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Review

Rapid high-performance liquid chromatographic methods that screen for aromatic compounds in environmental samples

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ABSTRACT

In order to monitor the quality of coastal waters that provide habitats for living marine resources, samples of sediment and biota must be analyzed to assess the degree and distribution of anthropogenic contamination. Analytical time and costs can be greatly reduced by first employing methods that screen for contaminants before selecting samples for rigorous analyses. In this paper, we review the applications of rapid high-performance liquid chromatographic (HPLC) methods to screen for aromatic compounds in sediment, bile and tissue samples. These methods have been used to assess damage to natural resources after the Exxon Valdez oil spill. In addition, the bile screening method has also been used to evaluate contaminants in fish sampled for a national monitoring program. The rapid screening of sediment or bile provides an estimate of contaminant concentrations that can then be confirmed in selected samples by more complicated and expensive analyses by gas chromatography–mass spectrometry (GC–MS). Furthermore, HPLC and GC–MS chromatographic patterns from sediment and bile can provide information about the source of contamination, *e.g.*, crude oil, diesel fuel or pyrogenic contaminants. We also discuss the important role screening methods will play in the future in assessing the quality of aquatic habitats, the safety of seafood, and other important issues related to anthropogenic contamination.

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1. INTRODUCTION

Maintaining the quality of our coastal waters is essential to providing a habitat in which important living marine resources can live and reproduce. However, in many urban estuaries, anthropogenic contaminants have degraded the quality of the water, sediment and biota. A major challenge confronting environmental scientists is developing accurate and cost-effective analyses for contaminants in marine biota and their habitats. In particular, reliable methods are needed to determine concentrations of toxic contaminants, *e.g.*, polynuclear and heterocyclic aromatic compounds (ACs) and their metabolites or polychlorinated biphenyls (PCBs), in a variety of environmental matrices. The data provided by these analyses can then be used by managers to evaluate problems as diverse as oil spills, marine mammal strandings, oil well fires, fish kills and chemical contamination in commercial seafood products. Often, analytical time and costs can be greatly reduced by first employing screening methods to rapidly estimate contaminant concentrations. Based on the results of the screening analyses, a subset of samples can then be selected for detailed analyses, *e.g.*, gas chromatography–mass spectrometry (GC–MS), that can confirm the presence and identity of the contaminants.

Both non-chromatographic [1–4] and chromatographic procedures [5,6] have been used to screen sediments for aromatic contaminants. Non-chromatographic procedures, such as normal [1] or synchronous scan [2–4] fluorescence spectrometry are of limited value because they do not separate out possible interfering compounds before measuring the analytes [7,8]. Even some chromatographic procedures, such as flash evaporation–pyrolysis–GC–MS, failed to eliminate all interferents [6]. Recently, however, Krahn *et al.* [9] reported a sensitive method that uses high-performance size-exclusion chromatography (HPSEC) to remove interferents before analyzing for AC analytes in sediments.

Choices of methods to screen for ACs in biota are more limited. Fish and marine mammals rapidly take up ACs (*e.g.*, naphthalene, benzo[*a*]pyrene) present in their environment, extensively metabolize most ACs in their livers, and then transfer a large proportion of the polar metabolites to bile for elimination [10–12]. As a result, the metabolic products, rather than the parent ACs, must be determined in these animals. Two methods that employ reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection have been developed in our laboratories to screen for metabolites of ACs in fish. The first, a bile screening method was developed to evaluate anthropogenic contamination by ACs in fish sampled from urban estuaries [13,14]. In addition, a method that estimates the concentrations of AC metabolites in fish tissues, *e.g.*, liver and muscle, has been developed recently to address the issue of seafood contamination [15], but has not yet undergone extensive field testing.

In this paper, we review applications of the sediment, bile and tissue screening methods to problem in the marine environment. These methods have been applied to establishing the extent of damage to natural resources after the 1989 Exxon Valdez oil spill in Prince William Sound, Alaska, USA. In addition, the bile screening method has been used ex-

tensively in the National Oceanic and Atmospheric Administration's (NOAA) National Status and Trends (NS&T) Monitoring Program to compare concentrations of AC metabolites in fish captured from sites in USA coastal waters. These applications have demonstrated that sediment or bile samples can be rapidly screened to select samples for confirmation of AC concentrations by the more complicated and expensive GC–MS analyses. In addition, HPLC and GC–MS chromatographic patterns from sediment and bile can often provide information about a possible source of contamination, *e.g.*, crude oil, diesel fuel or pyrogenic contaminants. We also describe analytical problems that need to be addressed in the future: rapidly determining the concentrations and toxicities of chemicals such as the coplanar PCB congeners or polychlorinated dibenzodioxins (PCDDs) and dibenzofurans (PCDFs); developing screening analyses that can readily be used in "real-time" in field applications; and improving immunoassay methods that test for specific chemicals or groups of chemicals.

2. METHODS TO SCREEN FOR AROMATIC CONTAMINANTS

2.1. Screening for metabolites of aromatic compounds in tissues

Enzymatic hydrolysis, methylene chloride extraction and hexane–potassium hydroxide partitioning were used with HPLC–fluorescence analysis to screen tissue samples for metabolites of ACs [15]. Briefly, tissues were homogenized and then treated with β-glucuronidase and aryl sulfatase to hydrolyze glucuronide and sulfate conjugates of AC metabolites. The hydrolysate was extracted with methylene chloride and this solvent was replaced with methanol. A portion of this methanol extract was diluted with hexane and the organic solution was partitioned with aqueous potassium hydroxide to extract the metabolites. The neutralized aqueous phase, containing AC metabolites, was analyzed by reversed-phase HPLC chromatography as described below for bile screening analyses.

TABLE 1

CONCENTRATIONS OF METABOLITES OF AROMATIC COMPOUNDS IN BILE DETERMINED BY THE HPLC SCREENING METHOD AT PHENANTHRENE WAVELENGTHS AND SUMS OF CONCENTRATIONS OF PHENANTHROLS DETERMINED BY GC–MS [16]

Bile sample	Concentration (ng/g wet mass)	
	Phenanthrene equivalents from HPLC	Sum of phenanthrols from GC–MS
Salmon	380 000	14 000
Pollock	90 000	2100
Reference pollock	5000	410

2.2. Screening for metabolites of aromatic compounds in bile

The method for bile analysis by HPLC with fluorescence detection is described in detail by Krahn and co-workers [13,14]. Briefly, bile was injected directly onto a reversed-phase C_{18} HPLC column and eluted with a linear gradient from 100% water (containing a small amount of acetic acid) to 100% methanol at a flow of 1.0 ml/min. Chromatograms were recorded at two or more fluorescence wavelength pairs where the parent compounds and metabolites fluoresce: naphthalenes and dibenzothiophenes at 290/335 nm (excitation/emission), phenanthrenes at 260/380 nm and benzo[*a*]pyrenes at 380/430 nm. The metabolite-containing portion of the chromatogram (> 7 min) was integrated and concentrations were calculated by normalization to an AC standard, *e.g.*, a phenanthrene standard was used to calculate concentrations of "phenanthrene equivalents" for chromatograms recorded at 260/380 nm (Table 1).

2.3. Screening for aromatic compounds in sediment

The method used to screen sediments for ACs features sonic extraction and HPSEC with fluorescence detection as described by Krahn *et al.* [9]. Briefly, sediment, sodium sulfate, activated copper and methylene chloride were mixed together in a centrifuge tube. The tubes were placed into a sonic

TABLE 2

CONCENTRATIONS OF AROMATIC COMPOUNDS IN SEDIMENT DETERMINED BY THE HPLC SCREENING METHOD AT BENZO[*a*]PYRENE (BaP) WAVELENGTHS AND SUMS OF CONCENTRATIONS OF HIGH-MOLECULAR-MASS AROMATIC HYDROCARBONS (HAHs) DETERMINED BY GC–MS [9]

	Concentration (ng/g wet mass)	
	BaP equivalents from HPLC	Sum of HAHs from GC–MS
South San Diego Bay	790	2300
Hunters Point	530	2400
Bodega Bay	6	5

bath and sonicated. The tubes were then centrifuged and the extracts were decanted into concentrator tubes. These steps were repeated twice and the combined extracts were concentrated by evaporation. A portion of the concentrated extract was injected onto a 100 Å HPSEC column, was eluted isocratically with methylene chloride at a flow of 2.5 ml/min for 12 min, and fluorescence was recorded at two or more of the wavelengths used for the bile analyses (above). The fraction containing ACs (> 8.2 min) was integrated and concentrations of AC equivalents were calculated in a manner similar to the bile screening method (Table 2).

2.4. Selecting the chromatographic mode

Although the bile, sediment and tissue screening methods are similar in many respects, HPLC columns and conditions were selected to best separate and detect the particular analytes to be determined by each method. For example, sediments often contain both pyrogenic and petrogenic ACs, possibly including petroleum-related components of high molecular mass (*e.g.*, asphaltenes), so a HPSEC column that separates analytes according to molecular size and shape was chosen for sediment screening. In contrast, ACs in bile are present almost entirely as polar metabolites due to the facile ability of many vertebrates to metabolize ACs in their livers and eliminate the metabolites via bile [10–12]. The ACs or their metabolites can also be transported to other tissues (*e.g.*, muscle) within the animal. The polar metabolites are best determined using a gradient elution from water to methanol on a reversed-phase C_{18} HPLC column, conditions under which the most polar compounds elute first. However, this reversed-phase column is not well-suited to the screening of sediment extracts, because many of the non-polar, high-molecular-mass compounds in the sediments can become irreversibly bound to the packing. Although the HPSEC column does not resolve AC metabolites well and, as a result, would not be a good choice for bile screening, this column can be used for separating endogenous substances in bile from the AC metabolites prior to GC–MS analysis [16].

3. VALIDATING THE SCREENING METHODS

3.1. Tissue screening method

To test the tissue screening method, rock sole (*Lepidopsetta bilineata*) were injected with Prudhoe Bay crude oil (PBCO) and samples of liver and muscle were screened for AC metabolites [15]. The concentrations of AC metabolites in both liver and muscle of these PBCO-exposed fish were linearly proportional to dose. In addition, when tissues from English sole (*Parophrys vetulus*) sampled from the Duwamish Waterway (an urban site in Seattle, WA, USA) and President Point (a non-urban reference site in Puget Sound, WA, USA) were screened, concentrations of polar metabolites of ACs were found to be about 15 times higher in livers of fish from the urban site compared to the reference site. Furthermore, concentrations of AC metabolites in muscle of fish from both sites were very low —*i.e.*, near the limit of detection. As demonstrated in laboratory studies with radiolabeled ACs, the parent compounds are extensively metabolized in liver and most of the metabolites are excreted into bile, thus limiting the accumulation of parent ACs or metabolites in extrahepatic tissues [12,17,18]. As a result, AC metabolites are not transported to edible tissues unless the animal is exposed to high concentrations of ACs, as demonstrated in dose–response studies with PBCO [15]. In the future, the tissue screening method needs to be further field-tested and validated for several fish and crustacean species, so that its application to important environmental issues, such as seafood contamination, can be assessed.

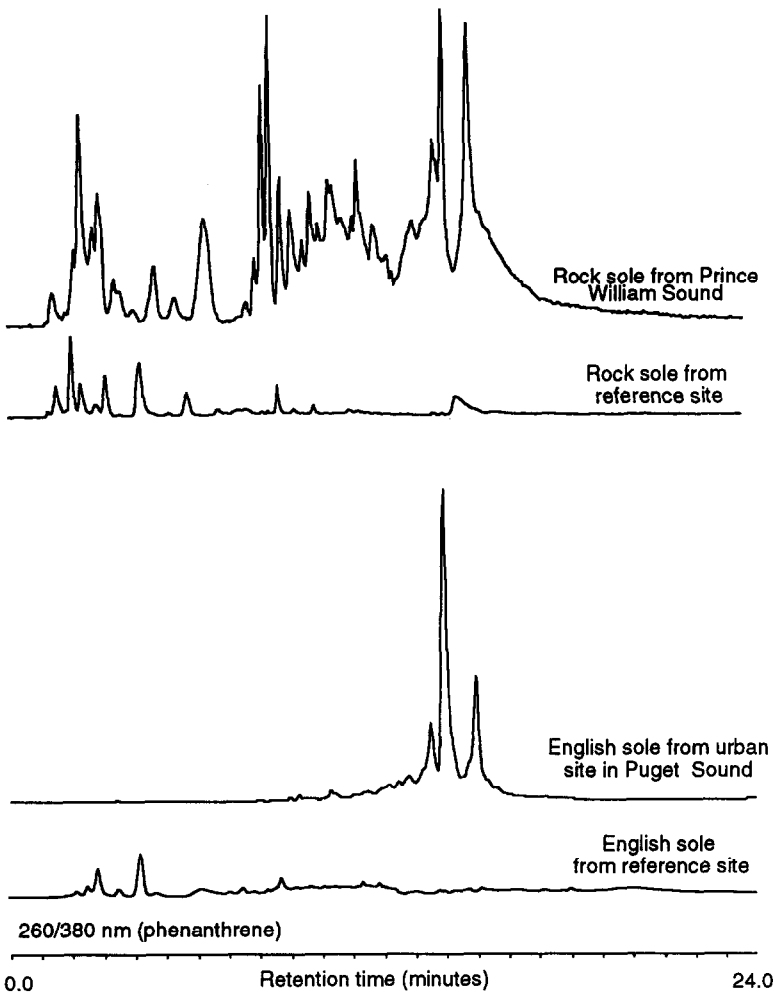

Fig. 1. Chromatograms from the HPLC–fluorescence screening of bile at phenanthrene wavelengths: from a rock sole from Prince William Sound, AK, USA, after the Exxon Valdez oil spill; from a rock sole from a reference (non-oiled) site; and from English sole captured from an urban site and from a non-urban reference site.

3.2. Bile screening method

Metabolites of ACs in bile elute from the reversed-phase HPLC column according to polarity and form a chromatographic pattern containing several sharp fused peaks (Fig. 1) [19]. Concentrations of AC metabolites were estimated by integration of the area (> 7 min) in the chromatograms where the metabolites of ACs elute (examples in Table 1) [13,14]. Detailed GC–MS analyses for individual AC metabolites were conducted on bile samples from fish captured from urban sites [19] and from Prince William Sound after the Exxon Valdez oil spill [16]. The concentrations of metabolites were then summed by classes (*e.g.*, the phenanthrols) for comparison to HPLC screening results from the same samples (examples in Table 1). Statistical comparison of the HPLC screening and GC–MS results from fish from the oil spill study showed an excellent correlation ($p \leqslant 0.0001$) between these methods [16]. Thus, bile screening was validated as a rapid method for determining the exposure of fish to ACs.

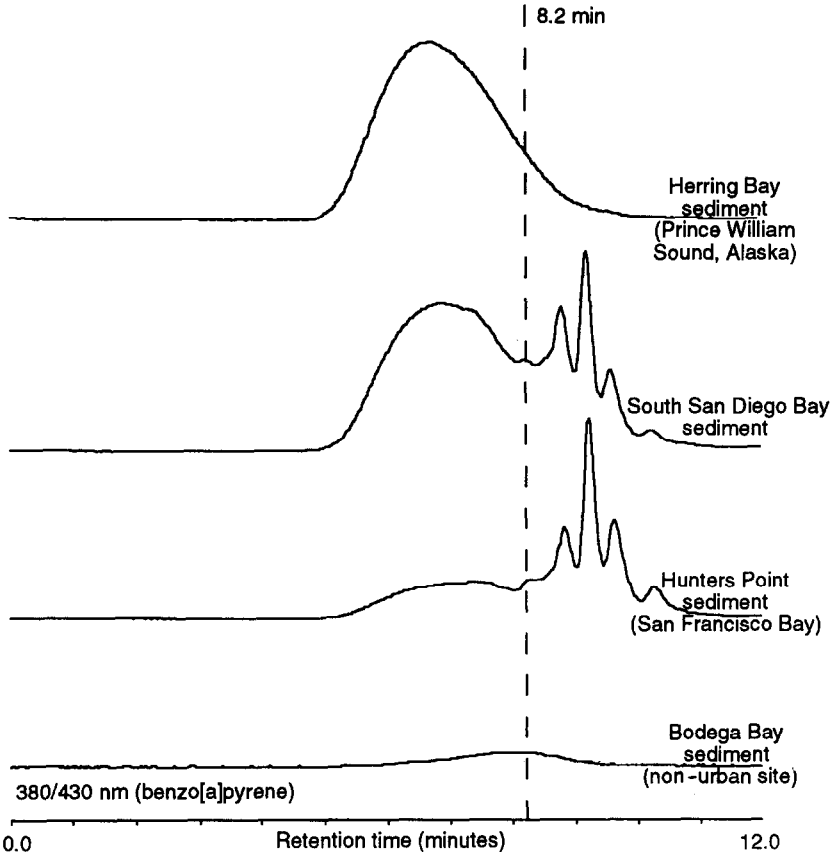

Fig. 2. Chromatograms from the HPLC–fluorescence screening of sediment at benzo[*a*]pyrene wavelengths: from Herring Bay, a site in the path of the Exxon Valdez spill; from the contaminated urban areas of South San Diego Bay and Hunters Point, CA, USA; and from the non-urban reference site of Bodega Bay, CA, USA. The retention time (8.2 min) at which integration of the AC fraction was begun is marked on the chromatograms and the peak.

3.3. Sediment screening method

Aromatic compounds elute from the HPSEC column according to molecular "volume" (largest first), forming a chromatographic pattern that is a continuum of broad peaks (Fig. 2) [9]. This pattern is in contrast to the sharp fused peaks found in chromatograms of AC metabolites in bile (Fig. 1). Concentrations of ACs were estimated by integrating that portion of the chromatogram (> 8.2 min) containing the ACs (examples in Table 2). To compare the results of screening with those from rigorous GC–MS analyses for ACs, a number of sediments from relatively uncontaminated sites and from sites in urban [9] or in oil spill areas [20] were screened and individual ACs were also determined by GC–MS in the same sediments (examples in Table 2). The AC concentrations from HPLC screening were highly correlated ($p \leqslant 0.0001$) with the sums of individual ACs determined by the GC–MS analysis of the same sediments [9,20]. Thus, the HPLC screening method was validated for use in estimating concentrations of ACs in contaminated sediments.

4. IDENTIFYING CONTAMINANT SOURCES

4.1. HPLC patterns from bile

Although HPLC chromatographic patterns from bile can be variable and somewhat difficult to interpret, these patterns can sometimes be used to

suggest a possible source of contamination, *e.g.*, pyrogenic or petrogenic contamination. For example, the chromatographic pattern from bile of a rock sole captured from Prince William Sound after the Exxon Valdez spill appeared very dissimilar to that of an English sole exposed to contaminants from an

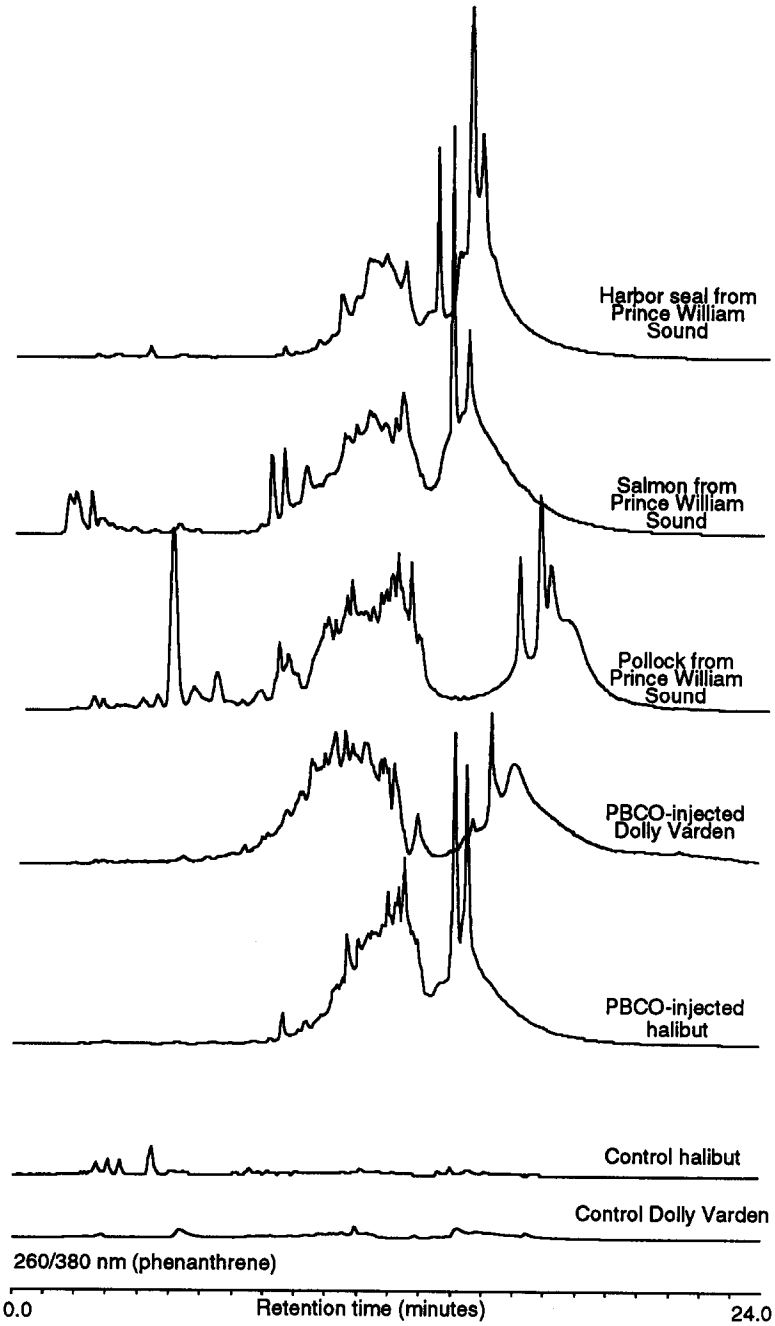

Fig. 3. Chromatograms from the HPLC–fluorescence screening of bile at phenanthrene wavelengths: from a harbor seal, a salmon and a pollock captured from Prince William Sound after the Exxon Valdez oil spill; from a Dolly Varden and a halibut injected with Prudhoe Bay crude oil (PBCO); and from a halibut and a Dolly Varden injected with carrier solvent (controls).

urban site (Fig. 1). However, even when the source of contamination in the fish was the same, *e.g.*, exposure to or injection with PBCO, some differences were apparent in the chromatographic patterns (Fig. 3). Chromatograms of bile from the PBCO-exposed fish exhibited two groups of peaks with a valley between them at *ca.* 13 min, but the retention times and intensities of individual peaks varied (Fig. 3). Variations of these sorts can occur in bile chromatographic patterns, possibly due to variations in the degree of exposure of individual animals to the oil or to species-specific differences in metabolism of the petroleum ACs [16]. For example, the same relative amount of weathered PBCO injected into two species of fish (halibut and Dolly Varden) resulted in HPLC chromatographic patterns of bile (Fig. 3) and proportions of each metabolite [16] that were similar, but not identical. In addition, physical factors from the chromatographic process itself, such as the chromatography column used, the condition of the column or the acidity of the mobile phase, can affect the appearance of a chromatogram in reversed-phase HPLC. For example, without the addition of a small quantity of acetic acid to the water in the mobile phase, the conjugated metabolites will elute earlier [13]. Therefore, due to both the variability of the metabolic process in various fish species and to the variability of the reversed-phase chromatography of the metabolites, the HPLC chromatographic pattern of bile can only suggest the source of contamination.

4.2. Relative proportions of bile metabolites by GC–MS

When bile samples were enzymatically hydrolyzed and their extracts were subjected to GC–MS analysis [16,19], the source of contamination suggested by HPLC screening could often be confirmed from the identities and the proportions of the individual metabolites determined by GC–MS. For example, bile of fish that were exposed to ACs from urban sites contained higher proportions of metabolites of the 4–6-ring ACs from pyrogenic sources [19] than did fish exposed to weathered PBCO [16]. Conversely, the bile of fish exposed to weathered PBCO [16] contained much larger proportions of metabolites of alkylated naphthalenes, phenanthrenes and dibenzothiophenes than were present in the urban fish [19]. Furthermore, the identification in bile of relatively large proportions of metabolites of the alkylated dibenzothiophenes that are recognized as marker compounds for PBCO [16,21] provided evidence for the exposure of the fish to PBCO [16]. Thus, the source of contamination suggested from the HPLC chromatogram can often be substantiated by examining the relative proportions of AC metabolites determined by the GC–MS analysis of bile.

4.3. HPLC patterns in sediment

HPLC chromatographic patterns of sediment extracts can also be used to suggest a possible source of AC contamination, *e.g.*, crude oil or pyrogenic ACs from urban sites. These chromatographic patterns are less variable than those from bile, because sediment screening measures the ACs themselves, with none of the confounding factors found in the bile chromatograms due to species-specific differences in degree of metabolism or excretion of metabolites. In addition, retention times in size-exclusion chromatography are stable over extended periods of time [20]. Thus, HPLC chromatograms from sediment are easier to interpret than those from bile. For example, a chromatogram of sediment from Herring Bay, a site in Prince William Sound that was in the path of the Exxon Valdez spill, showed a pattern very different from those of sediments from urban sites (South San Diego Bay or Hunters Point in San Francisco Bay) or the nonurban reference site of Bodega Bay (Fig. 2). The chromatogram of the Herring Bay sediment showed a continuous broad peak at BaP wavelengths and only a small portion of this peak was in the AC fraction (fraction after 8.2 min in which BaP and other ACs elute). In contrast, the chromatograms of the urban sediments exhibited the continuous peak before 8.2 min, but also had several well-defined peaks in the AC fraction (Fig. 2). In addition, the nonurban Bodega Bay sediment showed only low concentrations of contaminants by HPLC screening (Fig. 2).

When a particular contaminant source is suspected, *e.g.*, weathered PBCO in the sediment from Herring Bay, chromatograms can be recorded at several wavelengths and compared to those from the suspected source (Fig. 4) [20]. For example, the chro-

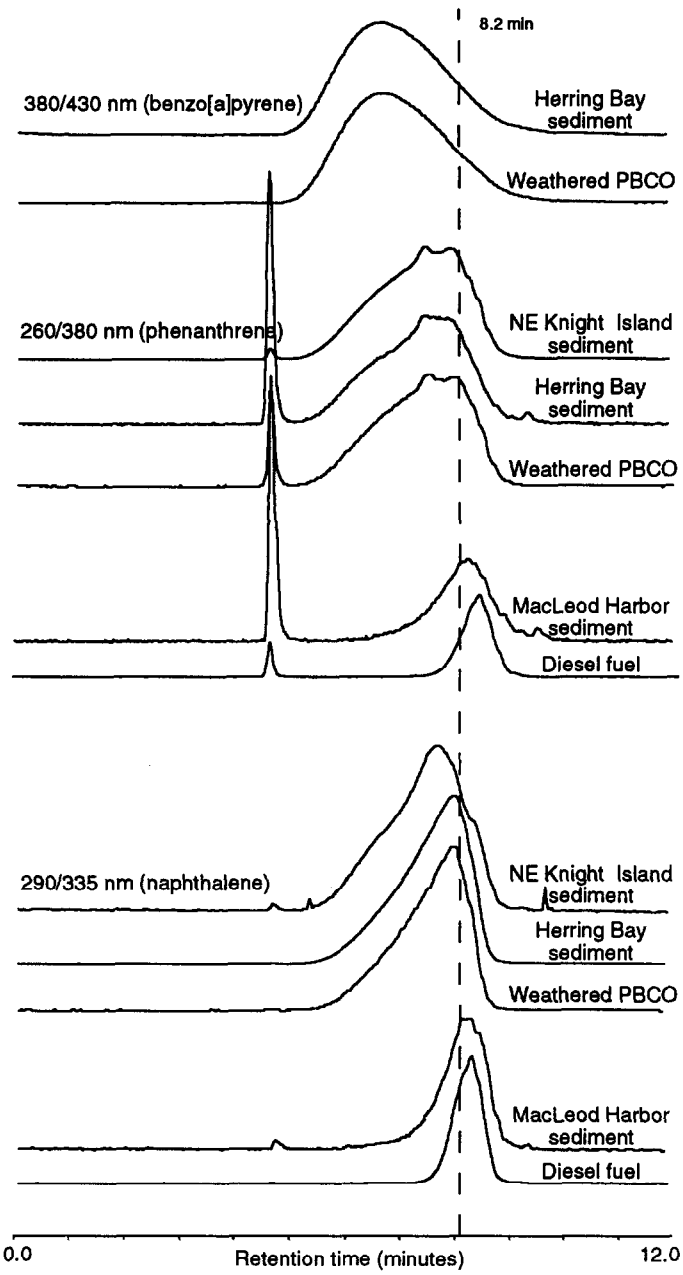

Fig. 4. Chromatograms from the HPLC–fluorescence screening of intertidal sediment from Prince William Sound: from two oiled sites (Herring Bay and Knight Island); from an unoiled site (MacLeod Harbor); and from some possible sources of contamination in the marine environment (diesel fuel and weathered Prudhoe Bay crude oil). Fluorescence was recorded at benzo[*a*]pyrene, phenanthrene and naphthalene wavelengths. To facilitate visual comparisons between chromatographic patterns of the Prince William Sound sediments and possible contaminant sources, the chromatograms in this figure have been electronically adjusted to the same height. Integration mark is as described in Fig. 2. The peak at 4.5 min (phenanthrene wavelengths) is the internal standard.

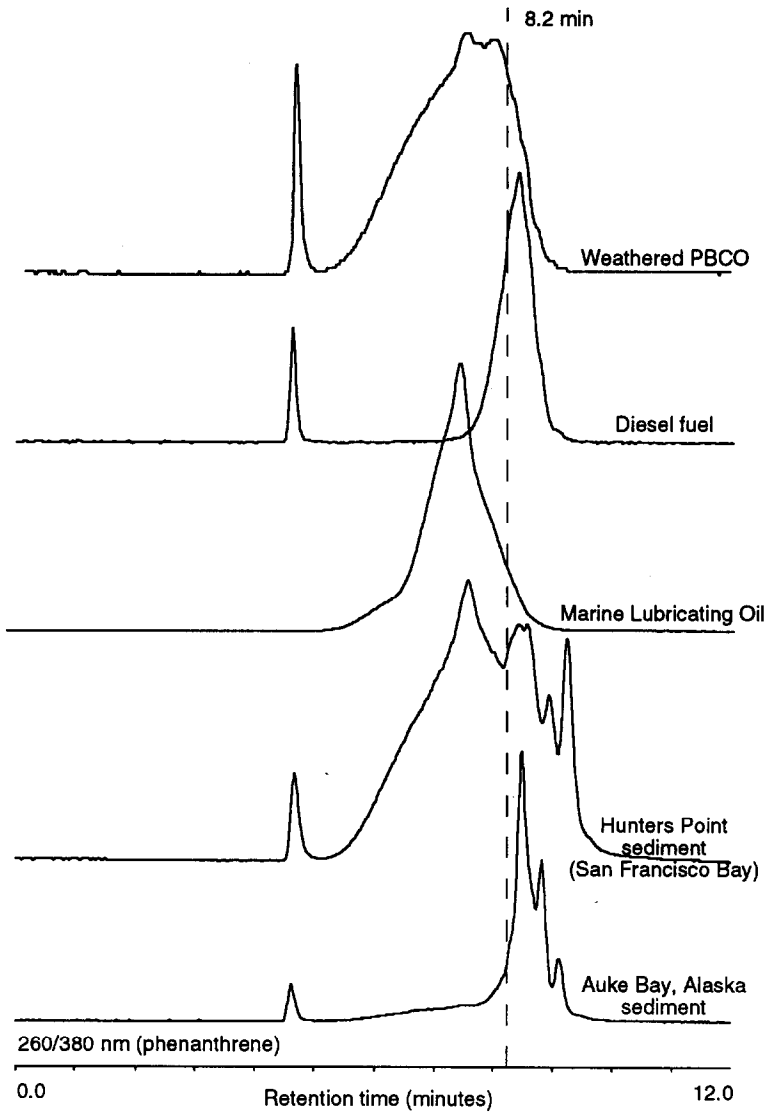

Fig. 5. Chromatograms from the HPLC–fluorescence screening of possible sources of contamination in the marine environment: weathered Prudhoe Bay crude oil (PBCO); diesel fuel; marine lubricating oil; and "urban" contamination in sediments from Hunters Point in San Francisco Bay, CA, USA and from Auke Bay, AK, USA. Fluorescence was recorded at phenanthrene wavelengths. Integration mark and internal standard peak are as described in Fig. 3.

matograms of Herring Bay and Knight Island sediments were nearly superimposable with those from weathered PBCO at all recorded wavelengths (Fig. 4). Furthermore, the chromatographic patterns (molecular size distributions) from the Herring Bay and Knight Island sediments were very different from those of other sources of contamination (*e.g.*, diesel fuel or marine lubricating oil) that might be found in Alaskan sediments (Fig. 5) [20]. Therefore, the HPLC chromatographic pattern provided evidence that PBCO was the likely contaminant source in certain Alaskan sediments. In contrast, results from screening sediment from MacLeod Harbor, a site not in the direct path of the spill, revealed low

concentrations of ACs and an HPLC chromatographic pattern that resembled that of diesel fuel (Fig. 4). Furthermore, chromatograms of some urban sediments showed evidence of both pyrogenic and petrogenic contaminants. For example, in the chromatogram of sediment from South San Diego Bay (BaP wavelengths; Fig. 2), the portion eluting before the AC fraction (<8.2 min) was similar to that from Herring Bay (Fig. 2) and weathered PBCO (Fig. 4), whereas several large peaks were detected in the fraction (> 8.2 min) where ACs, such as the 4–6-ring pyrogenic ACs, elute [9]. This chromatographic pattern is consistent with more than one source of contamination. Thus, contamination from multiple sources in urban estuaries may be more common than contamination from a single source. Contamination from a single source is likely only when that source (*e.g.*, crude oil or diesel fuel) is spilled into a relatively pristine environment, such as Alaska.

4.4. Relative proportions of aromatic compounds in sediment by GC–MS

To substantiate the identification of a contaminant source suggested by HPLC screening of sediment, individual ACs can be determined by GC–MS and the identities and proportions of the ACs in the sediment can be compared to similar characteristics of the probable sources. For example, when the AC fraction (> 8.2 min; Fig. 4) of the Herring Bay sediment was collected and analyzed by GC–MS, high proportions of 2–3-ring ACs typical of petroleum contamination, *i.e.*, alkylated naphthalenes, phenanthrenes and dibenzothiophenes were found [20]. The alkylated dibenzothiophenes have been identified as marker compounds for North Slope crude oils, such as PBCO [20,21]; hence, their presence in relatively large proportions in the Herring Bay sediment is an important factor in confirming PBCO as the contaminant source [20]. Furthermore, the pyrogenic 4–6-ring ACs were present in low or non-detectable amounts in this sediment, due to the low proportions of these ACs in PBCO [21]. In addition, the results from screening the MacLeod Harbor sediment were confirmed by GC–MS analysis. Although measurable concentrations of alkylated naphthalenes and phenanthrenes were found in MacLeod Harbor sediments, the concentrations of the alkylated dibenzothiophenes that are marker compounds for PBCO were below detectable limits [20]. These results are consistent with a diesel fuel —one refined from a crude oil low in dibenzothiophenes— as the contaminant source, not surprising in an area with commercial and recreational vessel traffic.

The AC fractions from sediments that exhibit "urban" HPLC chromatographic patterns, *e.g.*, South San Diego Bay and Hunters Point in San Francisco Bay, were also characterized by GC–MS [9]. High concentrations of several pyrogenic ACs (4–6 rings, *e.g.*, fluoranthene, pyrene and benzo[*a*]pyrene) that fluoresce at BaP wavelengths were found, as expected from the large peaks comprising the AC fraction of the chromatograms at BaP wavelengths (Fig. 2). In the reference sediment from Bodega Bay (north of San Francisco), GC–MS analyses found only low or non-detectable concentrations of ACs [9]. Thus, the presence of high proportions of pyrogenic ACs in the South San Diego Bay and Hunters Point sediments helped to distinguish these urban sediments from those of sediments contaminated primarily by petrogenic sources, such as crude oil or diesel fuel.

5. HPLC SCREENING OF BILE: URBAN SITES

5.1. Determining the exposure of adult fish to aromatic compounds

Bile screening was originally developed as a tool to rapidly estimate concentrations of metabolites resulting from the uptake and transformation of ACs by English sole exposed to environmental contaminants in Puget Sound [13,14]. These studies also revealed significant correlations between concentrations of AC metabolites in bile and prevalences of hepatic diseases (*e.g.*, neoplasms and megalocytic hepatosis) in fish. Because of this link between AC metabolites in bile and possible biological effects to the fish, bile screening has been used as a monitoring tool in the NS&T Program. This multiyear program is designed to measure concentrations of chemical contaminants in sediments and in tissues of bottom-dwelling fish species at selected sites in USA coastal areas and to determine prevalences of diseases as related to chemical contaminants in these fish. For example, tissues of white croaker

(*Genyonemus lineatus*) from several sites in the Los Angeles area contained much higher concentrations of organic contaminants, including metabolites in bile, than did fish from a non-urban reference site [22]. In addition, a number of liver lesions (*e.g.*, neoplasms, basophilic foci of hepatocellular alteration and megalocytic hepatosis) were detected in croaker from the Los Angeles sites, but only a low prevalence of a single lesion type (nuclear pleomorphism) was found at the reference site [22]. Other NS&T sites, including certain sites in San Diego Bay, San Francisco Bay and Puget Sound, were also found to have a variety of fish species with high concentrations of AC metabolites in bile [23,24] and with various pollution-associated pathological conditions (*e.g.*, liver lesions or fin erosion) [23].

Many of the ACs detected in the NS&T fish are known to be carcinogenic in fish and mammals, but these compounds exert their effect only after metabolic activation [12]. Hence, the presence of metabolites of ACs in fish bile can be used as an indicator of exposure of these animals to potential carcinogens that may have been activated through metabolism. As a result, the statistical correlation between AC metabolites in bile of the fish and tumors and other diseases in their livers demonstrates that bile screening is an indicator of potential risk factors for environmentally caused diseases. Moreover, the simplicity of the bile screening method is particularly important, because as Melancon *et al.* [25] conclude, "Techniques that measure important metabolites simply and at low cost are obviously desirable in monitoring programs."

5.2. Determining the exposure of juvenile salmon to aromatic compounds

In a pilot study conducted to assess the potential for the uptake of toxic chemicals by downstream migrant juvenile chinook salmon (*Oncorhynchus tshawytscha*) in the Duwamish Waterway (an urban estuary in Seattle, WA, USA), concentrations of AC metabolites in bile were estimated by screening to determine the uptake of ACs by the salmon [26]. Although the potential exposure of benthic and sedentary species to contaminants can be estimated from concentrations of contaminants in sediments, for migratory species, *e.g.*, salmon or marine mammals, bile screening is the only means of rapidly determining exposure to ACs. The mean concentrations in bile of AC metabolites that fluoresce at BaP wavelengths were significantly higher in salmon from the Duwamish Waterway than in salmon from a reference estuary or the hatchery (Table 3). Concentrations of other toxic compounds, such as PCBs, were also determined in the fish and also found to be higher in the salmon from the urban estuary [26]. This study demonstrated that the juvenile salmon bioaccumulated substantial concentrations of ACs and other toxic chemicals during their brief residency in the urban estuary. However, possible effects of exposure to these chemicals on the health and survival of the salmon are unknown. Consequently, a more detailed, multiyear study is currently underway.

TABLE 3

CONCENTRATIONS OF FLUORESCENT AROMATIC COMPOUNDS, MEASURED AT 380/430 nm (BENZO[*a*]PYRENE WAVELENGTHS), IN BILE OF JUVENILE SALMON CAPTURED FROM A HATCHERY AND FROM URBAN AND NON-URBAN WATERWAYS [26]

Site	Concentrations of fluorescent aromatic compounds, mean ± S.D. (ng/g bile)
Duwamish Waterway	1300 ± 430[a], 6 composites (n = 17, 17, 18, 19, 22, 27)
Kalama Creek Hatchery	150 ± 90, 3 composites (n = 10, 10, 10)
Nisqually River (reference)	50 ± 10, 3 composites (n = 20, 21, 21)

[a] Significantly different ($p < 0.05$) from the hatchery or reference fish.

6. HPLC SCREENING OF BILE: OIL SPILL SITES

6.1. Columbia River oil spill

The spill of a relatively small amount of high-density residual and industrial oil into the Columbia River provided an opportunity to test the bile screening method with fish exposed to oil in the field. Within five days after the spill, mean concentrations of metabolites in the bile of white sturgeon (*Acipenser transmontanus*) captured 57 miles (1 mile = 1609 m) downstream from the spill were significantly higher than those of sturgeon captured upriver from the spill site [27]. The bile screening method provided a rapid and cost-effective means of evaluating, in part, the impact of an oil spill on fish. Thus, this study provided a basis for using the bile screening method in assessing the exposure of fish and marine mammals to oil from the Exxon Valdez spill in Prince William Sound, Alaska in March, 1989.

6.2. Monitoring for PBCO in commercial fish species after the Exxon Valdez spill

Bile and tissue samples from many species of fish were collected and analyzed after the Exxon Valdez oil spill [28,29]. Clearly, the spill raised immediate concerns about the possible exposure of commercially important fish species to petroleum hydrocarbons. The halibut season was due to open on May 15, 1989, less than two months post spill, so NOAA and the International Halibut Commission immediately conducted a joint survey to determine if Pacific halibut (*Hippoglossus stenolepsis*) were being exposed to the petroleum hydrocarbons from the oil spill [30]. Bile from halibut (n = 81), screened for metabolites of petroleum-related ACs, had mean concentrations of AC metabolites that were similar to those from fish sampled before the spill from a pristine site. In addition, GC–MS analyses of edible tissue from these fish revealed concentrations of ACs that were below detection limits. Thus, the finding from the analyses of both bile and edible flesh indicated that these halibut were not exposed to appreciable concentrations of crude oil.

Native Alaskans were also concerned that their seafood might be contaminated by the crude oil after the Exxon Valdez spill. NOAA, in cooperation with Exxon and the Alaska Department of Fish and Game, analyzed edible flesh from fish and shellfish collected from native fishing grounds [31]. The principal use of bile screening for this study was to assess exposure of the fish to ACs and to set priorities for GC–MS analyses of the corresponding edible flesh. Screening analyses found elevated concentrations of metabolites of petroleum ACs in the bile of several fish species from sites in the path of the spill [31]. In contrast, results from tissue screening analyses, conducted on edible flesh from several of the fish having the highest concentrations of bile metabolites, found that concentrations of AC metabolites were near or below detectable limits [28]. Similarly, GC–MS analyses of the edible flesh showed relatively low concentrations of ACs (the majority of the samples had concentrations < 30 ng/g), even in the fish that had the highest concentrations of AC metabolites in bile [31]. These relatively low concentrations of petroleum-related ACs in edible tissue are consistent with the efficient metabolism by the fish of most ACs and the elimination of metabolites, predominantly via the bile [17].

Information about the exposure to PBCO of another commercially important fish species was obtained about one year after the spill. Samples from pollock, a species that feeds in the water column, were collected during an annual survey conducted by the National Marine Fisheries Service in February and March 1990 at sites along the path of the spill [28,29]. Pollock were tested for exposure to petroleum-related contaminants by screening for AC metabolites in their bile. The PBCO concentrations in bile of pollock from 9 of the 14 sites surveyed, including all the sites within Prince William Sound, were significantly different (higher) from those in bile of pollock captured from a reference site in Southeastern Alaska (Table 4).

Because bile is a major excretory route in fish for the elimination of metabolites of lipophilic contaminants, metabolites of ACs in bile are considered a short-term indicator of exposure [12,32]. Therefore, the AC metabolites found in bile of the pollock were an indication that these fish had been exposed to ACs near the time of their capture, suggesting that crude oil was still present in the Alaskan waters where the pollock resided nearly one year after the spill. Moreover, when mean PBCO concentrations in pollock from each site sampled in

TABLE 4

CONCENTRATIONS OF FLUORESCENT AROMATIC COMPOUNDS, MEASURED AT 260/380 nm (PHENANTHRENE WAVELENGTHS), IN BILE OF POLLOCK CAPTURED IN PRINCE WILLIAM SOUND AND SHELIKOF STRAIT IN 1990 AND 1991.

Site[a]	Concentrations of fluorescent aromatic compounds, mean ± S.D. (ng/mg protein[b])				Distance from spill site (nautical miles, 1 nautic mile = 1852 m)
	1990	*n*	1991	*n*	
Mummy Island	7600 ± 4700[c]	20	1200 ± 350	13	54
Naked Island	6400 ± 3500[c]	17	1200 ± 700	5	20
Point Bazil	3100 ± 1800[c]	20	1700 ± 600	14	64
Goose Island	2800 ± 1900[c]	20	NS[d]		9
Cape Ugat	2300 ± 1200[c]	10	NS		295
Cape Uyak	2300 ± 920[c]	10	NS		315
Cape Paramanof	2300 ± 870[c]	10	NS		260
Tugidak Island	2100 ± 760[c]	8	NS		440
Kinak Bay	2000 ± 600[c]	13	NS		300
Cape Kekurnoi	2000 ± 770	9	NS		340
Portage Bay	1800 ± 550	10	1200 ± 410	10	380
Malina Point	1800 ± 810	14	NS		270
Sanak Island	1600 ± 620	20	1400 ± 400	10	710
Chirikof Island	1300 ± 530	10	NS		500
Port Gravina	NS		2300 ± 670[c]	15	NA[e]
Bay of Isles	NS		2200 ± 1100[c]	8	NA
Hogan Bay	NS		2100 ± 980[c]	14	NA
Port Fidalgo	NS		2100 ± 740[c]	10	NA
Naked Island East	NS		1400 ± 770	6	NA
Bogoslof	NS		1400 ± 140	10	NA
Sutwick Island	NS		1300 ± 790	11	NA
Trinity Islands	NS		1300 ± 300	10	NA
Kuliak Bay	NS		1200 ± 550	12	NA
Cape Ikolik	NS		1200 ± 300	11	NA
Katmai Bay	NS		1100 ± 270	10	NA
Sturgeon Head	NS		1100 ± 290	11	NA
Uganik Island	NS		1000 ± 290	10	NA
Reference site	1300 ± 550	14	1500 ± 240	13	out of spill area

[a] Sites located in Prince William Sound are shown in italics.
[b] The concentrations of fluorescent aromatic compounds have been normalized to the amount of bile protein.
[c] Significantly different by analysis of variance from the reference site.
[d] NS = Not sampled.
[e] NA = Not applicable.

1990 were compared to the distance of each site from the origin of the spill (Table 4), a very good inverse correlation resulted ($p \leq 0.001$). This correlation may be interpreted like a dose-response relationship, *i.e.*, as the distance from the origin of the spill increased, the concentrations of PBCO in the pollock decreased proportionally.

HPLC patterns indicative of metabolites of petroleum-related ACs were found in pollock with elevated concentrations of AC metabolites in bile (*e.g.*, compare the pollock from Prince William Sound with the PBCO-injected fish, Fig. 3). Furthermore, metabolites of petroleum-related ACs, *e.g.*, alkylated naphthols, phenanthrols and dibenzothiophenol marker compounds, were found in bile of the pollock by GC–MS [16], thus confirming the exposure of these fish to crude oil. However, individual ACs measured by GC–MS in fish muscle were present

only in low or non-detectable concentrations [28]. These data for fish muscle agree with results from previous studies [15,31] (discussed above); even in those fish that had the highest concentrations of metabolites in bile, AC concentrations in muscle were very low.

Because the pollock captured at several sites in 1990 had elevated concentrations of metabolites of petroleum ACs in bile, pollock were sampled again during the 1991 annual survey to determine if exposure to PBCO was continuing. Five of the sites that were sampled in 1990 were also sampled in 1991. Concentrations of AC metabolites in bile of pollock from these sites were much lower in 1991 compared to 1990 (Table 4). Of the remaining sites sampled in 1991, pollock from four sites in Prince William Sound had concentrations of AC metabolites in bile that were significantly different (higher) from those in bile of pollock from the reference site (Table 4), but still were relatively low compared to the 1990 Prince William Sound sites [28,29]. Concentrations of AC metabolites in bile of pollock from the other 1991 sites were statistically indistinguishable from those in fish from the reference site. Thus, two years after the spill, bile from pollock from most sites had concentrations of AC metabolites at background levels, although fish from four sites within Prince William Sound still exhibited exposure to oil.

6.3. Determining the exposure of marine mammals to PBCO

Samples from several species of marine mammals, collected after the Exxon Valdez oil spill in June–August, 1989, were obtained from dead animals in the spill area, as well as from animals killed by native Alaskan subsistence hunters [33]. For example, harbor seals (*Phoca vitulina*) were substantially affected by exposure to petroleum-related ACs from the spilled crude oil. The harbor seals captured in Prince William Sound were found to have elevated concentrations of AC metabolites in bile (Table 5) compared to similar measurements in bile of harbor seals obtained from the spill area outside Prince William Sound and from Ketchikan, a site in Southeastern Alaska unaffected by the spill. Furthermore, the HPLC chromatograms of the seal bile were similar to those of the fish exposed to PBCO (Fig. 3). When a sample of hydrolyzed bile from a harbor seal was analyzed for individual metabolites by GC–MS, petroleum-related ACs, including the dibenzothiophenol marker compounds, were found in high proportions [33]. Therefore, harbor seals from Prince William Sound were exposed to PBCO and this exposure may have contributed to the deaths of some of these animals.

TABLE 5

CONCENTRATIONS OF FLUORESCENT AROMATIC COMPOUNDS, MEASURED AT 260/380 nm (PHENANTHRENE WAVELENGTHS) IN BILE OF HARBOR SEALS CAPTURED AFTER THE EXXON VALDEZ OIL SPILL

Site[a]	Concentrations of fluorescent aromatic compounds, mean ± S.D. (ng/mg protein[b])	
	1990	*n*
Bay of Isles	6700 ± 7900	4
Herring Bay	1400 ± 1700	6
Seal Island	1200 ± 470	2
West Amatuli Island	430	1
Agnes Island	290	1
Applegate Rocks	220	1
Perl Island	160	1
Ushagat Island	80 ± 13	2
Perenosa Bay	40	1
Big Fort Island	10	1
Ketchikan	70 ± 45	2

[a] Sites in italics are located in Prince William Sound.
[b] The concentrations of fluorescent aromatic compounds have been normalized to the amount of bile protein.

7. HPLC SCREENING OF SEDIMENTS: URBAN SITES

In a study of creosote contamination in the Elizabeth River (VA, USA), the HPLC method was used to screen sediments for ACs [34]. Concentrations of ACs in sediments were found to decrease with the distance of the sampling sites from the site of an old creosote manufacturing plant. In addition, an excellent correlation was found between summed concentrations of ACs in the sediments determined by GC–MS and those estimated by the HPLC screening method ($r = 0.92, p \leq 0.001, n = 6$ sites) [34], thus again demonstrating that sediment screening is a useful tool for rapidly assessing relative amounts of AC contaminants in sediments from polluted sites.

Our laboratory obtained some supposedly uncontaminated sediment from Auke Bay (near Juneau, AK, USA) for the purpose of conducting toxicity bioassays. To assure that the sediment was actually uncontaminated, replicate samples were analyzed by the HPLC screening method [20]. The HPLC pattern at phenanthrene wavelengths was suggestive of pyrogenic contamination, because the pattern was most similar to that of sediment from Hunters Point, a contaminated site in San Francisco Bay (Fig. 5). Moreover, the phenanthrene equivalents calculated for the Alaskan sediment were 17 000 ± 3300 ng/g (n = 2), a relatively high level of contamination compared to other sediments analyzed previously [20].

To substantiate this information, individual ACs were determined in the Alaskan sediment by GC–MS analyses. High concentrations of ACs, particularly the pyrogenic ACs, were found (sum of total ACs was 3700 ± 930 ng/g, wet mass, n = 2 and the sum of 4–6-ring ACs was 3500 ± 770 ng/g) [20]. Although this sediment had been collected from a site thought to be uncontaminated, the HPLC screening assay correctly predicted its high level of contaminants. As a result, another sediment sample was obtained for the bioassay study. Accordingly, the sediment screening method has proven to be a valuable and often-used tool in our research program.

8. HPLC SCREENING OF SEDIMENTS: SPILLED OIL

Following the Exxon Valdez spill, hundreds of sediment samples were collected to determine the degree and distribution of the oiling. Because analyzing all these samples by GC–MS would be excessively expensive and time-consuming, priorities for analyses needed to be set. Therefore, the HPLC screening method was used to determine concentrations of PBCO in more than 400 sediment samples from a large number of sites in the Exxon Valdez spill area and many were found to be contaminated by PBCO [20]. Formerly, information about oil contamination had to be obtained by GC–MS [35,36]. However, the expense of the GC–MS method limits the number of analyses that can be made. In contrast, sediment screening is a rapid and cost-effective tool with which to obtain data that can be used to plan the sampling of biota and to devise or evaluate site cleanup strategies. Furthermore, the screening method can increase the confidence in the results by providing sufficient data for adequate statistical analyses.

9. PROSPECTS FOR THE FUTURE OF SCREENING METHODS

One of the biggest challenges confronting environmental scientists today is to devise state-of-the-art techniques that are both accurate and practical. These methods must also be cost-effective, so that sufficient data can be collected to decrease the uncertainty in the conclusions while maintaining reasonable analytical costs. In addition, a rapid turnaround of analytical results is essential, so that environmental managers can evaluate the data and take action to solve the problem. In this regard, the utility and practicality of the HPLC bile and sediment screening analyses were recently demonstrated on board the NOAA research vessel Mt. Mitchel during the Arabian Gulf Project. Estimates of petroleum contamination in sediment and fish were available rapidly, allowing modification of the sampling strategy based on these results. In the future, miniaturization of instruments may allow such analyses to be conducted from a portable field laboratory without need for a support vessel or laboratory.

Future effort also needs to be expended on methods to rapidly determine the concentrations of coplanar PCBs, PCDDs, PCDFs and other toxic chlorinated compounds. These highly toxic compounds usually comprise only a small proportion of a total extract that also contains other, less toxic compounds, *e.g.*, the other (non-coplanar) PCBs, the DDTs, their breakdown products and chlorinated pesticides. Recent progress has been made toward developing rapid HPLC methods to separate the coplanar PCBs, PCDDs and PCDFs from coextracted compounds [37–39] and to screen for toxic PCBs in tissue samples [40]. Toxicity can then be estimated from the concentrations of these compounds via the "toxic equivalency factors" proposed by Safe [41]. However, these estimated toxicities need to be tested against a biological assay, *e.g.*, the rat hepatoma cell line bioassay, that accounts for possible interactions among the toxic chlorinated compounds and correlates with toxic

effects *in vivo*. A good correlation of the bioassay and the screening method would demonstrate the potential of the latter for providing data that rapidly and accurately estimate potential toxicity. As a result, the toxicities of a large number of samples could be determined by screening and then the results could be confirmed in selected samples by more laborious and expensive analytical or bioassay procedures.

Another expanding field —immunoassays based on monoclonal antibodies for detecting toxic substances such as the PCDDs— is in the preliminary stages of development [42]. Although the results show that monoclonal antibodies can be generated to small organic haptens such as PCDDs, the test needs to be made more specific. At present, certain of the PCDDs, PCDFs and one of the toxic PCBs are all recognized by the test. However, such an assay should be useful as a preliminary screening tool to determine those samples needing further characterization by standard analytical procedures. Ideally, future monoclonal antibodies will be selective and sensitive detectors of particular chemical contaminants and these procedures will be readily adapted as field assays.

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