LC, liquid chromatography system; $C_{18(2)}$ end-capped octadecylsilane bonded; mm, millimeter; µm, micrometer; Å, angström; mM, millimolar; TBAP, tetrabutylammonium phosphate; initial, initial starting conditions; min, minutes; UV-Vis, ultraviolet-visible; nm, nanometers; excitation, fluorescence excitation wavelength; emission, fluorescence emission wavelength; ng/mL, nanograms per milliliter; PIS, Procedural internal standard]

LC	Agilent 1100 series HPLC G1313A Autosampler G1312A Binary Solvent Delivery System G1316A Column Heater					
	Free hydroxylated PAHs		**PAH-glucuronides**		**Free phenolphthalein and -glucuronide**	
Column	150 mm x 4.6 mm Phenomenex Luna $C_{18(2)}$ 3 µm (100Å) (with Phenomenex Luna $C_{18(2)}$ 4 mm x 2 mm guard cartridge)					
	Time (min)	**Solvents Milli Q water—methanol (percent)**	**Time (min)**	**Solvents Milli Q water (5mM TBAP)—methanol (5mM TBAP) (percent)**	**Time (min)**	**Solvents Milli Q water (5mM TBAP)—methanol (5mM TBAP) (percent)**
Gradient flow profile						
	Initial	40:60	Initial	60:40	Isocratic	50:50
	3	60:40	3	60:40	20	50:50
	30	0:100	30	0:100		
	40	0:100	50	0:100		
	45	40:60	55	60:40		
	55	40:60	65	60:40		
Flow rate (mL/min)		0.14		0.20		0.20
Detection	Agilent 1100 series G1314A Variable Wavelength Detector G1321A Fluorescence Detector ChemStation Revision B.03.02 Software					
UV-Vis parameters	**Time (min)**	**Wavelength (nm)**	**Time (min)**	**Wavelength (nm)**	**Time (min)**	**Wavelength (nm)**
	Initial	254	Initial	254	Initial	220
	55	254	55	254	20	220
Fluorescence parameters						
	Time (min)	**Excitation/emission (nm)**		**Excitation/emission (nm)[a]**		
1-hydroxynaphthalene	Initial	290 / 380	Complete	270 / 320		
2-hydroxynaphthalene			Run	(fluorene-glucuronides)		
2-hydroxyfluorene	19.5	270 / 320		330 / 380		
9-hydroxyfluorene				(pyrene-glucuronides)		
9-hydroxyphenanthrene	25.0	256 / 380				
1-hydroxypyrene	28.2	330 / 380				
1-hydroxychrysene	31.2	268 / 380				
3-hydroxybenzo(a)pyrene	34.2	380 / 420				
triphenylamine (PIS)	37.5	289 / 365				
	40.0	290 / 380				

[a] Glucuronide analysis for hydroxyfluorenes or hydroxypyrenes only.

without the contribution from bile samples, and PB injections were used to establish the method LODs and LOQs. The LOD of an analyte was defined as three times the background noise at the retention-time and integrated over the average peak-width of the analyte; LOQ was defined as 10 times this background noise (Keith and others, 1983; Keith, 1991). Instrumental detection limits based on solvent blanks yielded LOD and LOQ values that were several-fold lower than the method detection limits based on procedural blanks. Negative control Black Carp (*Mylopharyngodon piceus*, BC) bile was not used to establish LOD and LOQ values because of the inherent trace concentrations of PAH metabolites identified during methods development.

Analysis for Bilirubin

A stock solution of mixed bilirubin isomers (30 micrograms per milliliter, µg/mL) was prepared in 5 millimolar (mM) aqueous potassium hydroxide (KOH) and stored in an amber vial. Two solutions of methanol:5 mM aqueous KOH:bilirubin (5:4:1 volume:volume, v:v) were prepared and potential photoisomerization and conversion of bilirubin was studied as a function of time under ambient conditions in the dark and in direct laboratory lighting. In addition, the effects of ascorbic acid antioxidant preservation on the stability of bilirubin (Brodersen, 1979) were investigated by repeating the above experiments with bilirubin solutions prepared in 50 mM aqueous potassium ascorbate (as a basic solution containing ascorbic acid). Studies were conducted using a 5-hour storage time by automated periodic scanning of a wavelength range from 200 to 800 nm repeatedly to monitor photoisomerization and using a 2-hour storage time to monitor ascorbic acid preservation, using an LS50-B Spectrofluorometer (Perkin-Elmer Inc., Waltham, Massachusetts).

The time-dependent loss of bilirubin from photolytic degradation is shown in figure 2*A*. The losses observed from storage at alkaline pH in ambient light and in the dark were similar to those described by Brodersen (1979). Losses of analyte at an alkaline pH and in the presence of the antioxidant ascorbic acid were pronounced under both dark and ambient light storage as shown in figure 2*B*. The decrease in bilirubin absorbance, monitored at 451 nm, was not accompanied by increases in maxima at any other wavelength, including the bilirubin oxidation product biliverdin with an absorption maximum of 380 nm.

Measurements of bilirubin in BC negative control and LMB positive control bile matrices were performed to determine the feasibility of bilirubin measurements in bile matrices. The BC bile was diluted 50-fold and the LMB bile 200-fold with 5 mM aqueous KOH and stored in amber vials. Samples were diluted in methanol:5 mM aqueous KOH:bile (5:4:1 v:v) immediately prior to analysis by automated periodic scanning of a wavelength range from 200 to 800 nm . Quantification was performed using a linear calibration curve of absorbance measured at 451 nm. Bilirubin was determined in selected field samples collected for methods development and validation by using undiluted bile in a solution of methanol:5 mM aqueous KOH:bile (5:4:1 v:v). All samples were prepared and immediately measured to minimize bilirubin degradation. In all investigations, spectra were monitored for an additional absorption maximum at 380 nm, indicative of biliverdin.

Spectrometric analysis for bilirubin in BC negative control bile and positive control LMB bile displayed the typical bilirubin absorption maximum at 451 nm; however, analysis of bile from field samples did not indicate a similar maximum at 451 nm, or a maximum at 380 nm for the biliverdin oxidation product. This may be a result of the prolonged holding-times of the samples, or exposure to light during field collection and laboratory analysis. Because of this behavior, bilirubin concentrations were not reported; instead, the OH-PAH concentrations were normalized to bile protein content, which was demonstrated to be a more robust and reproducible normalization of bile PAH metabolite concentration.

There was no available information about possible mixed-function oxidase activity induction that could be a cause of the observed bilirubin behavior; also, there was no indication that there was similar photo-oxidation of PAH bile metabolites. Generally, investigators working with PAH metabolites in fish bile have had problems with bilirubin analysis in bile; this is why the protein measurement was included as an alternative normalization method.

Hydrolysis of Phenolphthalein-β-Glucuronide and PAH-Glucuronides and -Sulfates

Phenolphthalein-β-glucuronide was used to monitor glucuronidase/sulfatase enzyme hydrolysis. Initial HPLC investigations used methanol–water mobile phases on C_{18}; however, the glucuronide exhibited poor peak shape and retention characteristics and an isocratic ion-pairing method was developed exhibiting adequate separation between the two components and providing acceptable glucuronide peak shape.

Under both ambient (20 degrees Celsius, ºC) and incubation (37 ºC) temperatures near quantitative hydrolysis was achieved in 16 hours for both lower (1.14 µg) and higher (11.4 µg) concentration glucuronide solutions. Using 10 microliters (µL) of the dilute enzyme solution (0.2 units sulfatase and 10 units glucuronidase activity), about 94 percent of the glucuronide was converted to phenolphthalein, though small but measurable amounts of glucuronide remained. Quantitative hydrolysis of the glucuronide to phenolphthalein (> 99 percent) was achieved in two hours by increasing the enzyme volume to 500 µL (10 units sulfatase and 500 units glucuronidase activity) and incubating the solution at 37 ºC for 3 hours; these were the optimized conditions selected for the final method to ensure completion of hydrolysis under potentially variable field sample compositions.

Recoveries of OH-PAHs fortified into negative control bile were quantitative (> 99 percent) indicating negligible loss of the free analytes through all preparation steps including

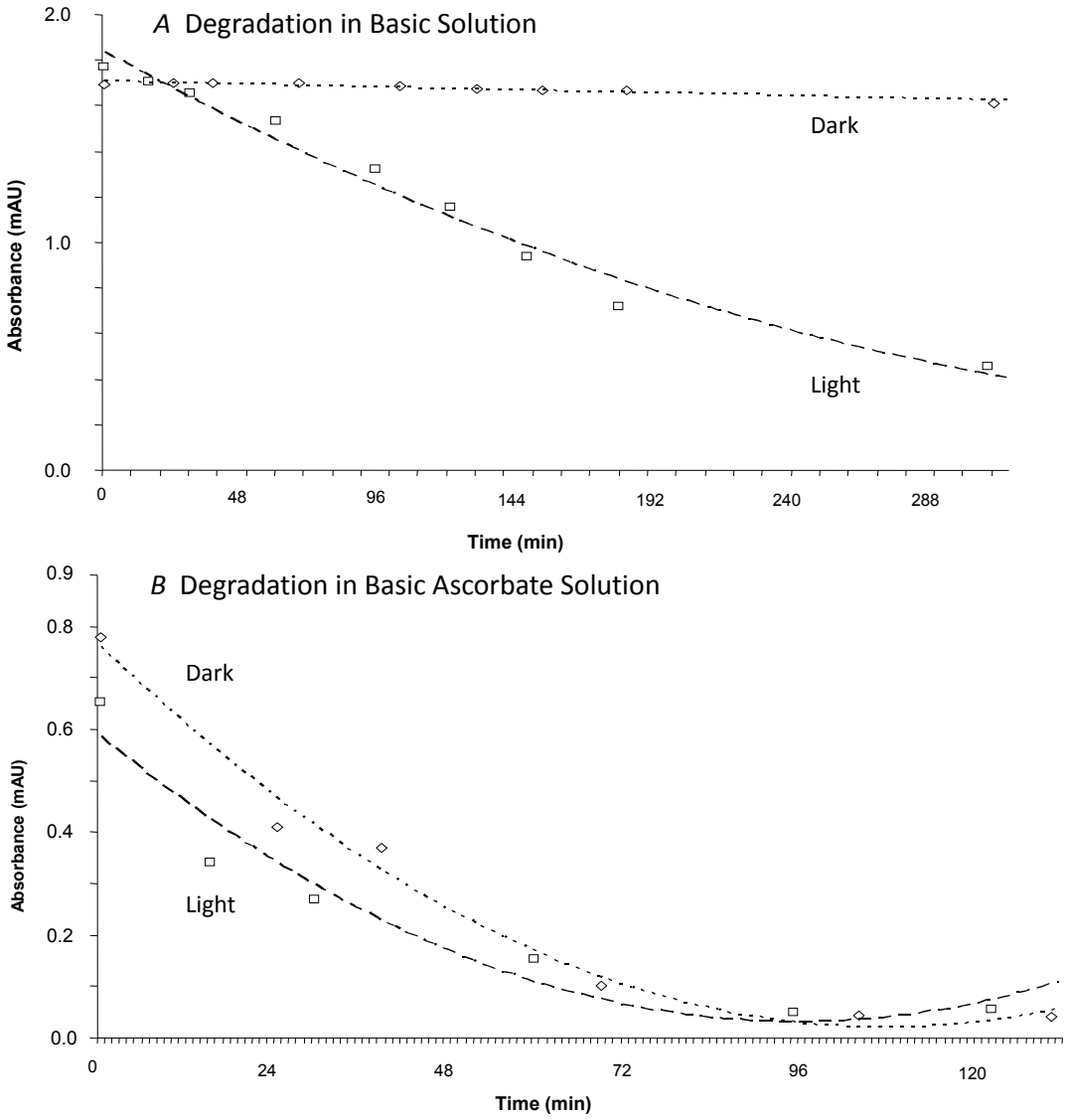

Figure 2. Degradation of bilirubin under dark and fluorescent laboratory lighting. *A*, degradation in basic solution and *B*, degradation in basic ascorbate solution. (Absorbance measured at 451 nm, mAU, milli-absorbance units).

hydrolysis. Recoveries of OH-PAHs from biologically incorporated PAH glucuronides that were hydrolyzed from positive control bile also were quantitative (> 99 percent) under the optimized conditions. The conditions for phenolphthalein-β-glucuronide hydrolysis to the phenolphthalein product were verified by analysis of replicate analyses of phenolphthalein-β-glucuronide fortified negative control bile. Phenolphthalein-β-glucuronide hydrolysis samples were included in the overall method validation and were used to monitor hydrolysis in each analytical set. Hydrolysis was monitored by recovery of phenolphthalein, compared to a glucuronide-fortified methanol:water (1:1 v:v) blank, corrected for the molar-mass differences between phenolphthalein-β-glucuronide and phenolphthalein. The analyses of phenolphthalein-β-glucuronide and phenolphthalein were performed independently using the isocratic ion-pairing HPLC method (table 2).

Control Materials

Bile samples from individual PAH-dosed LMB were characterized by HPLC-F to ensure the presence of working levels of PAH metabolites (glucuronides and sulfates), and indicated that all samples contained several PAH metabolites, presumably as glucuronides based on chromatography (fig. 3). The crude samples were then pooled and diluted to a final volume of 10 mL with water. The contents were mixed thoroughly and transferred to an amber glass vial. This was denoted as the positive control LMB bile and was used throughout the study to monitor method performance. Prior to analysis of field samples, 10 replicates of this positive control bile were analyzed following glucuronide hydrolysis for OH-PAHs and protein as part of the method validation. When not in use the positive control material was stored at -20 ºC.

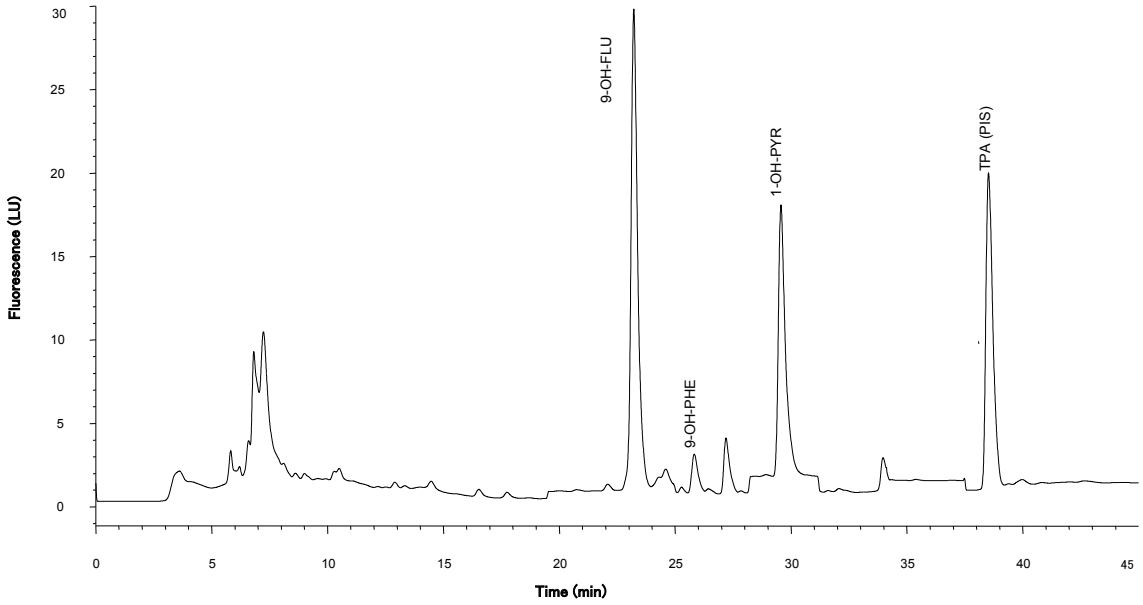

Figure 3. PAH metabolites as free OH-PAHs in largemouth bass positive control bile. (LU, luminescence units). 9-OH-FLU, 9-hydroxyfluorene; 9-OH-PHE, 9-hydroxyphenanthrene; 1-OH-PYR, 1-hydroxypyrene; TPA, triphenylamine; PIS, procedural internal standard.

Protein ranged from 1,200 to 3,000 µg/mL in the individual LMB bile samples. Analyses of 20 replicates of the 10 mL composited and diluted positive control bile resulted in an average protein concentration of 2,860±190 µg/mL with a variance of 6.7 percent. Four of the targeted analytes were identified in the positive control bile, and three analytes, 2-hydroxyfluorene, 9-hydroxyphenanthrene, and 1-hydroxypyrene were quantified (table 3). The storage time for the LMB positive control bile was monitored for 1 year and no losses of PAH metabolites (as quantified by OH-PAHs) were observed and no increases in background interferences were noted.

A BC bile material, containing very low or negligible OH-PAH concentrations, was provided by the Columbia Environmental Research Center (CERC) and was used as a negative control bile. Gall bladders were collected from BC and stored in amber glass vials at -20 ºC. Bile from individual BC was composited and replicate analyses conducted as part of the methods validation. Because the bile OH-PAH background in the BC was lower than in the LMB, the BC negative control bile was used throughout the study to monitor analyte recovery and background.

Part II—Determining PAH Metabolites in Bile from Fishes at Platforms and Natural Areas

Study Sites and Sample Collection

In an earlier phase of this research, heavy metal burdens in fishes were characterized at platforms and natural areas in the Southern California Bight (Love and others, 2003, 2009; Steinberger and others, 2004). The previously selected sites and species of interest were used here to provide continuity. Personnel from the Marine Science Institute (MSI) of the University of California – Santa Barbara, conducted the sampling effort for this study. Sampling corresponded to previously selected groupings of a platform, natural rocky reef, and natural soft sediment habitat in six geographic areas outlined in earlier work (fig. 4). Specimens of three species residing at platforms and composing the regional background within the Santa Barbara Channel and within the San Pedro Basin were collected.

Adult Pacific sanddab (*Citharichthys sordidus*, CS), benthic-dwelling flatfish, were collected from the oil platform shell mound and soft sediment natural sites away from the platforms. Kelp rockfish (*Sebastes atrovirens*, SA) and kelp bass (*Paralabrax clathratus*, PC) were collected from midwater depths at the platforms and from rocky reefs selected as natural sites. The three species are opportunistic carnivores with diets that include a variety of invertebrates and small fishes.

The fish were collected by MSI staff using scuba diving or hook and line. Kelp bass were collected at sites where kelp rockfish were not available and were used as surrogates for the kelp rockfish because of their similar trophic status. At least five fish of each species were collected at each site; additional collection trips were necessary to collect some species at sparsely populated sites and were conducted within a few days of the initial sample collection date. The fish were immediately placed on ice and returned to the MSI laboratory for gall bladder excision. Following removal, the gall bladders

Table 3. Concentrations of protein and PAH metabolites as hydroxylated-PAHs from composited positive control largemouth bass bile from methods validation and from field analytical sets.

[PAH, polyaromatic hydrocarbon; µg/mL, micrograms per milliliter; cv, coefficient of variation (variance); n, number of samples; —, not applicable.]

Analyte	Methods validation		Analytical sets	
	Concentration (µg/mL)	CV (percent, n)	Concentration (µg/mL)	Recovery (percent, n=10)
Protein	2,860	6.7 (20)	2,690–3,750	94–131
2-hydroxyfluorene	3,590	2.1 (10)	2,960–4,230	82–118
9-hydroxyphenanthrene	590	4.6 (10)	480–690	81–117
1-hydroxypyrene	6,550	2.3 (10)	5,830–7,680	89–117
1-hydroxychrysene	<120	—	<120	—

were placed in an appropriately labeled storage container and transferred to either a -20 ºC or a -80 ºC freezer. The frozen samples were labeled with the collection site identifier, wrapped in aluminum foil, placed in a ziplock bag, and packaged on dry ice for overnight transport to CERC, along with chain of custody forms and any additional, pertinent sample collection documentation. Following arrival, the samples were inventoried and stored in a -20 ºC freezer at CERC when not in use.

Preparation and Collection of Bile

Gall bladder samples were removed from the -20 ºC freezer and allowed to reach ambient temperature while still wrapped in foil. Individual gall bladders were removed from the storage container and placed on solvent-washed, air-dried aluminum foil. Any residual liver tissue was carefully removed from the outside of the bladder using forceps and a stainless steel scalpel. The bile duct was located, clamped with forceps, and cut with a scalpel. If sufficient bile was available, the fluid was expressed from the bladder into an amber glass vial with insert.

A minimum of approximately 20 µL of bile fluid was required using the methods developed here. During development of sample processing methods for bile from field samples, it was determined that most of the Pacific sanddab gall bladders were very small, only 2–3 mm in diameter, and did not express sufficient volumes of bile for protein and PAH metabolite analyses. Therefore, an alternate method for sampling bile from very small gall bladder samples was developed, consisting of placing the entire gall bladder in an amber vial, adding 25 µL of water, and homogenizing the entire bladder to recover a diluted bile fluid sample. The general effect of this alternative (dilution) method on measurement error was to slightly increase the LOD and LOQ for those samples by less than twofold, as noted in the tables of results.

Of the 65 Pacific sanddab gall bladders, 61 required alternate sampling for bile. Five of the 61 kelp rockfish gall bladders also contained no recoverable bile, and 2 additional kelp rockfish samples contained some, but < 20 µL, bile; these 7 samples also required alternate sampling. The samples requiring alternate sampling for bile are denoted in the tables. The remaining 4 Pacific sanddab samples, 54 kelp rockfish samples, and all 14 kelp bass samples had sufficient volumes of bile to provide adequate amounts for protein and OH-PAH analyses. The bile for these samples was collected by expelling fluid through the bile duct directly into amber vials. All samples requiring alternate sampling for bile are denoted in the '*Bile method'* column of the supporting information, tables S1, S2, and S3.

Quality Control

Field samples were processed in sets and each set included QC measures to monitor the integrity of the sample preparation methodology: procedural blanks, negative and positive control materials, negative control materials fortified with either OH-PAHs or PAH metabolites (from the positive control material), and replicated samples. During all sample and standard handling steps, exposure to light was avoided or minimized to reduce losses from photodegradation of PAH metabolites. The results of monitoring these quality control measures are summarized in the supporting information, table S4.

Overall recoveries for protein measurements in quality control samples ranged from 91 to 131 percent. Protein concentrations in the positive control ranged from 2,690–3,750 µg/mL bile and the relative variability of replicate analyses of bile from field samples for each set ranged from 0.6 to 4.0 percent. Hydrolysis was monitored by recovery of phenolphthalein from the corresponding glucuronide in separately spiked control samples. The analyses of phenolphthalein-β-glucuronide and phenolphthalein were performed independently from the sample analyses, using isocratic, ion-pairing HPLC with UV-Vis detection (table 2). The recoveries through the hydrolysis step ranged from 96 to 108 percent for all analytical sets.

Triphenylamine was added to each sample as the procedural internal standard (PIS) and was processed through the entire sample preparation scheme. Concentrations of OH-PAHs were corrected for PIS recoveries, with recoveries ranging from 56 to 103 percent.

Background interferences were considered as any responses producing an increased detector response within the analyte window and were monitored using procedural blank and BC negative control hydrolyzed bile samples. Potential

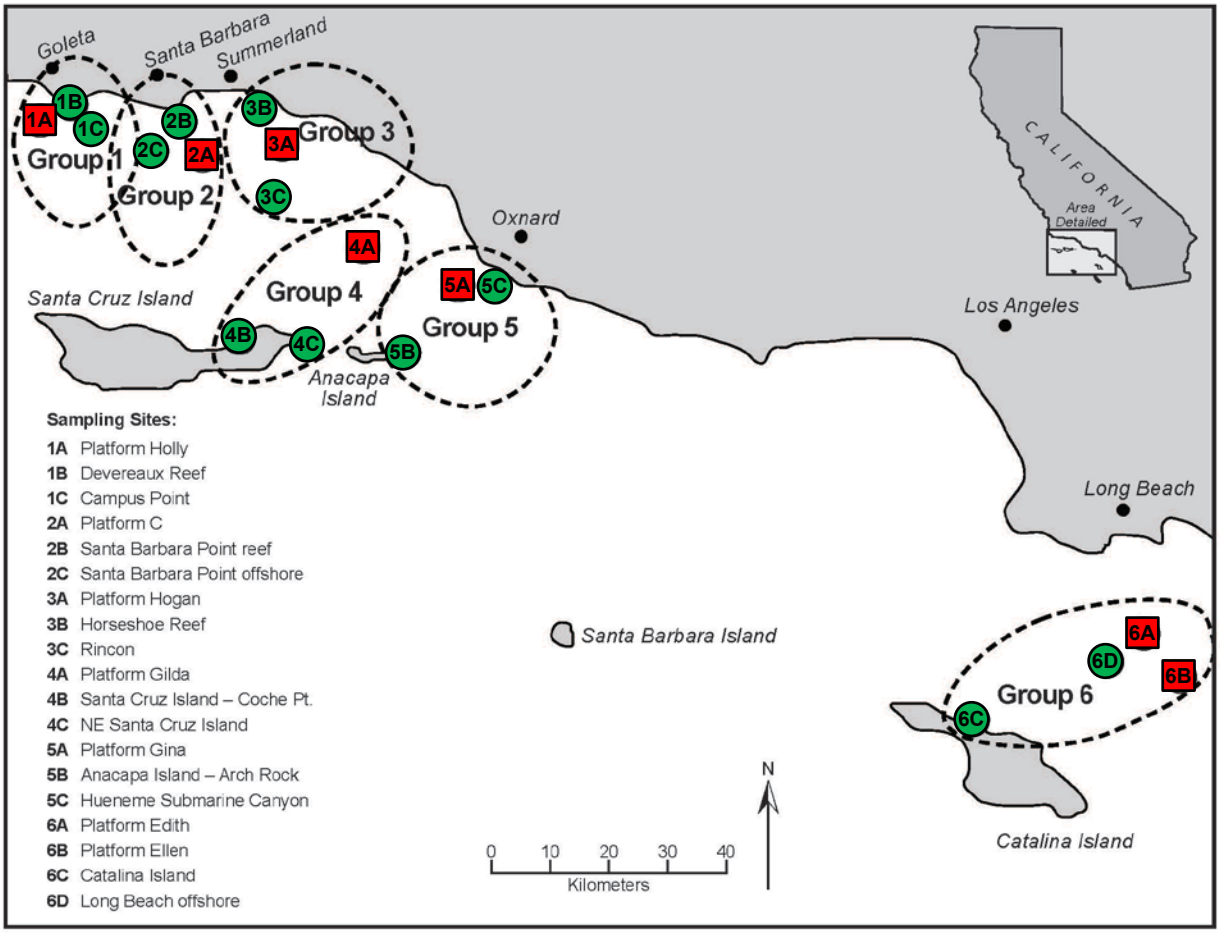

Figure 4. Platform (red squares) and natural area (green circles) site locations in the Southern California Bight.

sources of background included OH-PAHs that are inherent in the method, photolyzed PAHs from the laboratory environment, extraneous (non-OH-PAH) compounds, or nonchemical sources of method variability. Typical HPLC-F chromatograms of backgrounds from procedural method blanks, BC negative control hydrolyzed bile, and fortified negative control hydrolyzed bile samples are shown in figure 5.

Negligible amounts of interference were introduced from the method and only slight amounts of background interference were detected in the BC negative control hydrolyzed bile sample. The chromatographic responses from the BC hydrolyzed bile were identified as low concentrations of PAH metabolites biologically incurred in the carp used for the control material. Any method background responses measured in the procedural blanks were subtracted prior to reporting bile OH-PAH concentrations in field samples; however, negative control background response was not subtracted because these signals represented inherent OH-PAH responses.

Potential analyte peaks detected in hydrolyzed bile from field samples were identified by comparison to known standards using relative retention time (RRT) to that of the procedural internal standard. The percent difference between RRTs for peaks in the fortified negative control hydrolyzed bile and positive control hydrolyzed bile samples was < 0.5 percent. Analyte peaks in the hydrolyzed bile from field samples with differences in RRT of ≤ 1.5 percent those of the corresponding standard analyte were considered tentatively identified and were confirmed by comparison of fluorescence spectra to that of authentic standards.

Three quantifiable PAH metabolites, as OH-PAHs, in the LMB positive control hydrolyzed bile were identified during method validation (table 3). These free OH-PAHs were then monitored in each of 10 subsequent analytical sets and compared to the results from the validation as a QC measure.

Recoveries of free OH-PAHs in the fortified BC negative control bile samples are reported in table 4. With the exception of 3-OH-benzo(*a*)pyrene, recoveries were within 59 to 120 percent. Analyte concentrations in field samples were corrected for the appropriate laboratory-fortified control bile recoveries for reporting purposes, with the exception of 3-OH-benzo(*a*)pyrene, which was not corrected for recovery. The loss of 3-OH-benzo(*a*)pyrene is discussed below.

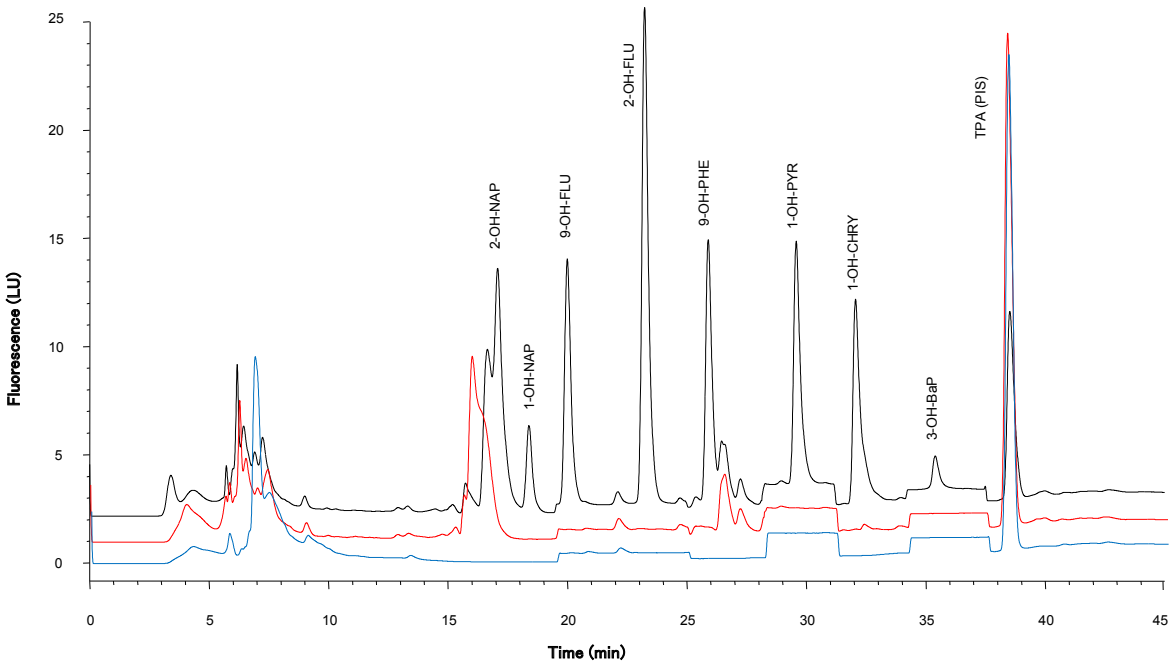

Figure 5. Comparison of HPLC-F chromatograms of procedural blank (blue – lower line), black carp negative control bile (red – middle line), and negative control bile fortified with hydroxy-PAHs (black – upper line). (LU, luminescence units). 2-OH-NAP, 2-hydroxynaphthalene; 1-OH-NAP, 1-hydroxynaphthalene; 2-OH-FLU, 2-hydroxyfluorene; 9-OH-FLU, 9-hydroxyfluorene; 9-OH-PHE, 9-hydroxyphenanthrene; 1-OH-PYR, 1-hydroxypyrene; 1-OH-CHYR, 1-hydroxychrysene; 3-OH-BAP, 3-hydroxybenzo(*a*)pyrene; TPA, triphenylamine; IS, internal standard.

Table 4. Recovery of hydroxylated PAHs fortified into composited black carp negative control bile from analytical field sets.

[PAHs, polyaromatic hydrocarbons; n, number of samples.]

Analyte	Analytical sets
	Recovery (percent, n=10)
1-hydroxynaphthalene	91–109
2-hydroxynaphthalene	93 – 107
2-hydroxyfluorene	98 – 120
9-hydroxyfluorene	94 – 108
9-hydroxyphenanthrene	59 – 100
1-hydroxypyrene	72 – 106
1-hydroxychrysene	85 – 101
3-hydroxybenzo(*a*)pyrene	3 – 54

Replicate analyses of selected field samples were performed for each set. Because of the lack of detectable concentrations of environmentally incorporated analytes in most of the field samples, reproducibility of the method was only confirmed for bile from selected field samples as presented in table 5.

Table 5. Reproducibility of triplicated analyses of hydroxylated PAHs from analytical field sets.

[PAHs, polyaromatic hydrocarbons; µg/mL, micrograms per milliliter; cv, coefficient of variation (variance); n, number of samples.]

Analyte	Set number	Analytical sets	
		Concentration (µg/mL)	CV (percent, n=3)
2-hydroxynaphthalene	4	84 – 104	12
2-hydroxyfluorene	5	300 – 350	8
	10	220 –230	4
9-hydroxyfluorene	5	220 – 290	14

Instrumental detection limits (IDLs), and limits of detection and quantification (LOD and LOQ) were calculated using responses from solvent blanks and procedural blanks,

respectively. The concentrations of analytes in the lowest calibration standard were prepared to have levels of just above the observed solvent blank IDL. The calculated (solvent only) IDL values and (procedural blank) LOD and LOQ values are presented in table 6.

Table 6. Instrumental detection limits and method limits of detection and quantification for hydroxylated PAHs.

[PAHs, polyaromatic hydrocarbons; IDL, instrumental detection limit; LOD, limit of detection; LOQ, limit of quantification; ng/mL, nanograms per milliliter; ng/mg, nanograms per milligram of protein.]

Analyte	IDL (ng/mL)[a]	LOD (ng/mL)[b]	LOQ (ng/mL)[b]	Range of protein normalized LOD (ng/mg protein)[c]
1-hydroxynaphthalene	0.8	62	210	0.07 – 12
2-hydroxynaphthalene	0.1	26	87	0.2 – 22
2-hydroxyfluorene	0.1	18	59	0.002 – 5
9-hydroxyfluorene	0.3	72	240	0.01 – 25
9-hydroxyphenanthrene	0.1	11	38	0.03 – 4
1-hydroxypyrene	0.1	5	18	0.1 – 3
1-hydroxychrysene	0.6	120	390	0.01 – 42
3-hydroxybenzo(*a*)pyrene	0.2	22	72	0.6 – 8

[a] Concentration in ng/mL of standard solution directly analyzed.

[b] Concentration in ng/mL of bile processed through the method.

[c] Range of protein-normalized LODs in ng/mg of protein were very dependent on total protein and volume of bile.

Investigation of the Procedural Loss of 3-Hydroxybenzo(*a*)pyrene

Recoveries of 3-OH-benzo(*a*)pyrene from the method validation were acceptable, ranging from 89 to 92 percent. However, excessive losses of only this compound were observed early in the analyses of QC samples associated with sets of field samples. The recoveries of 3-OH-benzo(*a*)pyrene were low and variable in the fortified BC negative control bile processed with each analytical set. Experiments were immediately conducted to determine the cause(s) for this loss and the associated increase in variability. Potential areas for concern included adsorption during filtration, photolysis under fluorescent lighting, degradation under the selected enzymatic conditions, and analyte interference in the excitation and emission pair window from biogenic materials in the bile.

Recoveries of all analytes, including 3-OH-benzo(*a*) pyrene, through the initial filtration procedure were excellent ranging from 107 to 115 percent. Aqueous solutions of OH-PAHs were exposed to fluorescent laboratory lighting for various periods from 1 to 3 hours (normal preparatory handling times). Recoveries ranged from 82 to 87 percent, were not biased toward 3-OH-benzo(*a*)pyrene and were not correlated with exposure time to light, indicating that direct photolysis was unlikely. Recoveries through the hydrolysis procedure were not affected by changes in enzyme solution concentration or by extended exposure to both the hydrolysis solution and fluorescent lighting. Recoveries were slightly more variable for hydrolysis in negative control bile and ranged from 86 to 114 percent. A single instance of an unacceptably low recovery (64 percent) was noted for 3-OH-benzo(*a*)pyrene under hydrolysis conditions in the absence of bile; however, this was not reproducible. No causes of loss of 3-OH-benzo(*a*)pyrene were identified; therefore, interpretations of results for 3-OH-benzo(*a*)pyrene reported here should be made with caution.

Confirmation of PAH-Glucuronides by Ion-Pairing High-Performance Liquid Chromatography

Very large fluorescence responses were observed in HPLC separations during the routine analysis of hydrolyzed bile samples. These responses were found to elute early in the chromatographic analysis and were mainly detected in the current (hydroxynaphthalene) fluorescence window. PAH glucuronides and sulfates are known to elute earlier than their hydrolyzed forms; therefore, the potential for these responses to be residual PAH-glucuronides resulting from incomplete hydrolysis was investigated. This was confirmed by monitoring both the production of OH-PAHs and the loss of PAH-glucuronides resulting from hydrolysis.

The LMB positive control bile was considered the most likely sample to be affected by incomplete hydrolysis of glucuronides as it contains the greatest concentrations of biologically incorporated OH-PAH glucuronides encountered in this study. This positive control material was used to determine the potential for residual glucuronides to remain after the hydrolysis step. Likewise, 2-fluorene-glucuronide was selected as the most likely residual glucuronide candidate because this hydrolysis product has the greatest concentration in the positive control material and was monitored using the same excitation and emission wavelength pair as the hydrolyzed product: 270 nm and 320 nm.

A nonhydrolyzed sample of the positive control bile was separated by HPLC-F using the OH-PAH gradient program (without ion-pairing reagents, table 2) and with routine excitation and emission wavelength detection of hydroxyfluorenes (fig. 6*A* – black). The early eluting responses of the

hydroxyfluorene-glucuronides from 8.5 to 11 minutes in the region of the hydroxynaphthalene detection window are typical of the responses in many of the field samples and of the suspected residual PAH glucuronides. Note that there is no detectable response for 2-OH-fluorene (23.2 minutes). After hydrolysis, the positive control bile sample was separated under the same conditions (fig. 6*A* – red). The responses from about 8.5 to 11 minutes are no longer present and 2-OH-fluorene (23.2 minutes) was quantitatively recovered, confirming complete hydrolysis of 2-OH-fluorene-glucuronide in bile.

Direct analysis of glucuronides was investigated to confirm that the signal in the early-response window did not result from other residual PAH metabolites after hydrolysis. Ariese and others (1997) reported HPLC separation of glucuronides in bile without hydrolysis using ion-pairing mobile phases containing tetrabutylammonium phosphate (TBAP). A nonhydrolyzed

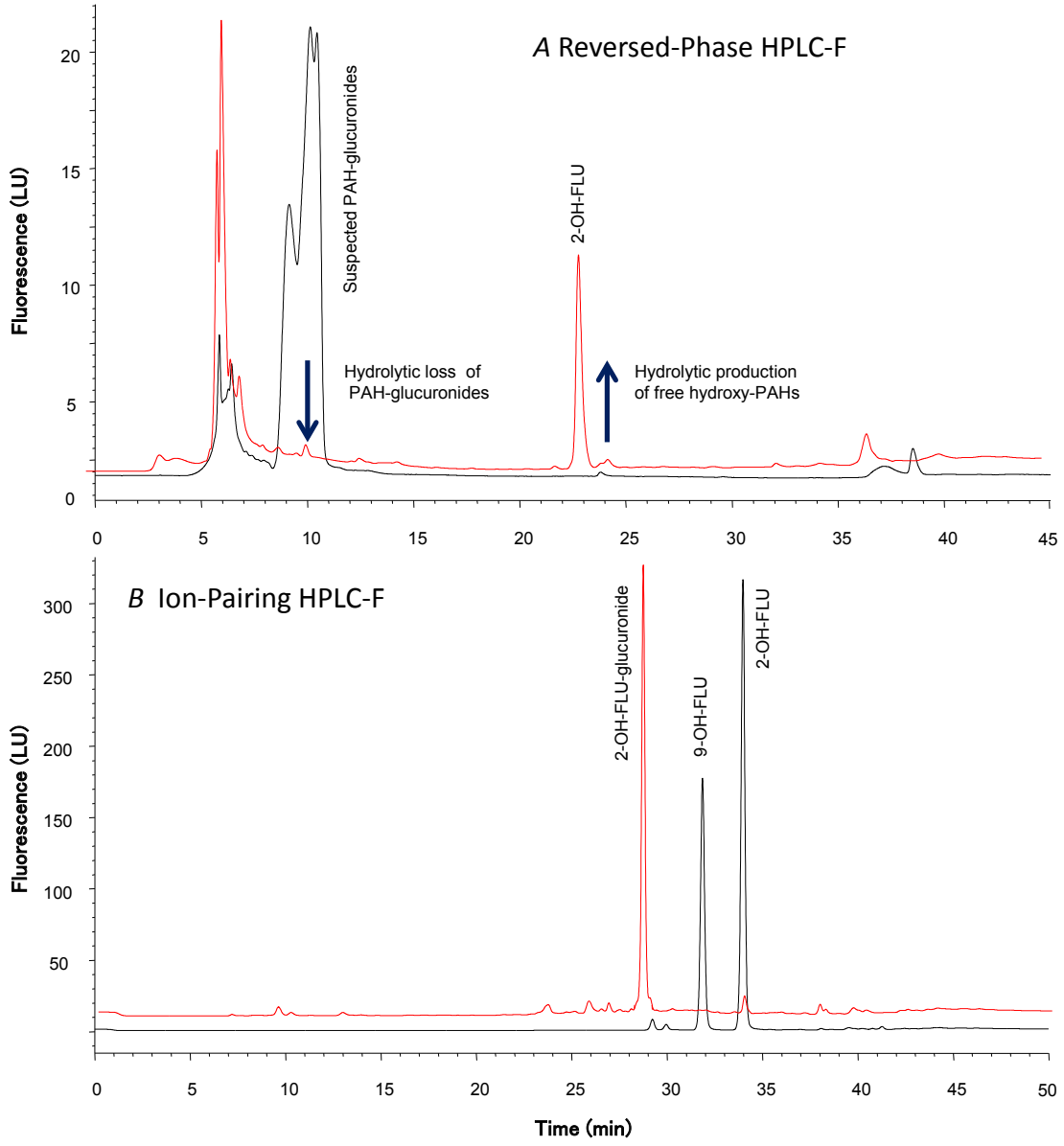

Figure 6. Comparisons of *A*, reversed-phase HPLC-F chromatograms of non-hydrolyzed (black) and hydrolyzed (red) largemouth bass positive control bile, indicating hydrolytic loss of glucuronides and production of free OH-Fluorene; and *B*, ion-pairing HPLC-F chromatograms of nonhydrolyzed OH-PAH-glucuronides in largemouth bass positive control bile (red) and free OH-fluorenes standard (black). (LU, luminescence units). 2-OH-FLU, 2-hydroxyfluorene; 2-OH-FLU-glucuronide, 2-hydroxyfluorene glucuronide; 9-OH-FLU, 9-hydroxyfluorene.

sample of positive control bile was separated by HPLC using a TBAP ion-pairing water—methanol solvent gradient (table 2 and fig. 6*B* red), with only hydroxyfluorene excitation and emission wavelength detection; a standard showing the relative elution of the free 9-OH- and 2-OH-fluorenes is overlaid for comparison (fig. 6*B* – black). The 2-OH-fluorene-glucuronide at a retention time of 14.6 minutes exhibited adequate peak shape and retention. Upon hydrolysis, the corresponding 2-OH-fluorene peak was quantitatively produced and the corresponding glucuronide was no longer present (not shown).

Field samples with previously quantifiable concentrations of OH-fluorenes (after hydrolysis) were screened for additional amounts of fluorene-glucuronides. Selected non-hydrolyzed field samples were separated by the ion-pairing method and hydroxyfluorene excitation and emission detection to determine fluorene-glucuronides. Any unidentified responses were further investigated by acquiring spectra using broadband fluorescence at 220–380 nm (excitation) and 300–500 nm (emission).

As a representative example, in the previous analysis of hydrolyzed field sample (HOLLY-CS-1), 9-OH-fluorene and 2-OH-fluorene were quantified at 250 and 320 ng/mL bile, respectively. The prevalence of high levels of early-eluting components and the smaller hydroxyfluorene responses may be seen in the HPLC-F chromatogram (fig. 7*A*); essentially no fluorene-glucuronide response (arrow at 28.6 minutes) was seen in the corresponding nonhydrolyzed sample separated by IP-HPLC-F (fig. 7*B*). Most early-eluting fluorescing components again elute early in the ion-pairing separation, prior to the 2-OH-fluorene-glucuronide, confirming that the OH-fluorene-glucuronides were quantitatively hydrolyzed and that these OH-fluorene-glucuronides were not contributing to the suspected early-eluting component responses. Similar analyses for pyrene-glucuronides again determined no residual glucuronides remaining after hydrolysis. No spectral similarities were determined between the major unidentified components and either hydroxy-PAHs or PAH-glucuronides.

Potential inhibition of glucuronide hydrolysis by bile components also was examined by hydrolysis of a field sample (HOLLY-SA-1) fortified with LMB positive control bile. Recoveries of free 2-OH-fluorene, 9-OH-phenanthrene, and 1-OH-pyrene ranged from 86 to 116 percent indicating the absence of any bile components in the field sample that were capable of inhibiting hydrolysis, suppressing conversion of conjugated forms to free OH-PAHs, or interfering with quantification.

Effect of Increased Sample Volume on Detection of OH-PAHs

The volumes of bile from most of the fish collected in the field were very small and the levels of PAH metabolites in the samples were very low. To increase the probability of detecting any potential PAH exposure to these fish, larger volumes of bile were analyzed wherever possible, on the hypothesis that increasing the sample volume will increase analyte concentration response relative to background response and decrease the method detection and quantification limits. Several samples were of sufficient volume to be reprocessed using larger volumes. The original analyses required only 10 µL of bile; those samples with sufficient volume were re-processed using either 30 µL or, if possible, 50 µL of bile.

The accuracy of using larger sample volumes was demonstrated by comparing quantifiable concentrations of 2-OH-fluorene in field samples. Concentrations of 2-OH-fluorene in field sample HOGA-SA-2 were 74 ng/mL at 10 µL and 67 ng/mL at 30 µL (the maximum available sample volume); similarly, concentrations in field sample HOLLY-SA-1 were 224 ng/mL at 10 µL and 237 ng/mL at 50 µL. The analyses of larger sample volumes did not sufficiently decrease the limits of detection or quantification to levels such that additional concentrations of OH-PAHs (previously below the detection limits) became reportable for any sample. Those samples previously exhibiting responses just below the method detection limits (at smaller sample volumes) produced similarly low responses at larger sample volumes, suggesting that the responses were not from OH-PAHs but from nonspecific background interference(s) from the samples.

Part III—Concentrations of Bile PAH Metabolites in Field Samples

This Study

Concentrations of OH-PAHs (ng/mL bile), protein concentrations, and protein -normalized OH-PAH concentrations in bile collected from field samples are available in the supporting information (tables S1, S2, and S3) for Pacific sanddab, kelp rockfish, and kelp bass from natural areas and from offshore oil-platforms. The concentrations of OH-PAHs in all samples were very low, ranging from less than the limits of detection (5 to 120 ng/mL bile) to a maximum of 320 ng/mL bile. Some analyte responses were just detectable, but below the method LOQ. Because of the consistently low OH-PAH concentrations found in bile samples from this study, these potential OH-PAHs were reanalyzed, where possible, using larger volumes of bile. These analyses confirmed that the previous responses were not the result of OH-PAHs, but rather that the responses resulted from background interferences.

The OH-PAH concentrations measured in fishes from natural sites and from platforms are summarized in table 7. The OH-PAH concentrations have been normalized to protein content and reported as nanograms of OH-PAH per milligram of protein to allow comparison among fishes at different stages of feeding. Concentrations were low at natural areas and at platform sites. Because of the small number of samples with quantifiable concentrations of OH-PAHs, there were no readily observable differences in the concentrations or profiles of PAH metabolites between platform sites and natural sites. There were insufficient numbers of positive samples, those with OH-PAH concentrations greater than the limit of quantification, to indicate that the potential clustering of the few reported concentrations was specific for

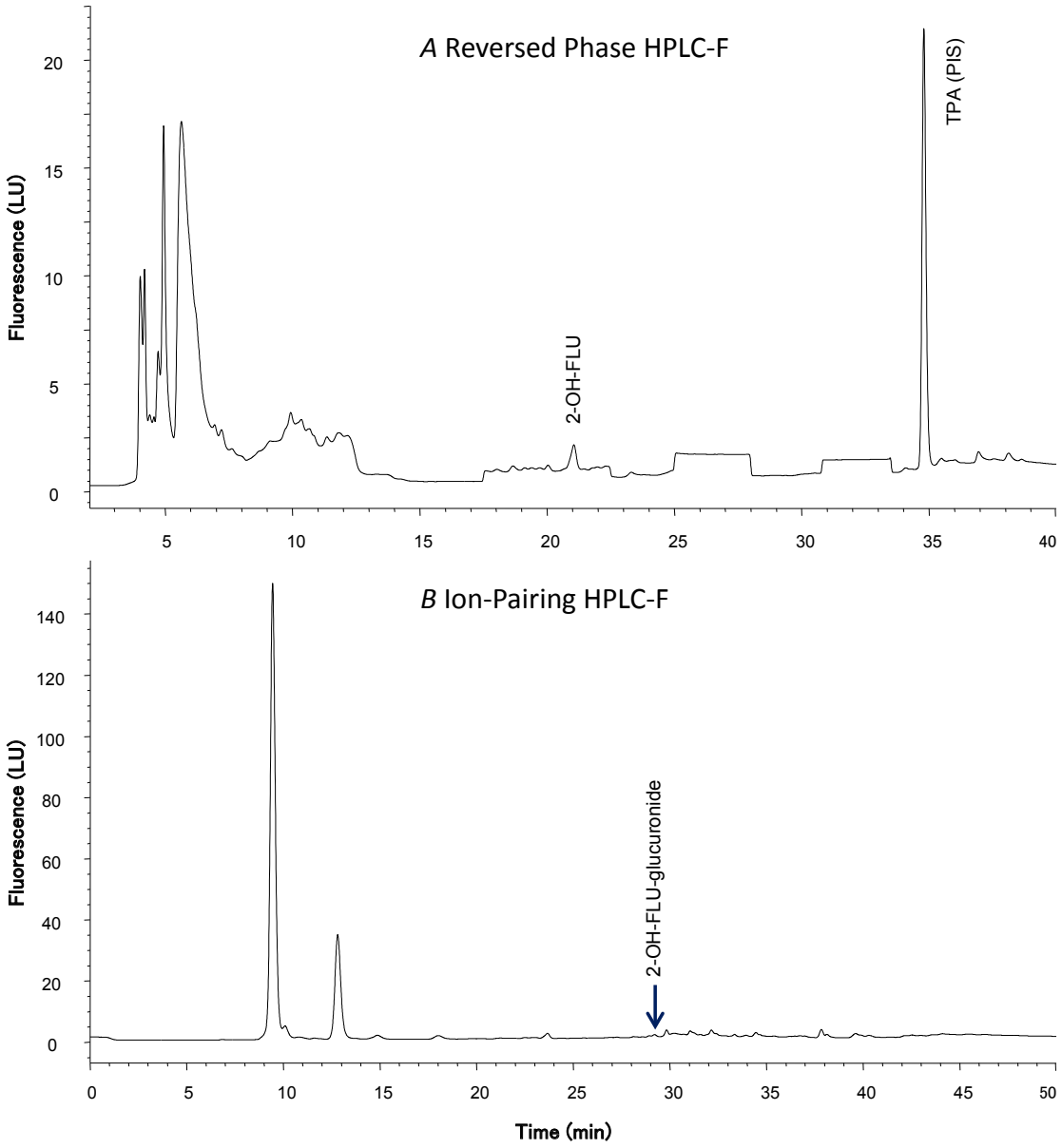

Figure 7. Comparisons of *A*, reversed-phase HPLC-F chromatograms of hydrolyzed bile from sample HOLLY-CS-1, showing high levels of early-eluting components and smaller 2-OH-fluorene response; and *B*, ion-pairing HPLC-F chromatograms of the corresponding nonhydrolyzed sample demonstrating the lack of 2-OH-fluorene-glucuronide. (LU, luminescence units) . 2-OH-FLU, 2-hydroxyfluorene; 2-OH-FLU-glucuronide, 2-hydroxyfluorene glucuronide; TPA, triphenylamine; PIS, procedural internal standard.

either sites or species (table 7). Few conclusions can be made about the reported OH-PAH concentrations:

- Analytes were detected and quantified at a specified concentration in some samples.
- Analytes were not detected in some samples at the specified LODs.
- Comparisons among different samples are confounded by the very low concentrations of OH-PAHs in the bile and by the widely varying amounts of bile protein and bile volume, which resulted in LODs for some samples that were higher than quantified values for other samples.

Table 7. Concentrations of targeted hydroxylated PAHs in bile of fish collected from natural sites and platform sites in the Southern California Bight.

[PAHs, polyaromatic hydrocarbons; ng/mg, nanograms per milligram of protein; All values are adjusted for surrogate recoveries and procedural blank sample background values; 1-OH-NAP, 1-Hydroxynaphthalene; 2-OH-NAP, 2-Hydroxynaphthalene; 2-OH-FLU, 2-Hydroxyfluorene; 9-OH-FLU, 9-Hydroxyfluorene; 9-OH-PHE, 9-Hydroxyphenanthrene; 1-OH-PYR, 1-Hydroxypyrene; 1-OH-CHRY, 1-Hydroxychrysene; 3-OH-BaP, 3-Hydroxybenzo(*a*)pyrene; parentheses indicate number of individuals with detectable concentrations and used in calculating the average.]

Natural Sites	Species	Number of Samples	1-OH-NAP (ng/mg)	2-OH-NAP (ng/mg)	2-OH-FLU (ng/mg)	9-OH-FLU (ng/mg)	9-OH-PHE (ng/mg)	1-OH-PYR (ng/mg)	1-OH-CRY (ng/mg)	3-OH-BaP (ng/mg)
Campus Point	Pacific sanddab	5	--	--	--	--	--	--	--	--
Rincon	Pacific sanddab	5	--	22 (1)	--	--	--	--	--	--
Hueneme Natural Site	Pacific sanddab	5	--	--	--	--	--	--	--	--
Northeast Santa Cruz	Pacific sanddab	5	--	--	--	--	--	--	--	--
Platform Edith Natural Site	Pacific sanddab	5	--	--	--	--	--	--	--	--
Isla Vista-Devereaux	Kelp rockfish	5	--	--	--	--	--	--	--	--
Santa Barbara Point Natural Site	Pacific sanddab	6	--	--	--	--	--	--	--	--
	Kelp rockfish	12	--	9 (1)	--	--	--	1.4 - 2.1 (3)	--	--
Horseshoe Reef	Kelp rockfish	7	--	--	--	--	--	--	--	--
	Kelp bass	4	--	--	8 (1)	--	--	--	--	--
East Anacapa Island Natural Site	Kelp bass	5	--	--	8 (1)	--	--	--	--	--
Coche Point	Kelp rockfish	6	--	--	7 - 9 (1)	--	--	--	--	--
Catalina Island Natural Site	Kelp rockfish	6	--	--	--	--	--	--	--	--
					Platform Sites					
HOLLY	Pacific sanddab	5	--	--	32 (1)	25 (1)	--	--	--	--
	Kelp rockfish	5	--	--	20 – 31 (2)	--	--	--	--	--
C	Pacific sanddab	8	--	--	--	--	--	--	--	--
	Kelp rockfish	4	--	--	--	--	--	--	--	--
HOGAN	Pacific sanddab	5	--	--	--	--	--	--	--	--
	Kelp rockfish	6	--	--	13 (1)	--	--	--	--	--
GINA	Pacific sanddab	5	--	14 (1)	--	--	--	--	--	--
	Kelp bass	5	--	5 (1)	--	--	--	--	--	--
GILDA	Pacific sanddab	5	--	23 (1)	--	--	--	--	--	--
	Kelp rockfish	5	--	--	--	--	--	--	--	--
EDITH	Pacific sanddab	1	--	--	--	--	--	--	--	--
	Kelp rockfish	5	--	--	--	--	--	--	--	--
ELLEN	Pacific sanddab	5	--	--	--	--	--	--	--	--
Protein-normalized Limits of Detection (LODs) for samples without detectable peaks.										
Minimum LOD (ng/mg protein)			0.69	0.16	0.01	0.01	0.03	0.13	0.01	0.58
Maximum LOD (ng/mg protein)			12.	22.	25.	4.9	3.9	3.3	42.	7.7
Average LOD (ng/mg protein)			2.8	5.9	4.9	0.76	1.2	0.56	9.7	2.3

Comparison to Other Studies

1-Hydroxypyrene, suggested for use as a bioindicator of PAH exposure of fish (Vuorinen and others, 2003, 2006), was not detected in fish samples from any of the platform sites (7 platforms, 76 fish). Low levels of 1-hydroxypyrene were detected in 3 of 12 kelp rockfish; all from the same natural reef site (Santa Barbara Point, table 7). The most prevalent OH-PAH, 2-hydroxyfluorene, was detected at very low levels in 4 fish (1 kelp rockfish, 1 Pacific sanddab, and 2 kelp bass) from 4 of 16 1 from 2 platforms and 3 of 15 fish from 3 natural sites. The greatest concentrations of 2-hydroxyfluorene were found in fish from platform HOLLY (3 of 10 fish samples) and were only about 3-fold above the levels found in fish from Horseshoe Reef, East Anacapa Island, and Coche Point natural sites; the concentrations at these sites were very low and the concentration differences among these sites were negligible. To place this study in perspective, recent investigations into relations between biochemical endpoints and exposure of fishes to PAHs from oil platforms and natural oil seeps were reviewed and information about method limits of detection and quantification and concentration ranges of PAH metabolites in various field locations were summarized (table 8).

The methods developed in this study were comparable or more sensitive than those methods recently reviewed (table 8). Jonsson and others (2003) compared HPLC-F and GC/MS methods and reported that HPLC-F was equivalent to gas chromatography-mass spectrometry (GC/MS) for lower molecular weight PAHs and that HPLC-F was much more sensitive than GC/MS for higher molecular weight PAHs. Hydrolysis, derivatization, and GC with high-resolution mass spectrometry was reported by Johnson-Restrepo and others (2008). Method detection limits were not reported, though using lowest concentrations from the ranges reported suggests that LOD were several-fold more sensitive than the GC/MS method reported by Jonsson and others (2003) and may approach the sensitivity of HPLC-F methods. HPLC-F detection limits reported in a study of bile reference materials (that are no longer available) were about 10- to 20-fold greater than the most sensitive method and about 10- to 20-fold less than the least sensitive method. The lack of consistent reporting conventions is problematic; normalization to bilirubin or protein content is not strictly followed making comparisons of sensitivity difficult. Because lower detection limits can be achieved using very dilute bile from recently feeding fish (by minimizing background interferences) such reported detection limits appear more sensitive; however, diluted bile results in diluted PAH metabolite concentrations, which tends to counter any increases in method sensitivity. A comparison of samples from this study requiring alternate preparation by dilution to other samples not requiring dilution indicated that LODs differed by less than twofold and were not consistently lower for either preparation method.

The PAH metabolite concentration ranges reported in this study are among the lowest reported in the recently reviewed literature (see table 8) and are approaching those concentrations reported in fish from pristine ocean sampling (Goysøyr and others, 2009). Nonprotein-normalized PAH metabolite concentrations in fish from open ocean sites along transects from Peru to Polynesia ranged from < 0.5 to 3.4 ng/mL bile; the detection limits imposed on data from this study (5 to 120 ng/mL bile) preclude a more detailed comparison. The methods developed and validated in this study anticipated a range of samples, from very low-level ocean sites to potentially contaminated samples from oil platforms and natural seeps, therefore a compromise was made between sensitivity and the potential for background interference, and this was reflected in the final detection limits. The instrumental detection limits reported here are at or below the instrumental detection limits reported for the most sensitive method reviewed, the open-ocean study by Goysøyr and others (2009). Modification of the present method could readily achieve these lower detection limits by increasing bile volume, provided larger fish were collected, decreasing reagent volumes, applying additional cleanup steps, and increasing the fraction of the bile applied onto the HPLC-F system. Appropriate quality control measures would be necessary to verify that additional sensitivity, as defined by the signal to noise ratio, and not only additional nonspecific signal was achieved with field sample matrices.

Relative Potential for PAH Exposure of Fishes Resident at Platform Sites and Natural Areas near Seeps

Discharges from 23 oil platforms operating in the Southern California Bight in 1996 and 2000 were estimated from available data by Steinberger and others, 2004. Volumes of produced water (high salinity brine commingled with oil or gas or both in a well) were about 5×10^9 L and constant over time; amounts of associated solids decreased several-fold, from 12 metric tons in 1996 to 3 metric tons in 2000. These inputs into the Bight were considered minor compared to inputs from nearby coastal publicly owned treatment works. In contrast, releases of heavy crude from natural seeps offshore of Goleta, California, were estimated at 20–25 metric tons per day by Farwell and others (2009). Platform Holly and two designated natural sites, Campus Point and Isla Vista-Devereau, are located in this natural seep area off Coal Oil Point (not shown). In a recent study of the fate of heavy crude, Farwell and others (2009) reported that sediment contamination resulted from fallout from the persistent surface plumes from natural seeps and that coastal currents modulated the fallout distribution with a period of 0.4 to 5 days. The mean direction of coastal currents is westward and shoreward along the mainland coast in this vicinity. The effects of heavy crude from natural seeps on sediment-dwelling organisms in the area studied by Farwell were estimated to be from 0.3×10^6 to 3×10^6 metric tons; the equivalent of 8 to 80 spills of the Exxon Valdez accident of 1989 (Farwell and others, 2009).

The potential for exposure of fishes to PAH contamination in the Southern California Bight is dependent on the

Table 8. Recent reports of hydroxylated PAHs in bile of Fishes: concentration ranges and method detection limits.

[PAHs, polyaromatic hydrocarbons; HPLC-F, high-performance liquid chromatography-fluorescence detection; GC/MS, gas chromatography – mass spectrometry; GC/HRMS, gas chromatography – high-resolution mass spectrometry; ng/g, nanograms per gram; ng/mL, nanograms per milliliter; ng/mg, nanograms per milligram of protein; n/a, not applicable; LOD, limit of detection; equiv, equivalence; BCR, European Union reference material code; ring, number of aromatic rings.]

Location	Species	Hydroxy-PAH Concentration Range	Detection Limits	Results	Reference
Baltic Gulf of Finland Villinki	perch	HPLC-F 14 – 43 ng/g bile 6 – 14 ng/g bile (reference site)	n/a	1-hydroxypyrene may be used as a bioindicator for exposure.	Vuoreinen and others, 2003
Baltic Sea	perch flounder eelpout	HPLC-F 20 – 700 ng/g 15 – 95 ng/mg-protein	n/a	1-hydroxypyrene detected in all samples.	Vuoreinen and others, 2006
Severn Estuary Bristol Channel	common eel European flounder	n/a	n/a	Distribution of OH-PAHs in all samples: 1-hydroxypyrene 74-84 percent 1-hydroxyphenanthrene 2-8 percent 1-hydroxychrysene 2-15 percent 3-hydroxybenzo(a)pyrene < 3 percent	Ruddock and others, 2002
Baltic Sea	Perch Salmon flounder eelpout	HPLC-F 1-hydroxypyrene <LOD – 1200 ng/mL bile	HPLC-F 2-hydroxynaphthalene 477 ng/mL bile 1,2-dihydroxychrysene 209 ng/mL bile 1-hydroxyphenanthrene 224 ng/mL bile 1-hydroxypyrene 20.2 ng/mL bile 3-hydroxybenzo(a)pyrene 41.5 ng/mL bile	n/a	Vuontisjärvi and others, 2003
Northwest Mediterranean	red mullet sea cucumber	HPLC-F 1-hydroxypyrene equiv 210 – 4194 ng/mL naphthol equiv 23 – 117 µg/mL benzo(*a*)pyrene equiv 34 – 1259 ng/mL 1-hydroxypyrene equiv 576 – 2927 ng/mL naphthol equiv 43 – 378 µg/mL benzo(*a*)pyrene equiv 18 – 224 ng/mL Total PAH 15 – 159 ng/mL (0.26 – 3.16 ng/mg protein)	n/a	n/a	Escartin and others, 1999

Table 8. Recent reports of hydroxylated PAHs in bile of Fishes: concentration ranges and method detection limits.—Continued

[PAHs, polyaromatic hydrocarbons; HPLC-F, high-performance liquid chromatography-fluorescence detection; GC/MS, gas chromatography – mass spectrometry; GC/HRMS, gas chromatography – high-resolution mass spectrometry; ng/g, nanograms per gram; ng/mL, nanograms per milliliter; ng/mg, nanograms per milligram of protein; n/a, not applicable; LOD, limit of detection; equiv, equivalence; BCR, European Union reference material code; ring, number of aromatic rings.]

Location	Species	Hydroxy-PAH Concentration Range	Detection Limits	Results	Reference
n/a	Reference Materials	BCR 720 (HPLC-F) 2-hydroxynaphthalene - nd 1-hydroxyphenanthrene 544 ng/mL bile 1-hydroxypyrene 51782 ng/mL bile 3-hydroxybenzo(*a*)pyrene 80 ng/mL bile BCR 721 (HPLC-F) 2-hydroxynaphthalene - nd 1-hydroxyphenanthrene - nd 1-hydroxypyrene 2196 ng/mL bile 3-hydroxybenzo(a)pyrene - nd	HPLC-F 2-hydroxynaphthalene 24±13 ng/mL bile 1-hydroxyphenanthrene 20±12 ng/mL bile 1-hydroxypyrene 14±25 ng/mL bile 3-hydroxybenzo(*a*)pyrene 14±8 ng/mL bile GC/MS 2-hydroxynaphthalene 30±6 ng/mL bile 1-hydroxyphenanthrene 60±14 ng/mL bile 1-hydroxypyrene 70±23 ng/mL bile 3-hydroxybenzo(*a*)pyrene 3,000±2,000 ng/mL bile	n/a	Jonsson and others, 2003
Colombia Cartenga Bay Caimanera Marsh Totumo Marsh	Lisa	GC/MS Cartenga Bay 2-ring 9 ng/mL bile 3-ring 220 ng/mL bile 4-ring 940 ng/mL bile 5-ring 47 ng/mL bile Caimanera Marsh 2-ring 5 ng/mL bile 3-ring 24 ng/mL bile 4-ring 150 ng/mL bile 5-ring - nd Totumo Marsh 2-ring 2 ng/mL bile 3-ring 16 ng/mL bile 4-ring 45 ng/mL bile 5-ring - nd	n/a	23 hydroxylated PAHs determined by hydrolysis, derivatization, and GC/HRMS.	Johnson-Restrepo and others, 2008
Pacific Ocean Crossing Peruvian coast Polynesia	dorado wahoo mackerel tuna jackfish	HPLC-F 1-hydroxyphenanthrene < 1 – 3.4 ng/mL bile 1-hydroxypyrene < 0.5 – 1.4 ng/mL bile 3-hydroxybenzo(a)pyrene < 2 ng/mL bile	HPLC-F 1-hydroxyphenanthrene < 1 ng/mL bile 1-hydroxypyrene < 0.5 ng/mL bile 3-hydroxybenzo(a)pyrene < 2 ng/mL bile	Open ocean sampling	Goksøyr and others, 2009

Table 8. Recent reports of hydroxylated PAHs in bile of Fishes: concentration ranges and method detection limits.—Continued

[PAHs, polyaromatic hydrocarbons; HPLC-F, high-performance liquid chromatography-fluorescence detection; GC/MS, gas chromatography – mass spectrometry; GC/HRMS, gas chromatography – high-resolution mass spectrometry; ng/g, nanograms per gram; ng/mL, nanograms per milliliter; ng/mg, nanograms per milligram of protein; n/a, not applicable; LOD, limit of detection; equiv, equivalence; BCR, European Union reference material code; ring, number of aromatic rings.]

Location	Species	Hydroxy-PAH Concentration Range	Detection Limits	Results	Reference
Northwest Mediterranean	seabream	Fixed fluorescence screening	Fixed fluorescence screening	General screening comparison to EROD activity.	Insausti and others, 2009
Besos fishing grounds	mullet	2-ring 3000 – 1000000 ng/mL bile	2-ring 50 ng/mL instrumental		
Vilanova fishing grounds	bogue	3-ring 24000 – 69000 ng/mL bile	3-ring 100 ng/mL instrumental		
	flounder	4-ring 340 – 660 ng/mL bile	4-ring 0.05 ng/mL instrumental		
	hake	5-ring 550 – 1200 ng/mL bile	5-ring 1.2 ng/mL instrumental		
	grenadier				
	codling				
	rosefish				

location- and time-integrated concentrations and profiles of PAHs contacted by the fish and their food webs. Exposure of marine fish communities to petroleum hydrocarbons and their concomitant PAHs in the outer continental shelf is likely to follow a similar cycle of events, regardless of the nature of the point source: platform or natural seep. Straughan and others (1982) presented the following working hypothesis based on a review of population, community, and ecosystem studies at Coal Oil Point, California.

Initial exposure to large volumes of petroleum results in near or total destruction of all organisms; this is followed by a stimulatory period of rapid growth of lower trophic level biota, with different species having different cycle times; and finally concludes in a returning of the area to normalcy. Because of the patchy distribution of seeps (and platforms), the general effect is a mosaic of small units that are at different stages of this cycling process. The overall effect is the enrichment in some communities in these areas.

Peterson and others (1996) proposed an explanation for the typical failure to detect evidence of exposure or of sub-lethal impacts on fishes at drilling operations:

> (…the failure to detect evidence of exposure or sublethal impacts on fishes and most larger invertebrates is a joint consequence of their mobility over the relevant scales of environmental change and their negligible exposure to hydrocarbons and other contaminants.)

Effects from exposure to hydrocarbons and PAHs near platforms have been observed in benthic organisms (mostly polychaetes, nematodes, and crustaceans) at distances of about 1,000 (meter) m in shallow waters with depths of only a few meters (Armstrong and others, 1979); however, the distances for effects drop-off sharply as water depth increases. Osenberg and others (1992) reported effects in outplanted mussels were measurable only out to 100 m from platforms at depths of 10 to 12 m. Effects included depressed mussel densities, decreased survivorship, and lower allometric values indicative of inhibited growth. In an assessment of impacts from drilling in Southern California's Arguello field from 1986 to 1990, Steinhauer and others (1994) concluded that the most likely sources for exposure to PAHs were from natural seeps. Biochemical endpoints in California halibut (*Paralichthys californicus*) exposed to heavy crudes (from natural seeps) were determined to be less sensitive than the same endpoints previously reported for PAHs from urban sources (Seruto and others, 2005); it was hypothesized that this insensitivity may be unique to natural petroleum sources because of higher concentrations of lower molecular weight PAHs or the presence of uncharacterized inhibitors.

In this study, PAH exposure as evidenced by OH-PAH metabolites in resident fish populations at platforms is not observably different than in fish from nearby natural areas. Concentrations of hydroxylated PAHs in all fish samples were very low, ranging from less than the limits of detection (5 to 120 ng/mL bile or 0.03 to 42 nanograms per milligram protein,

ng/mg protein) to a maximum of 320 ng/mL bile or 32 ng/mg protein. 1-Hydroxypyrene, suggested for use as a dosimeter of PAH exposure of fish, was not detected at any of the platform sites. Among all fish samples, low levels of 1-hydroxypyrene were detected in only 3 of 12 kelp rockfish collected from a natural reef site off Santa Barbara. The most prevalent OH-PAH, 2-hydroxyfluorene, was detected at very low levels in only 7 fish sampled from 2 of 7 platforms (4 of 64 fish) and 3 of 12 natural areas (3 of 76 fish), and the concentration differences among sites varied by less than threefold.

Acknowledgments

The authors acknowledge James Candrl of the U.S. Geological Survey for the preparation of the positive and negative control bile materials developed for this study.

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Supporting Information

Table S1. Hydroxylated PAH metabolite concentrations in bile from fishes collected at natural reefs near seeps and at platform sites in the Southern California Bight, Summer 2008. The Excel file may be downloaded from *http://pubs.usgs.gov/of/2012/1248/downloads/supplemental_tables.xlsx*.

Table S2. Protein-normalized hydroxylated PAH metabolite concentrations in bile from fishes collected at natural reefs near seeps and at platform sites in the Southern California Bight, Summer 2008. (Excluding protein-normalized estimates of the limits of detection or quantification. The Excel file may be downloaded from *http://pubs.usgs.gov/of/2012/1248/downloads/supplemental_tables.xlsx*.

Table S3. Protein-normalized hydroxylated PAH metabolite concentrations in bile from fishes collected at natural reefs near seeps and at platform sites in the Southern California Bight, Summer 2008. (Including protein-normalized estimates of the limits of detection or quantification). The Excel file may be downloaded from *http://pubs.usgs.gov/of/2012/1248/downloads/supplemental_tables.xlsx*.

Table S4. Summary quality-control statistics monitored for hydroxylated PAH metabolite concentrations in bile from fishes collected at natural reefs near seeps and at platform sites in the Southern California Bight, Summer 2008. The Excel file may be downloaded from *http://pubs.usgs.gov/of/2012/1248/downloads/supplemental_tables.xlsx*.

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