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Mass Spectrometric Analysis for Aromatic Compounds in Bile of Fish Sampled after the *Exxon Valdez* Oil Spill

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■ After the *Exxon Valdez* oil spill, the exposure of marine organisms to petroleum had to be determined. Gas chromatography/mass spectrometry was used to identify metabolites of aromatic compounds (ACs), such as alkylated naphthols, phenanthrols, and dibenzothiophenols, in the hydrolyzed bile of five salmon (*Oncorhynchus gorbuscha*) and four pollock (*Theragra chalcogramma*) captured in Prince William Sound several months after the oil spill. These metabolites were not found in control fish sampled from areas not impacted by the oil. The metabolites were identified by comparison to those from the hydrolyzed bile of a halibut (*Hippoglossus stenolepis*) which had been injected with weathered Prudhoe Bay crude oil. The dibenzothiophenols are proposed as promising marker compounds for identifying the exposure of fish to certain crude oils. In addition, a high-performance liquid chromatographic method to screen bile for metabolites of ACs was validated for use in estimating the exposure of fish to petroleum.

Introduction

Following the spill of 11 million gallons of Prudhoe Bay crude oil (PBCO) from the *Exxon Valdez* into Prince William Sound (PWS), AK, in March 1989, analyses to determine oil exposure in the biota along the path of the spill were essential. The degree of exposure of marine organisms to oil is often assessed by measuring their body burden of petroleum-related aromatic compounds (ACs), because ACs are potentially harmful to the animals (*1*). However, fish and marine mammals extensively metabolize most ACs in their livers and then the metabolites are excreted, predominantly into bile (*2–6*). A rapid screening method for bile, which determines the metabolites as fluorescent ACs (FACs), has proven useful in estimating the exposure of fish and marine mammals to petroleum (*7, 8*). But this screening method is limited to providing relative concentrations of FACs in bile; individual metabolites are not identified and quantitated. Accordingly, detailed chemical analyses are needed to determine the concentrations of individual metabolites of petroleum-related ACs in selected bile samples and, thus, to support the results of the semiquantitative bile screening method showing exposure of marine organisms to spilled oil.

The specific individual metabolites that result from the uptake and metabolic conversion of petroleum ACs in fish have not been well-characterized. Two previous studies identified only a few individual AC metabolites in the livers (*2*) or bile (*3*) of fish that had been exposed to no. 2 fuel oil, a distillate fraction of petroleum that contains only a portion of the ACs found in crude oil. In a more detailed study, several individual metabolites were identified by gas chromatography/mass spectrometry (GC/MS) in the bile of fish captured from urban sites (*9, 10*). However, urban sediments generally contain high proportions of products from the combustion of fossil fuels, such as unsubstituted, four- to six-ring ACs. In contrast, PBCO contains a variety of one- to three-ring alkylated ACs, as well as the alkylated dibenzothiophenes typical of the North Slope crude oils (*11–14*). But because spilled oil is degraded in time by physical, chemical, and microbial processes, the aromatic fraction of the weathered oil will be dominated by those ACs (e.g., highly alkylated naphthalenes, phenanthrenes, and dibenzothiophenes) that are most resistant to weathering (*15, 16*). Therefore, metabolites of these resistant ACs should be found in bile of fish exposed to weathered crude oil; in this paper, we report the results of our efforts to characterize such compounds.

Initially, the products resulting from the metabolism of PBCO by fish were identified by GC/MS in the enzymatically hydrolyzed bile of a halibut (*Hippoglossus stenolepis*) and a Dolly Varden (*Salvelinus malma*) injected with weathered PBCO. Subsequently, many of these metabolites were determined in the hydrolyzed bile of five pink salmon (*Oncorhynchus gorbuscha*) and four pollock (*Theragra chalcogramma*) collected in PWS several months after the oil spill. Finally, the bile screening method was validated by demonstrating a strong statistical correlation between concentrations of FACs determined by screening and the sums of metabolite concentrations determined by GC/MS in oil-exposed fish.

Experimental Section

Chemicals. A sample of PBCO was obtained from the oil remaining in the hold of the *Exxon Valdez*, and the weathered PBCO sample was collected from PWS 11 days after the spill occurred. 2,6-Dimethyl-3-naphthol, 6-methyl-2-naphthalenemethanol, and *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene were prepared in our laboratories (*17*).

Reference Standard. A GC/MS standard, containing reference compounds dissolved in methanol, was prepared (listed in order of GC elution, ng/μL): 2,6-dibromophenol (surrogate standard, 7.38), hexamethylbenzene (GC internal standard, 7.56), 1-naphthol (15.66), 2-hydroxybiphenyl (15.72), 6-methyl-2-naphthalenemethanol (2.76), 4-methyl-1-naphthol (15.42), 2,6-dimethyl-3-naphthol (2.82), *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (3.36), 9-fluorenol (15.54), phenanthrene-d_{10} (HPLC internal standard, 6.00), 9-phenanthrol (15.06), 9-anthracenemethanol (14.76), and 1-pyrenol (13.80).

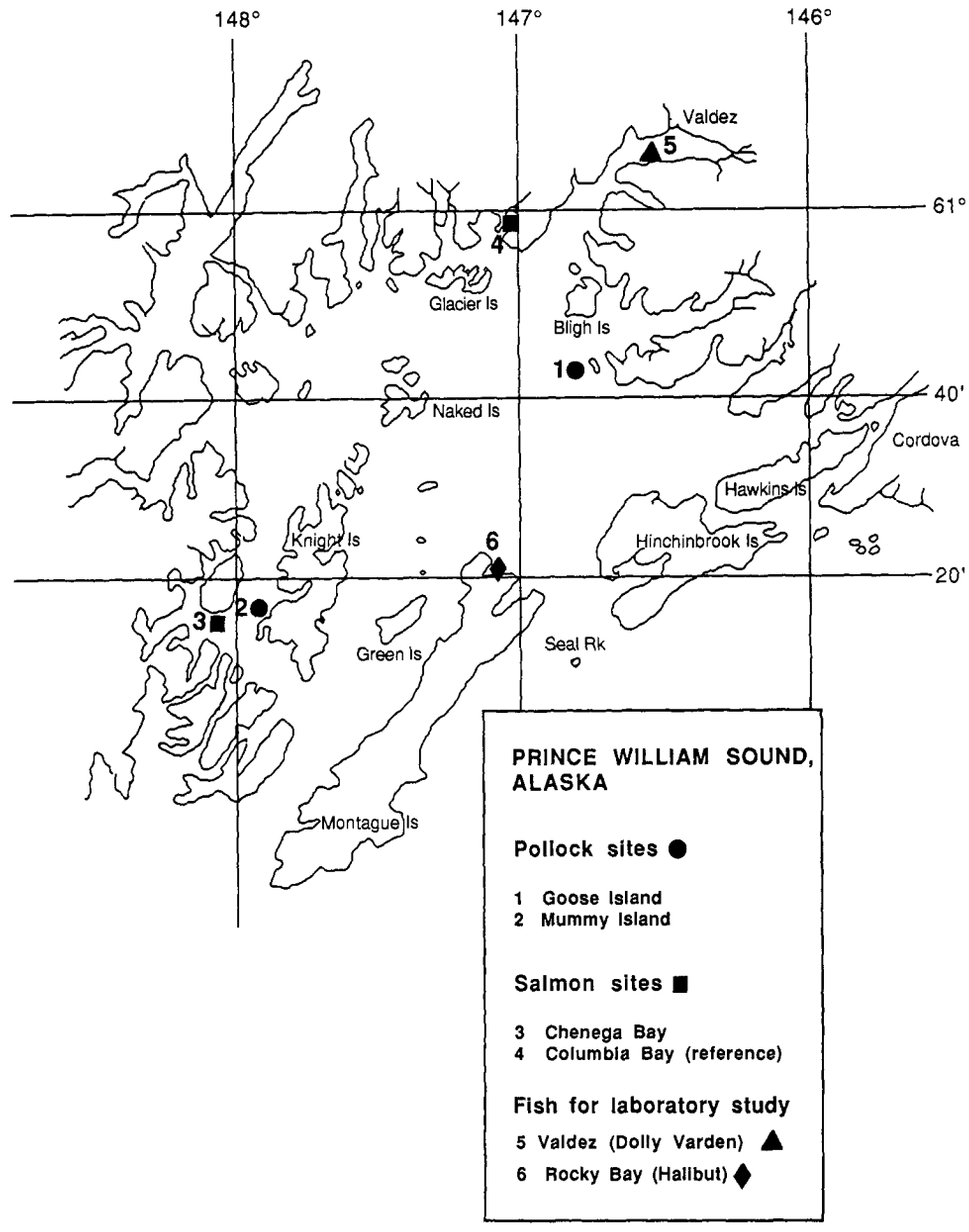

Figure 1. Chart of Prince William Sound showing the sites of capture for salmon, pollock, and fish (halibut and Dolly Varden) injected with weathered Prudhoe Bay crude oil. The relatively uncontaminated site (reference site) from which pollock were captured, Seymour Canal, is located in southeastern Alaska near Ketchikan (not shown).

Injection of Fish with PBCO. Halibut (*Hippoglossus stenolepis*) were captured by long line from a relatively uncontaminated site (reference site) in PWS (Figure 1). The halibut were injected, into the dorsal anterior musculature, with (a) 1.0 μL/g of fish wt of a 1:1 (v:v) mixture of weathered PBCO dissolved in a carrier or (b) with 0.5 μL/g of fish wt of the carrier alone. The carrier was a 1:1 (v:v) mixture of acetone and Emulphor. The fish were maintained in flow-through seawater aquaria and were not fed after injection. After 48 h, the fish were killed by severing the spinal column; bile was then removed from the gall bladders and frozen. The above experiment was repeated using Dolly Varden char (*Salvelinus malma*) captured by gill net from a site in PWS not impacted by oil (Figure 1). Bile samples were selected for this study from the following fish: an oil-injected halibut (wt) (2500 g), a carrier-injected halibut (5400 g), an oil-injected Dolly Varden (368 g), and a carrier-injected Dolly Varden (378 g).

Field Collection of Fish. Adult pink salmon (*Oncorhynchus gorbuscha*) were captured in PWS (Figure 1) by hook and line at an oiled site (Chenega Bay) ~5 months postspill and by gill net from a site relatively unimpacted by oil (Columbia Bay) ~4 months postspill. Adult pollock (*Theragra chalcogramma*) were captured by trawl ~1 year after the spill from areas that had been oiled (Mummy Island and Goose Island) in PWS (Figure 1) and from a relatively uncontaminated site, Seymour Canal, in southeast Alaska. Bile was collected from the gall bladders and the samples were frozen. Fish from the field were then selected for this study on the basis of a number of criteria. FAC concentrations determined by bile screening were available for approximately 300 salmon and 200 pollock collected within 1 year of the spill. Because we intended to confirm the results of bile screening by analyzing for individual bile metabolites by GC/MS, we selected bile samples with a range of FAC concentrations. The minimum amount of bile that was suitable for both screening and GC/MS analysis was 125 μL.

Hydrolysis of Bile and Extraction of Metabolites. A modification of the enzymatic hydrolysis and extraction method of Varanasi, Nishimoto, Reichert, and Eberhart

Table I. Ions of Metabolites (m/z) of Aromatic Compounds Scanned by Sequenced Selected Ion Monitoring[a]

compound	molecular ion (M^+)	ion 2	ion 3	ion 4	ion 5	segment	scan start time, min
2,6-dibromophenol	250	252				1	10
hexamethylbenzene		147				1	10
phenanthrene-d_{10}	188	184				1, 2	10
C_2 naphthols	172	157	143	129		1, 2	10
C_3 naphthols	186	171	143	129		1, 2	10
C_1 dibenzothiophenols	214	185	184	165		3, 4	31
C_2 dibenzothiophenols	228	213	185	184	165	4	36
C_3 dibenzothiophenols	242	227	185	184	179	4, 5	36
C_1 phenanthrols	208	179	178	165		4	36
C_2 phenanthrols	222	193	179	178	165	4, 5	36
C_3 phenanthrols	236	193	192	178		5, 6	41

[a] For the naphthols, dibenzothiophenols, and phenanthrols, the fragment ions are identified as follows: $M^+ - 15 = CH_3$; $M^+ - 29 = CHO/C_2H_5$; $M^+ - 30 = CH_2O/C_2H_6$; $M^+ - 43 = C_2H_3O/C_3H_7$; $M^+ - 44 = C_2H_4O/C_3H_8$; $M^+ - 57 = C_3H_5O/C_4H_9$. A table of the other ions scanned and the start time for each segment is available in the supplementary material.

(*18*) was used. Each bile sample (100 μL) was treated with 2000 units of β-glucuronidase (containing 25 units of arylsulfatase activity) in 0.4 M acetate buffer (1 mL; pH 5). A surrogate standard, 2,6-dibromophenol (100 μL, 24.6 ng/μL) was added. The samples were incubated in a warm water bath at 40 °C for 2 h and then extracted with methylene chloride (1 mL) and methanol (100 μL). After extracting two additional times with methylene chloride (1 mL each), the extracts were combined and sodium sulfate (0.1 g) was added. The combined extracts were transferred to concentrator tubes and the solvent was evaporated to 1 mL. Phenanthrene-d_{10} (HPLC internal standard; 50 μL, 60 ng/μL) was added and the solvent was further evaporated to 400 μL under a stream of nitrogen gas.

HPLC Cleanup of Bile Extracts. A modification of an HPLC cleanup technique (*19*, *20*) was used to separate the metabolites from any biogenic material in the extracts from bile hydrolysis. The procedure was modified by slowing the solvent flow rate to 5 mL/min and by calibrating to collect a fraction (containing the metabolites) from the beginning of the biphenyl elution to the end of 1-pyrenol elution (approximately 19–30 min). A 250-μL portion of the 400-μL extract from hydrolysis was injected onto the HPLC column, the fraction was collected, and the solvent was evaporated to 1 mL. Methanol (0.500 mL) was added, the solvent was evaporated to 1 mL, and hexamethylbenzene (GC internal standard; 30 μL, 25.2 ng/μL) was added; evaporation was continued under a stream of nitrogen gas until the volume was reduced to 30 μL.

GC/MS Analysis of Bile Extracts. The extracts from hydrolyzed bile were analyzed by GC/MS as described earlier (*9*), except that the GC/MS system now included a 5970 Hewlett-Packard mass selective detector (MSD), a 59940A Hewlett-Packard HP-UX Chemstation data system, a 5890 Hewlett-Packard GC, and a 7673B autosampler. The mass spectrometer was scanned using a sequenced selected ion monitoring (SSIM) descriptor at ~1 scan/s. The GC run time was divided into segments in which different sets of ions were scanned (see Table I; also, Table A-I in the supplementary material has a complete list of the ions scanned in each segment). For full-scan spectra, the mass spectrometer was scanned from 45 to 450 amu at approximately 1 scan/s.

Identification and Quantitation of Metabolites. Full-scan GC/MS analyses were conducted on bile from the oil-injected halibut. Numerous metabolites of ACs were tentatively identified by comparing retention times and mass spectra for each metabolite to (a) reference standards or (b) mass spectra from the mass spectral library. This library includes spectra of metabolites from a previous study in which fish were injected with unsubstituted ACs (*10*). In addition, identification of some metabolites, particularly those in the C_2–C_3 alkylated ACs, was based on the molecular ion and on fragmentation patterns. Subsequently, SSIM was used to increase sensitivity in analyzing for metabolites of ACs in the bile of the halibut and the other fish species studied. Several major ions in the spectrum of each metabolite were selected for scanning in the SSIM mode (Table I). [Full scan and SSIM mass spectra of a C_2 dibenzothiophenol are compared in the supplementary material (Figure A-1).]

The concentrations of the metabolites were calculated using single-point response factors and were corrected for the recovery of the surrogate standard. When no commercially available reference standard was available, metabolites were quantitated using a GC/MS response factor for an isomer (i.e., the C_1 naphthol isomers were quantitated by the response factor for 4-methyl-1-naphthol, the C_2–C_3 naphthols by 2,6-dimethyl-3-naphthol, the fluorenols by 9-fluorenol, the phenanthrols by 9-phenanthrol, the fluoranthenols/pyrenols by 1-pyrenol). In addition, when no isomer or reference standard was available, the concentrations of certain metabolites were calculated using a GC/MS response factor of 1 (i.e., dibenzofuranols, dibenzothiophenols, and dibenz[*a*]anthracenols/chrysenols). As a result, the concentrations determined for many of the metabolites were semiquantitative.

The accuracy of quantitation of certain metabolites may also be limited by their dehydration in the GC. For example, phenanthrene is preferentially metabolized to a dihydrodiol in many fish (*21*, *22*). However, the dihydrodiol dehydrates in the GC resulting in two phenols (*10*). Although derivatization of dihydrodiols to trimethylsilyl ethers prevents dehydration during the GC analyses and thus should allow quantitation of the dihydrodiol itself, the molecular ion of the derivative is small and the identification of the metabolite is difficult (*9*).

Matrix Spikes. To determine recovery efficiencies, reference standards were added to bile from a halibut from a reference site (matrix spike; $n = 3$); hydrolysis, extraction, HPLC cleanup, and GC/MS analyses were then conducted as described above. Reference standards (ng) were as follows: 1-naphthol (1044), 2-hydroxybiphenyl (1048), 6-methyl-2-naphthalenemethanol (184), 4-methyl-1-naphthol (1028), 2,6-dimethyl-3-naphthol (188), *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (224), 9-fluorenol (1036), 9-anthracenemethanol (984), and 1-pyrenol (920).

HPLC/Fluorescence Screening of Bile. A previously reported HPLC method (*23*) was used with fluorescence detection at excitation/emission wavelengths where two-ring (290/335 nm; naphthalene standard), three-ring (260/380 nm; phenanthrene standard), or four- and five-

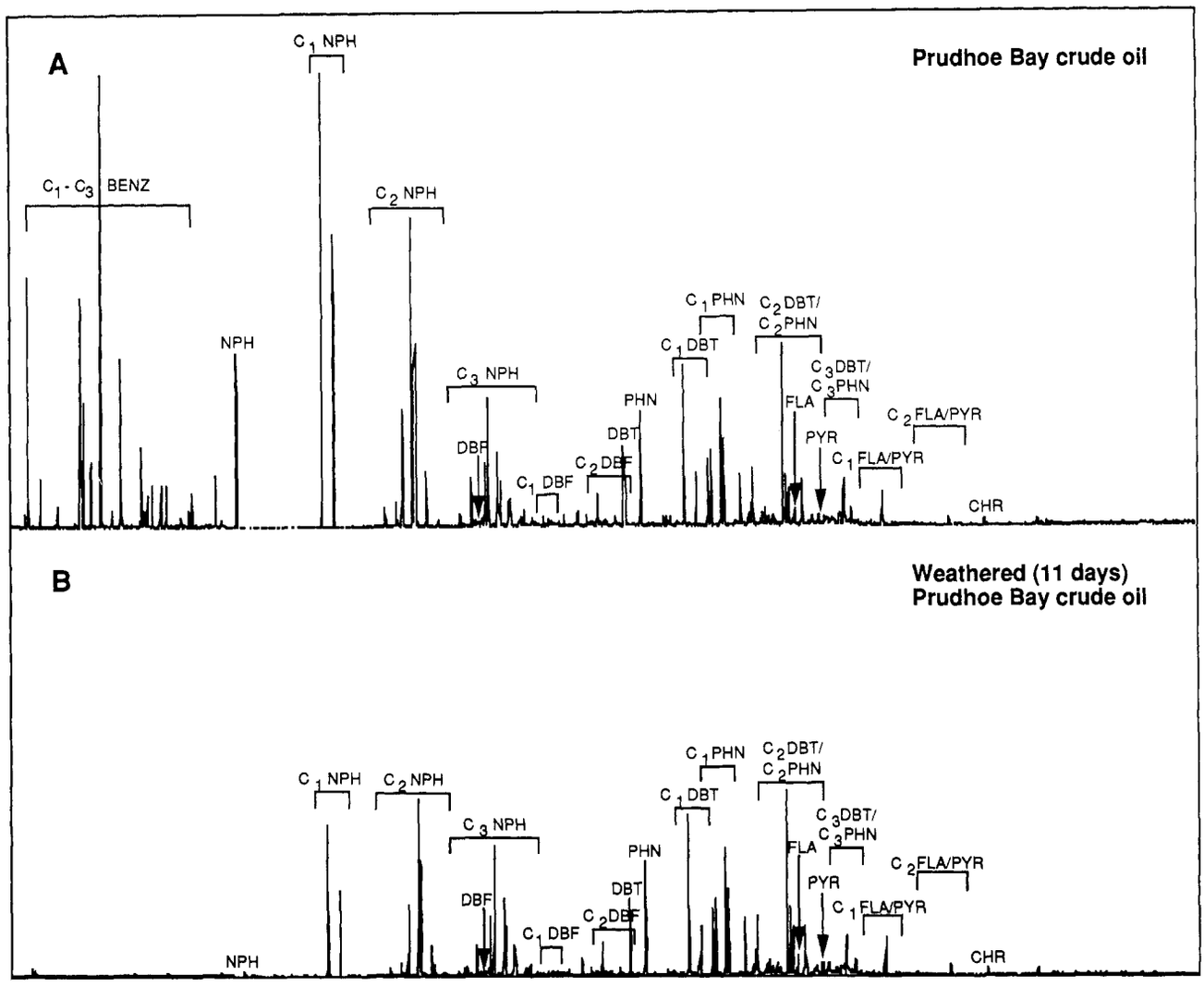

Figure 2. Full-scan GC/MS chromatogram of Prudhoe Bay crude oil (A) taken from the *Exxon Valdez* and (B) collected from a beach in Prince William Sound 11 days after the spill. Abbreviations: BENZ, benzene; NPH, naphthalene; DBF, dibenzofuran; PHN, phenanthrene; DBT, dibenzothiophene; FLA, fluoranthene; PYR, pyrene; CHR, chrysene.

ring [380/430 nm; benzo[*a*]pyrene (B*a*P) standard] ACs fluoresce to quantitate total fluorescent ACs metabolites in (naive) bile.

Statistical Analyses. The relationship between concentrations of bile metabolites determined by GC/MS and concentrations measured by screening of the same bile samples was evaluated by correlation (*24*). The concentrations obtained by each method were first log-transformed to improve homogeneity in the variances.

Results

Chromatograms of Prudhoe Bay Crude Oil. GC/MS chromatograms of the aromatic fraction of PBCO and weathered PBCO are shown in Figure 2. PBCO contained large proportions of highly alkylated ACs with one to three rings (e.g., benzenes, naphthalenes, and phenanthrenes; Figure 2A). After only 11 days of weathering, the composition of the crude oil had changed dramatically; major losses of low molecular weight ACs, particularly the one- and two-ring ACs, had occurred (Figure 2B). Less severe losses were noted for the alkylated phenanthrenes and alkylated dibenzothiophenes in the weathered PBCO.

Identification and Quantitation of Metabolites in Hydrolyzed Bile. Mass chromatograms, such as those shown for the molecular ions of alkylated phenanthrols (Figure 3A,B) and dibenzothiophenols (Figure 3C,D), were graphed for each homologous series of metabolites in each fish. Then, mass spectra (Figure 4) and relative retention times of metabolites identified in the oil-exposed halibut were used to establish the identity of the corresponding metabolites in the oil-injected Dolly Varden and in the environmentally exposed fish sampled from PWS. As many of the metabolites as feasible were identified and quantitated in the bile from three of the fish: 158 in the oil-injected halibut, 119 in salmon A, and 54 in the pollock A. Alkylated naphthols, fluorenols, phenanthrols, dibenzofuranols, and dibenzothiophenols comprised the majority; smaller numbers of alkylated fluoranthenols/pyrenols and benz[*a*]anthracenols/chrysenols were also identified (see Tables II and III for selected metabolites and Table A-II in the supplementary material for a complete list).

In the remainder of the fish, including the oil-injected Dolly Varden, a limited number of bile metabolites were quantitated to illustrate intra- and interspecies differences in relative proportions and concentrations of the metabolites. The 22 metabolites selected were as follows: C_2 and C_3 naphthalenes ($n = 2$); C_1, C_2, and C_3 dibenzothiophenols ($n = 11$); C_1, C_2, and C_3 phenanthrols ($n = 9$). Concentrations of these metabolites are given for oil- and carrier-injected halibut, for salmon and for the method blank in Table II, and for oil- and carrier-injected Dolly Varden and for pollock in Table III. Bile samples from the reference fish and the carrier-injected fish were analyzed for

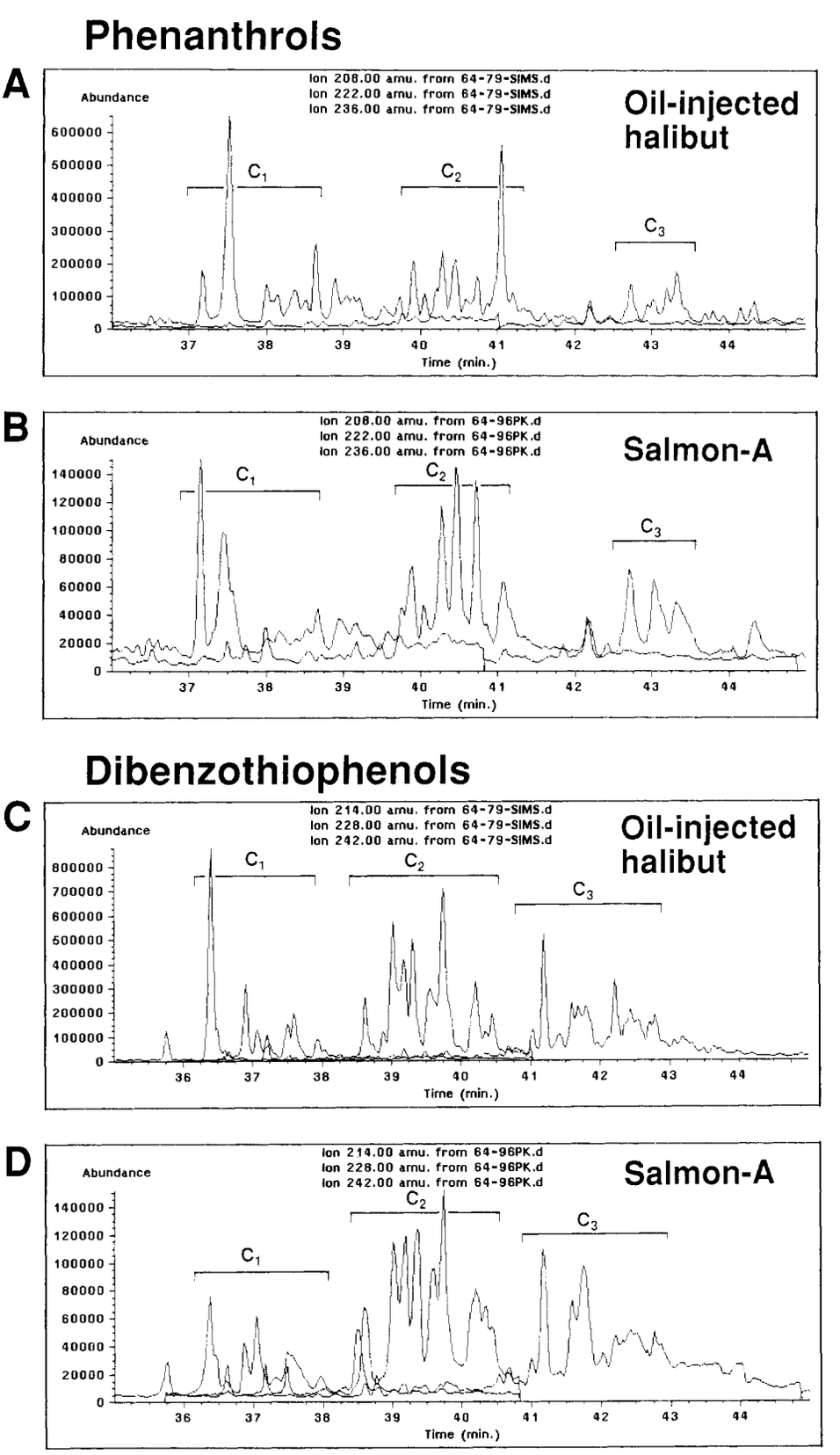

Figure 3. Mass chromatograms of the C_1–C_3 phenanthrols (m/z 208, 222, 236) from the bile of (A) the oil-injected halibut and (B) salmon A and the C_1–C_3 dibenzothiophenols (m/z 214, 228, 242) from the bile of (C) the oil-injected halibut and (D) salmon A.

all metabolites found in the oil-injected halibut. The salmon and pollock collected from reference sites, as well as the carrier-injected halibut and Dolly Varden, contained either low or undetectable concentrations of these metabolites (Tables II and III; detection limits for the other metabolites were similar to those shown). An exception was the C_1 phenanthrol (400 ng/g) found in the bile from the reference pollock (Table III); the source of this contaminant is unknown.

Both phenols and alcohols are formed from the metabolism of certain ACs (*3*, *17*, *25*). However, the structure of these isomeric hydroxy metabolites cannot be assigned solely from their GC/MS spectra; either reference standards are needed for comparison or the phenols must first

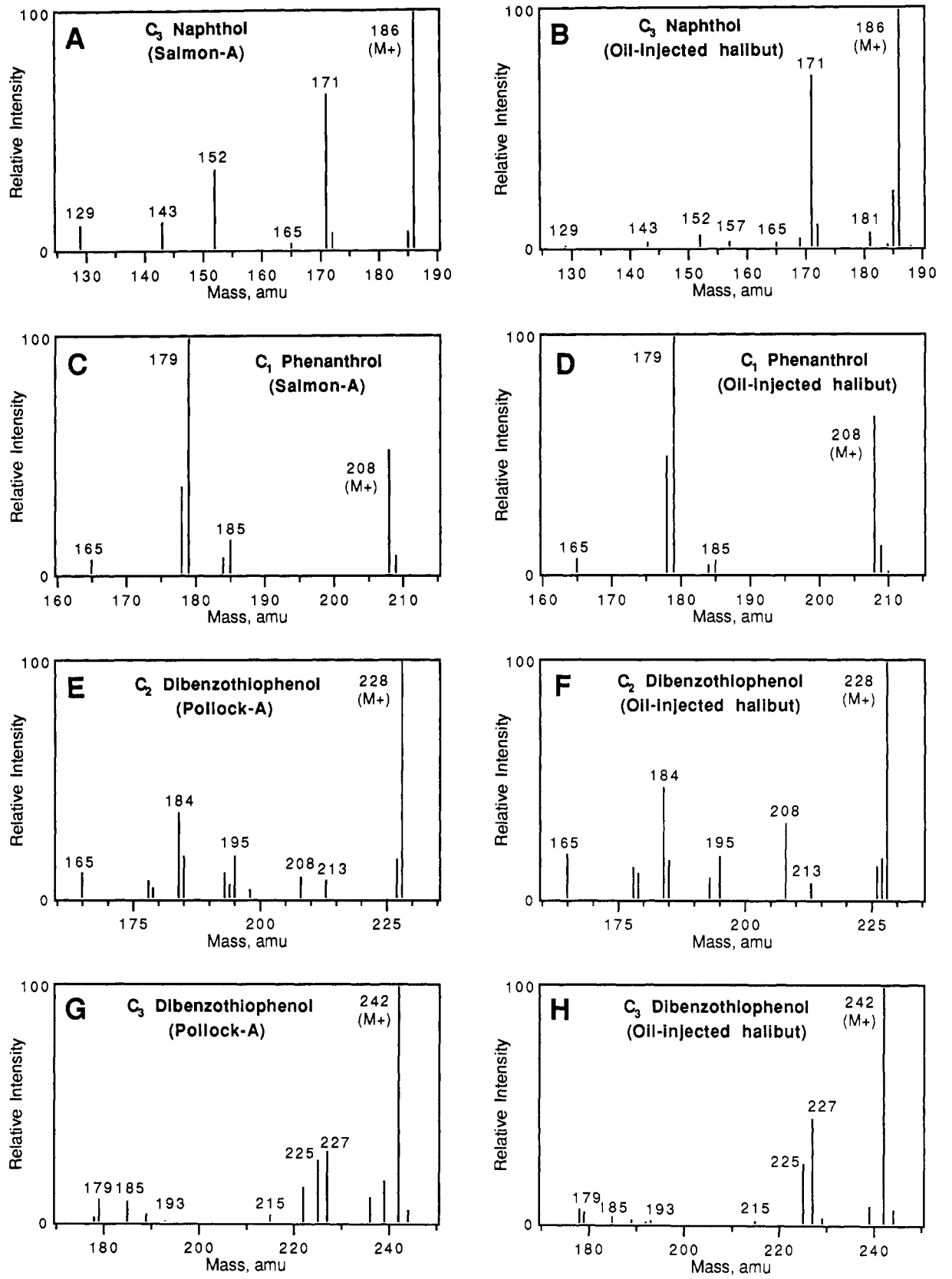

Figure 4. Sequenced selected ion monitoring (SSIM) mass spectra of bile metabolites: C_3 naphthol [relative retention time (RRT) based on phenanthrene-d_{10}, RRT = 1.065] in (A) salmon A and (B) the oil-injected halibut; C_1 phenanthrol (RRT = 1.409) in (C) salmon A and (D) the oil-injected halibut; C_2 dibenzothiophenol (RRT = 1.450) in (E) pollock A and (F) the oil-injected halibut; C_3 dibenzothiophenol (RRT = 1.546) in (G) pollock A and (H) the oil-injected halibut.

be separated from the alcohols on the basis of their acidity. Thus, we have arbitrarily identified most of these hydroxy metabolites as phenols (Tables II, III, and A-II), although a number of alcohols may have been formed.

Identification of Dibenzothiophenols. The dibenzothiophenols, important metabolites because they can serve as marker compounds in bile for detecting the exposure of fish to certain crude oils (see Discussion), have not been reported previously. The homologous series of C_0–C_3 dibenzothiophenols were identified in the bile of the

Table II. Relative Retention Times (RRT) Based on Phenanthrene-d_{10} (PHN-d_{10}) and Concentrations by SSIM GC/MS of Selected Metabolites of Naphthalenes, Dibenzothiophenes, and Phenanthrenes in Bile of (a) Halibut Injected with Prudhoe Bay Crude Oil and with Carrier, (b) Salmon Captured in Prince William Sound 5 Months after Oil Was Spilled into the Sound, and (c) the Method Blank

	RRT (PHN-d_{10})	concentration, ng/g wet wt: halibut oil-inj	halibut carrier-inj	salmon A	salmon B	salmon C	salmon D	salmon E	salmon ref	method blank
naphthols										
C_2	0.913	66	<7	310	87	1400	75	71	10	<10
C_3	1.065	1600	<7	960	210	2600	470	300	10	<10
dibenzothiophenols										
C_1	1.367	17000	55	6900	810	4900	1800	2800	56	<12
	1.385	5900	<30	2600	870	3500	940	890	33	<12
	1.391	2600	48	5100	690	12000	1900	1800	39	<12
C_2	1.465	14000	73	11000	2000	6000	3700	4400	<29	<12
	1.471	9800	45	10000	1900	6400	3700	4100	<29	<12
	1.475	11000	44	12000	2000	7700	5100	4300	49	<12
	1.492	21000	<30	15000	2700	8300	5400	5700	<29	<12
	1.509	9100	<30	11000	1900	8500	4100	4500	<29	<12
C_3	1.546	9000	<30	9100	1800	3200	3500	3900	<29	<12
	1.561	4200	<30	5800	3300	10000	4300	3300	<29	<12
	1.565	4600	<30	12000	2700	7300	6600	6100	<29	<12
sum of metabolites fluorescing at 290/335 nm[a]		**110000**	**360**	**102000**	**21000**	**82000**	**42000**	**42000**	**300**	**80**
phenanthrols										
C_1	1.409	14000	52	10000	1,300	18000	2800	3800	140	<27
	1.432	1200	<12	1400	150	1600	580	610	<11	<17
	1.451	3100	<12	1800	290	2800	1000	780	<11	<17
C_2	1.512	3200	22	3700	490	2000	1300	1300	18	<17
	1.519	2700	<12	4900	830	4300	1900	1900	44	<17
	1.529	1900	<12	4200	480	2200	1200	1200	17	<17
	1.541	6600	<12	3200	660	2800	1800	690	<11	<17
C_3	1.622	1400	<12	3300	640	1400	1600	1600	<11	<17
	1.626	3200	<12	3600	580	1200	1700	1900	<11	<17
sum of metabolites fluorescing at 260/380 nm[a]		**37000**	**120**	**36000**	**5400**	**36000**	**14000**	**14000**	**250**	**80**
2,4-dibromophenol rec, %		100	94	100	92	99	110	99	84	120

[a] When a concentration is below the limit of quantitation (LOQ; preceded by <), an amount equal to $^1/_2$ the LOQ is included in the sum of metabolites.

oil-injected halibut from their molecular ions (mass/charge (m/z) 200, 214, 228, 242) and their fragmentation patterns (Table I and Figure 4). The dibenzothiophenols were also identified in the field-exposed fish. For example, nine C_2 dibenzothiophenols isomers were present in bile of the oil-injected halibut (Figure 3C) and from four to nine isomers in the other species; e.g., nine isomers were present in salmon A (Figure 3D). However, the occurrence and relative proportions of these isomeric dibenzothiophenols were different among the individual fish (Figure 5; see Discussion).

Recoveries in Matrix Spikes. Percent recoveries (mean ± standard deviation) of the reference standards in the spiked matrices (n = 3) were as follows: 1-naphthol (110 ± 10), 2-hydroxybiphenyl (110 ± 6), 6-methyl-2-naphthalenemethanol (110 ± 29), 4-methyl-1-naphthol (79 ± 11), 2,6-dimethyl-3-naphthol (86 ± 5), *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene (140 ± 38), 9-fluorenol (120 ± 12), 9-anthracenemethanol (120 ± 21), 1-pyrenol (92 ± 11), and 2,6-dibromophenol (surrogate standard, 79 ± 6). In general, the polar phenols were more difficult to extract and to recover from chromatographic procedures than were the alcohols. Because recoveries of the analytes (above) were corrected for the surrogate standard (a phenol), the recoveries of the alcohols tended to be high.

Comparisons of Screening and GC/MS Methods. Bile samples from all the fish in the study were analyzed by the screening method of Krahn, Rhodes, Myers, Moore, MacLeod, and Malins (*26*); the FAC equivalents at phenanthrene (260/380 nm) and naphthalene (290/335 nm) wavelengths are reported in Table IV. The highest concentrations of FACs in bile were found in the halibut injected with PBCO. The salmon had the next highest levels, the oil-injected Dolly Varden and pollock had intermediate concentrations, and the reference fish had the lowest levels of all the fish. BaP equivalents were determined for the salmon (in ng/g wet wt): salmon A (2300), salmon B (1300), salmon C (1100), salmon D (830), and salmon E (870).

To make a comparison between the semiquantitative results obtained with the bile screening method and the quantitative results from the detailed GC/MS method, the metabolites in Tables II and III were divided into two groups by their fluorescence characteristics—those that fluoresce at 260/380 nm and those that fluoresce at 290/335 nm. Although no dibenzothiophenol standards were available, these compounds are presumed to fluoresce at 290/335 nm because dibenzothiophene fluoresces at this wavelength pair and phenolic metabolites usually fluoresce at wavelengths close to those of the parent compound. Therefore, the concentrations of the naphthols and dibenzothiophenols were summed to represent metabolites fluorescing at naphthalene wavelengths. Similarly, the phenanthrols were summed for phenanthrene wavelengths (Tables II and III). The total phenanthrene equivalents (analyzed by bile screening; Table IV) were highly correlated with the summed concentrations (GC/MS) of those

Table III. Relative Retention Times (RRT) Based on Phenanthrene-d_{10} (PHN-d_{10}) and Concentrations by SSIM GC/MS of Selected Metabolites of Naphthalenes, Dibenzothiophenes, and Phenanthrenes in Bile of (a) Dolly Varden Injected with Prudhoe Bay Crude Oil and with Carrier and (b) Pollock Captured in Prince William Sound 1 Year after Oil Was Spilled into the Sound

		concentration, mg/g wet wt							
		D. Varden		pollock					
	RRT (PHN-d_{10})	oil-inj	carrier-inj	A	B	C	D (repl 1)	D (repl 2)	ref
naphthols									
C_2	0.913	<14	<12	<0.8	<3	<0.8	<12	<15	<1
C_3	1.065	53	<12	<0.8	<3	<0.8	<12	<15	<1
dibenzothiophenols									
C_1	1.367	150	<15	220	150	170	58	87	<3
	1.385	350	<15	<2	<12	<2	15	<18	<3
	1.391	290	<15	<2	<12	<2	<14	<18	<3
C_2	1.465	250	<15	480	170	330	110	160	<3
	1.471	160	<15	250	90	130	48	53	<3
	1.475	290	<15	<2	<12	<2	28	63	<3
	1.492	160	<15	1100	210	560	150	200	<3
	1.509	270	<15	<2	<12	<2	37	45	<3
C_3	1.546	74	<15	350	120	110	33	38	34
	1.561	80	<15	170	69	140	21	23	<3
	1.565	96	<15	<2	<12	<2	38	38	<3
sum of metabolites fluorescing at 290/335 nm[a]		**2200**	**90**	**2600**	**880**	**1400**	**560**	**740**	**50**
phenanthrols									
C_1	1.409	370	<33	1,100	180	970	280	300	400
	1.432	93	<20	<1	<5	<1	<19	<25	<2
	1.451	580	<20	<1	<5	<1	<19	<25	<2
C_2	1.512	<23	<20	200	49	81	94	110	<2
	1.519	270	<20	290	67	160	140	130	<2
	1.529	91	<20	330	30	71	50	50	<2
	1.541	560	<20	<1	<5	<1	<19	<25	<2
C_3	1.622	<23	<20	190	55	69	79	25	<2
	1.626	<23	<20	<1	<5	<1	<19	<25	<2
sum of metabolites fluorescing at 260/380 nm[a]		**2000**	**100**	**2100**	**400**	**1400**	**680**	**670**	**410**
2,4-dibromophenol rec, %		100	110	110	98	110	110	89	93

[a] When a concentration is below the limit of quantitation (LOQ; preceded by <), an amount equal to $^1/_2$ the LOQ is included in the sum of metabolites.

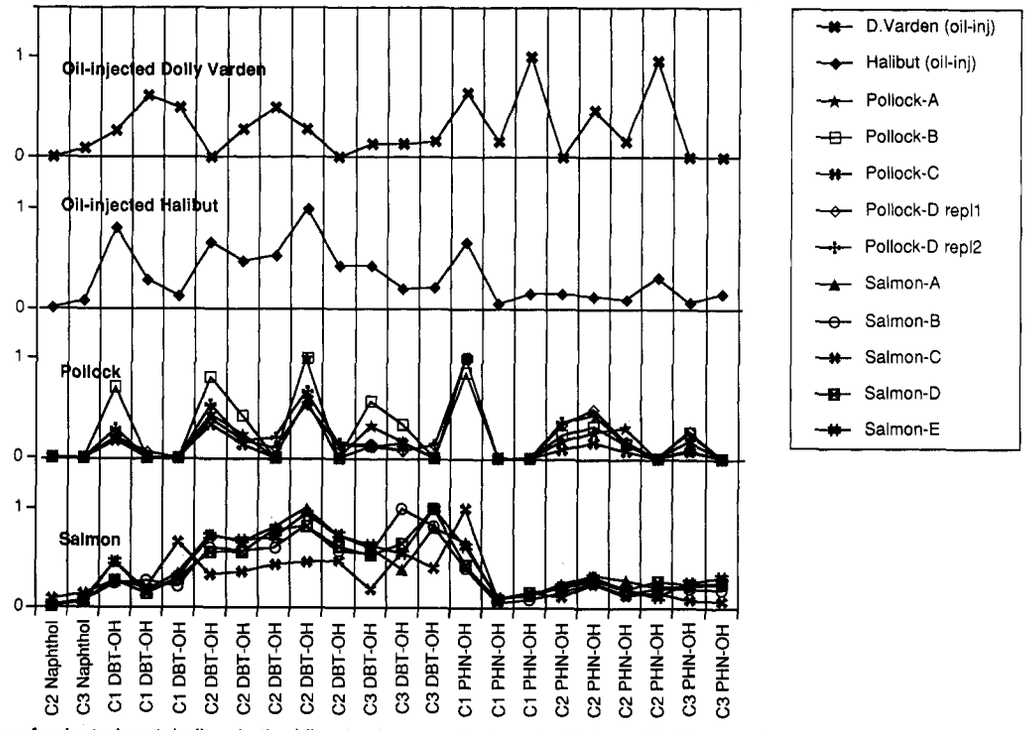

Figure 5. *Profiles of selected metabolites in the bile of salmon, pollock, and oil-injected halibut and Dolly Varden (see Tables II and III for RRT). Concentrations are normalized to the metabolite with the highest concentration. Abbreviations: DBT-OH, dibenzothiophenol; PHN-OH, phenanthrol.*

Table IV. Equivalents of Fluorescent Aromatic Compounds in Bile Determined by the HPLC Screening Method Described by Krahn et al. (*26*)

sample	equivalents of fluorescent aromatic compds,[a] ng/g wet wt: phenanthrene wavelengths (260/380 nm)	equivalents of fluorescent aromatic compds,[a] ng/g wet wt: naphthalene wavelengths (290/335 nm)
oil-injected halibut	1 300 000	3 800 000
solvent-injected halibut	4 000	30 000
oil-injected Dolly Varden	150 000	470 000
solvent-injected Dolly Varden	6 700	40 000
pollock A	90 000	340 000
pollock B	44 000	270 000
pollock C	68 000	280 000
pollock D (n = 2)	66 000	340 000
pollock reference	5 000	36 000
salmon A	370 000	1 800 000
salmon B	240 000	990 000
salmon C	380 000	2 600 000
salmon D	190 000	780 000
salmon E	380 000	1 700 000
salmon reference	5 000	44 000

[a] HPLC bile screening.

metabolites that fluoresce at phenanthrene wavelengths (260/380 nm; Figure 6A). A similar correlation was found for metabolites that fluoresce at naphthalene wavelengths (290/335 nm; Figure 6B).

Comparisons of Metabolite Profiles in Salmon and Pollock. For the fish in this study, each metabolite concentration (from Tables II and III) was divided by (normalized to) the concentration of the most abundant metabolite. The resulting normalized values were then plotted for each metabolite to provide a visual representation of the metabolite profiles (Figure 5). The profiles of the various pollock are similar, while those of the salmon are somewhat similar to each other, but dissimilar to the pollock profiles.

Discussion

The environmental exposure of the feral salmon and pollock in this study to weathered crude oil was supported by results from the analysis of their hydrolyzed bile showing high proportions of metabolites of those petroleum-related ACs that resist the weathering processes. In particular, homologous series of alkylated naphthols, phenanthrols, and dibenzothiophenols were found by GC/MS to be abundant in these fish captured in PWS from 5 to 12 months after the spill. The homologous series of dibenzothiophenols have not been reported previously. We propose that these metabolites can serve as marker compounds to indicate the exposure of fish to particular crude oils. For example, PBCO and other North Slope crude oils contain relatively high proportions of dibenzothiophenes, unlike most other Alaskan (e.g., Cook Inlet crude) and continental U.S. crude oils (*11–13*). Therefore, identification of high concentrations of homologous dibenzothiophenols in the bile of the pollock and salmon suggests a North Slope crude oil as the source of exposure. In future spills of high-sulfur crude oils, the presence of dibenzothiophenols in bile of fish can be used to help establish the source of exposure.

The results of this study have validated bile screening as a method for estimating petroleum exposure in fish. A statistical analysis of concentrations of metabolites of ACs in bile, determined both by bile screening (Table IV) and by GC/MS (Tables II and III), revealed highly significant

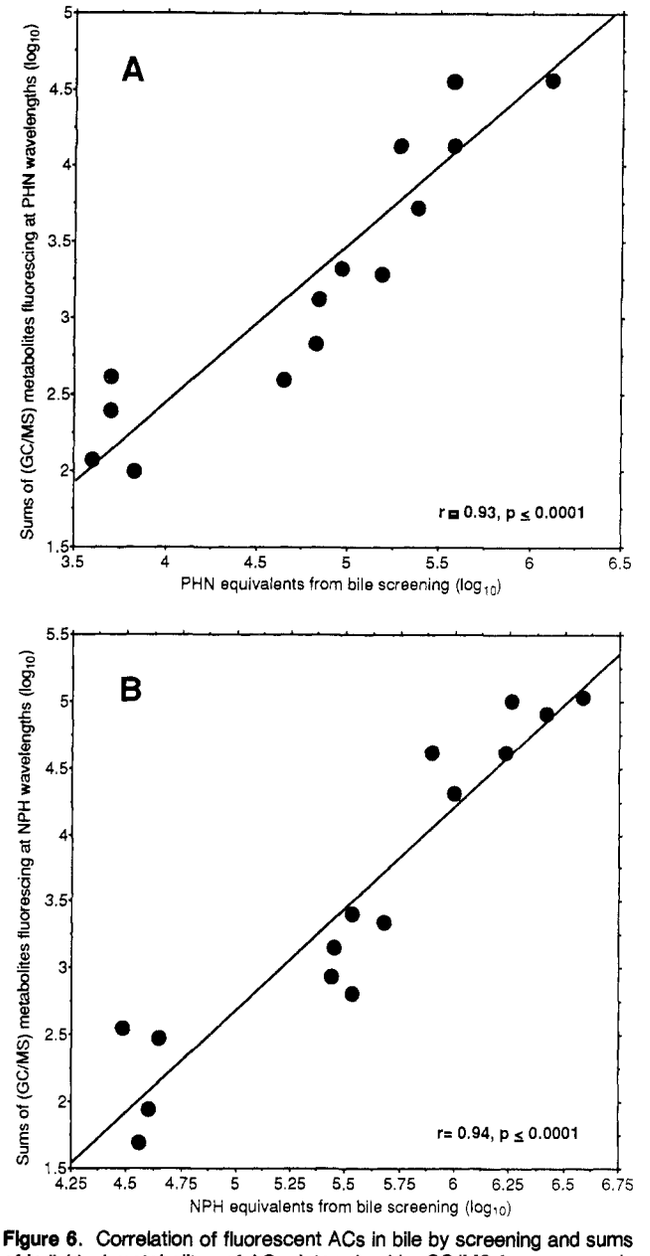

Figure 6. Correlation of fluorescent ACs in bile by screening and sums of individual metabolites of ACs determined by GC/MS for compounds fluorescing at (A) phenanthrene wavelengths and (B) naphthalene wavelengths.

correlations (Figure 6). These results agree with the results for bile samples analyzed by similar methods in our earlier study of fish exposed to urban pollution (*10*). Furthermore, we suggest the ratios of phenanthrene or naphthalene equivalents to benzo[*a*]pyrene (B*a*P) equivalents from bile screening can be used to differentiate the source of the AC contamination. The concentrations of four- and five-ring ACs that fluoresce at B*a*P wavelengths are much lower in crude oil than in urban sediments that have pyrogenic inputs. As a result, the naphthalene/B*a*P ratios should be higher in fish exposed to crude oil. For example, the ratios of naphthalene to B*a*P equivalents from the salmon (n = 5) in this study (mean ± standard deviation 1300 ± 600) were higher than similar ratios in English sole (n = 38) from a site in Puget Sound known to be contaminated with creosote (mean ± standard deviation 110 ± 62) (*26, 27*). Therefore, these results strongly support use of the bile screening method in oil spill situations to test large numbers of bile samples for exposure to petroleum. Then, concentrations of individual metabolites in selected

samples can be confirmed by GC/MS. This approach should ensure that exposure data will be available in a cost-effective and timely manner.

In the current study, the suite of petroleum-related metabolites that were identified in the oil-injected halibut and the Dolly Varden were also found in the environmentally exposed salmon and pollock, although the proportions of the metabolites varied (Figure 5). When different fish species are exposed to a particular AC (e.g., benzo[*a*]pyrene), the efficiency of metabolism and the types and proportions of metabolites produced may differ significantly, i.e., interspecies differences (*6*). Even when individual fish of the same species are exposed to an AC, the rates of bioconversion and excretion and, thus, the proportions of each metabolite may vary, i.e., intraspecies differences (*6*). In fact, we observed these variations in our field samples (Figure 5): the pollock, as a group, had relatively similar profiles, while those of the salmon were somewhat similar to each other, but dissimilar to the profiles of the pollock. In addition to species differences, exposure to oil with different degrees of weathering could account for some differences in the observed metabolite profiles.

Metabolites of ACs are rapidly excreted into bile for elimination. As a result, bile is considered a short-term indicator of contamination. For example, Collier and Varanasi (*28*) showed that 6 weeks after fish were injected with an extract from a contaminated urban sediment, concentrations of fluorescent ACs in their bile were reduced to ~15% of the highest measured concentration. Therefore, the presence of petroleum-related metabolites in the bile of the salmon and pollock captured from PWS (Tables II and III) is evidence of relatively recent exposure of these fish to crude oil.

Because analyses of the bile of fish from these two commercially important species provided evidence of their exposure to crude oil, other studies were conducted to analyze samples of edible tissue from selected salmon and pollock for petroleum-related contaminants (*8*, *29*). The study of the salmon showed that, even for the salmon containing the highest levels of FACs in bile (*8*), concentrations of parent ACs in the flesh were low (i.e., the sums of concentrations of ~300 ACs quantitated by GC/MS were generally <50 ng/g). The edible tissue of pollock also contained low or undetectable concentrations of ACs (*29*). These results from field-exposed fish are supported by laboratory studies that have shown that ACs are efficiently metabolized in liver and the metabolites are excreted via bile. As a result, the accumulation of ACs or their metabolites in edible tissue is generally low or below detection limits (*30–32*).

In summary, numerous metabolites of ACs have been determined by GC/MS in the hydrolyzed bile of five salmon and four pollock captured in PWS several months after PBCO was spilled from the *Exxon Valdez*. High proportions of alkylated phenanthrols, dibenzothiophenols, and other metabolic products of ACs that tend to persist after crude oil weathers were present in bile of the experimentally injected fish and field-exposed fish, but not in the carrier-injected or reference fish. Therefore, identification of these metabolites in bile provided evidence that the fish had been exposed to crude oil. In addition, alkylated dibenzothiophenols were suggested as promising marker compounds for identifying the source of crude oil. Furthermore, the bile screening method was validated for use in determining exposure of fish to petroleum, because concentrations determined by the screening method were highly correlated with those determined by GC/MS. In future studies, an assessment of exposure of fish from PWS and the Gulf of Alaska to the spilled oil will be obtained by conducting bile screening analyses on a large number of fish and confirming metabolite concentrations by GC/MS analysis of bile from selected fish. To complete the evaluation, sediments and stomach contents will also be analyzed for ACs.

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Supplementary Material Available

Tables of selected ion monitoring descriptors for metabolic ions from aromatic compounds (Table A-I) and of concentrations of metabolites of aromatic compounds in halibut, salmon, and pollock bile (Table A-II) and SSIM and full-scan mass spectra of a C_2 dibenzothiophenol metabolite (9 pages) will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper or microfiche (105 × 148 mm, 24× reduction, negatives) may be obtained from Microforms Office, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036. Full bibliographic citation (journal, title of article, authors' names, inclusive pagination, volume number, and issue number) and prepayment, check or money order for $19.00 for photocopy ($22.00 foreign) or $10.00 for microfiche ($11.00 foreign), are required.

Registry No. PHN, 85-01-8; 2,6-dibromophenol, 608-33-3; hexamethylbenzene, 87-85-4.

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Gas- and Particle-Phase Concentrations of α-Hexachlorocyclohexane, γ-Hexachlorocyclohexane, and Hexachlorobenzene in Ontario Air

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■ The airborne gas-phase and particle-phase concentrations of α-hexachlorocyclohexane (α-HCH), γ-hexachlorocyclohexane (γ-HCH), and hexachlorobenzene (HCB) were measured near Turkey Lake (in central Ontario) and at Pt. Petre (on the north shore of Lake Ontario) between the spring of 1987 and the spring of 1989 using a gas and particle (GAP) sampler which employs a multiannular diffusion denuder to trap the vapor-phase constituents. During October 1987 at Turkey Lake, HCB, α-HCH, and γ-HCH were all exclusively in the vapor phase. At Pt. Petre, during November and March when comparable temperatures prevailed, the vapor-phase components for HCB, α-HCH, and γ-HCH were 96.6%, 97.6%, and 100% respectively. The overall method detection limits for HCB, α-HCH and γ-HCH were 7, 14, and 15 pg/m^3. The α-HCH/γ-HCH ratio was always greater than 1.8 and generally between 6 and 10, indicating the detection of technical grade HCH and/or the environmental isomerization of the γ form to the α form.

Introduction

Long-range atmospheric transport and deposition is often implicated as being the primary mechanism to explain the presence of toxic contaminants in locales remote from known sources. Specific attention has recently focused on the semivolatile organic compounds (SVOCs) such as the chlorinated pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic compounds (PACs), and other medium to high molecular weight species (*1–15*). These compounds enter the atmosphere through volatilization during application of pesticides such as lindane, through the disposal of the byproducts from the chemical synthesis of other compounds, and as products of incomplete combustion that occur in sources such as incinerators. SVOCs exist in the atmosphere in the vapor phase, in the solid phase (as adsorbed components of suspended particulate matter), and associated with aerosols. The vapor/particle distribution ratio will strongly influence the residence time of a pollutant in the air and its removal from the atmosphere by wet and dry deposition processes. For those compounds which have an appreciable aqueous solubility, dissolution at the air/water interface (for example, at bodies of water or raindrops) can be expected to occur. The effectiveness of washout will depend upon the aqueous solubility of the individual vapors. Substances which have substantial particle-phase components may be removed primarily by dry deposition of particles, or by washout (*16–18*). Those compounds which are not removed from the atmosphere by air/water exchange processes and which do not adsorb or absorb to particles may

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