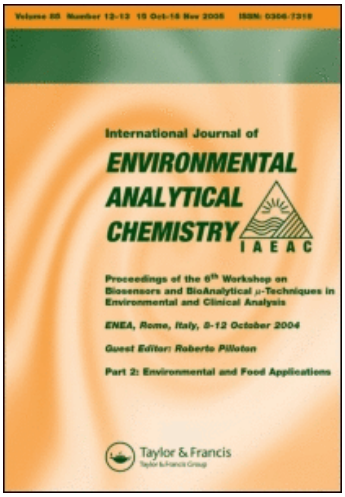

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Monocyclic Aromatic Hydrocarbons in Bile of Flounder Exposed to a Petroleum Oil

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To cite this Article Hellou, J. and Upshall, C.(1995) 'Monocyclic Aromatic Hydrocarbons in Bile of Flounder Exposed to a Petroleum Oil', International Journal of Environmental Analytical Chemistry, 60: 2, 101 — 111
To link to this Article: DOI: 10.1080/03067319508042868
URL: http://dx.doi.org/10.1080/03067319508042868

Intern. J. Environ. Anal. Chem., Vol. 60, pp. 101–111
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MONOCYCLIC AROMATIC HYDROCARBONS IN BILE OF FLOUNDER EXPOSED TO A PETROLEUM OIL

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(Received, 1 July 1994; in final form, 22 September 1994)

The bioaccumulation of contaminants in tissues of fish exposed to petroleum hydrocarbons is of concern because of the toxicity associated with polycyclic aromatic compounds (PAC). Exposure of Winter flounder (*Pseudopleuronectes americanus*) to several concentrations of Hibernia crude oil in sediments, for four months during the winter, resulted in a dose-response in the accumulation of hydrocarbons in muscle tissue and the elimination of metabolites (glucuronides and sulphates) through the gall bladder bile. Results of a multispectroscopic analysis using ultraviolet/fluorescence (uv/f) and gas chromatography-mass spectrometry (GC-MS) are presented. In muscle tissue, the monocyclic aromatic hydrocarbons were previously shown to predominate over alkylated PAC, while parental PAC were least detectable. Naphthalene and phenanthrene derivatives were more easily characterised as bile metabolites (GC-MS) than benzenoid derivatives which, according to uv/f analysis also represent a large fraction of the metabolites. The higher sensitivity of bile metabolites in determining exposure compared to free hydrocarbons in muscle tissue was confirmed in terms of the concentration of hydrocarbons in sediments.

KEY WORDS: Fish muscle, bile, monocyclic hydrocarbons, metabolites, petroleum.

INTRODUCTION

Fish can be exposed to organic contaminants present in the environment, under chronic or acute conditions. Polycyclic aromatic hydrocarbons (PAH) are a class of ubiquitous contaminants derived mainly from anthropogenic sources, that can be regarded as either of combustion origin or from petroleum products[1]. On the Grand Banks, offshore Newfoundland, there are oil fields slated for development in the near future. In the event of an oil spill, petroleum could persist in sediments inhabited by a variety of vertebrate and invertebrate species, for a period of time that would be determined by the location of the accident, atmospheric conditions and remediation action taken. As a preventive-predictive measure, a series of long-term exposure experiments are being carried out to investigate the fate and effects of that petroleum oil on local biota.

In the present study, winter flounder, *Pseudopleuronectes americanus,* were exposed to Hibernia crude oil in sediments, for four months during the winter. Exposure was done at five different concentrations ranging from 0.09–4.5 mg/g (total amount of oil in sediments, dry weight) at the beginning of the experiment, to 0.10–0.90 mg/g, at the end. A dose-response bioaccumulation was observed in muscle tissue[2], when PAH were measured in terms of fluorescence (chrysene units) or in terms of the concentration of 27

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parental and alkylated compounds (GC-MS). Concentrations were also measured in terms of fluorescence in liver tissues[2] where they were higher than in muscle, but did not display a significant increase with level of exposure.

The present study relates to the analysis of metabolites isolated from the gall bladder bile, as glucuronide and sulphate conjugates. Bile metabolites are now well recognized indicators of previous short-term exposure of vertebrates to PAH[3–5]. They are produced after enzymatic oxidation and conjugation of free PAH[6]. Short-term experiments have shown that bile metabolites are eliminated from fish, a few days following exposure to hydrocarbons[4,7–9].

The present investigation aimed to compare our previously reported bioaccumulation of PAH in muscle[2] to the elimination of glucuronides through the bile, (long-term accumulation versus short-term elimination[10]). A sensitive, semi-quantitative analytical approach (uv/f) was used, followed by more specific analyses (GC-MS) to identify the components within the mixtures. Special emphasis was placed on determining the presence of alkylbenzenes, since these monocyclic aromatic compounds were observed in muscle tissue where, according to synchronous fluorescence (SF) analyses, they appear to predominate.

EXPERIMENTAL

Between 18 and 20 male winter flounder, *Pseudopleuronectes americanus,* were placed in each of six tanks (300 L) containing sediments (45 kg) obtained from a remote area. Mean fish weight and length ranged from 243 to 280 g and 27 to 29 cm, respectively. One tank containing the sediments was used as a control, while 5, 25, 50, 100 and 250 ml of oil were added to sediments in five other tanks, prior to the addition of seawater and fish a week later. The fish and experimental tanks are referred to as E-5, 25, 50, 100 and 250, for simplification. Exposure was conducted for 4 months, during the winter (January to April, water temperature -1 to +3°C), when fish naturally fast and can be viewed as dormant[11,12].

Sediments were subsampled from each tank, at the beginning and end of the experiment and analyzed as detailed in Hellou *et al*[2]. At the termination of the experiment, the whole gall bladder was collected from each fish and stored at -20°C, until needed. Bile samples were processed as previously described by Hellou *et al*[13]. Briefly, one volume of bile was added to 15 volumes of ethanol:hexane (3:1, 40°C), to precipitate the proteins, the mixture was centrifuged and the solvent evaporated, at low temperature, under reduced pressure. The extract was rinsed three times with dichloromethane, to remove free organics and the remaining precipitate redissolved in water and analyzed by synchronous fluorescence (SF, $\Delta\lambda$ = 25 nm), as well as at the wavelength pairs characteristic of naphthalene (290/335 nm), chrysene (310/360 nm) and benzo(a)pyrene (380/430 nm). Enzymatic hydrolysis with β-glucuronidase (from limpets) in acetate buffer (0.3M, pH 4.8), 37°C, 20 hrs was followed by acidification and extraction with dichloromethane, followed by the above mentioned uv/f analysis. The organic layer was acetylated with acetic anhydride-pyridine-dimethylaminopyridine, 20°C, 18 hrs and analyzed by uv/f and GC-MS after solvent displacement.

GC-MS was performed on a Hewlett-Packard Series 5970 mass selective detector, connected to a Hewlett-Packard 5890 gas-liquid chromatograph and a Hewlett-Packard Series 300 data system, using a CP-Sil 5CB column (25 m × 0.2 mm, i.d.). The temperature program started at 80°C, for one minute and increased to 280°C, at 4°/min,

where it remained for 15 min. Using these conditions, cholesterol acetate displayed a retention time of 51.5 min and this biogenic standard was used to report relative indices.

RESULTS AND DISCUSSION

The chemical description of Hibernia crude oil has been reported earlier[2]. It is a light petroleum oil containing 42% saturates and 32% unsaturates, the rest being volatile and/or insoluble material. The resolved non-volatile unsaturated components of the fresh oil are dominated by alkylated naphthalenes (NA), phenanthrenes (PA) and dibenzothiophenes (DBT). Extraction of ions m/z = 91 and 105 a.m.u., indicated the presence of benzenoid derivatives ranging from less than C-6 to more than C-20 side chains, in the fresh oil. These components display a fingerprint similar to that of saturated hydrocarbon fractions: predominance of one isomer, in a repetitive manner, with minor more branched constituents. Some specific structures of more abundant alkylbenzenes have been previously identified in oils, where they form a large percentage of the total aromatic content[14,15].

The sediments collected from the tanks, at the beginning and termination of the experiments were also analyzed and concentrations previously reported in terms of the sum of alkylated PAH, parental PAH and DBT derivatives[2]. Figure 1 presents the normalised concentrations of individual PAC, determined in sediments at the end of the four months experiment. Results indicate that out of the parental PAH, phenanthrene dominates in the lower molecular weight range (up to three ring compounds), while chrysene predominates in the higher molecular weight PAH (four, five and six ring PAH). Out of the alkylated components (E-5 sediments being slightly different), C-2DBT, C-3PA and C-2 and C-3NA predominate. Of the parental and alkylated PAC analyzed after the four month experiment, parental hydrocarbons dominated in the lowest exposure, while alkylated components dominated in the higher exposure sediments.

Extraction of the benzenoid ions of m/z = 91 and 105 a.m.u. showed a maxium for the C-11 to C-13 benzenoid derivatives within the sediments mixture, with a higher abundance for the minor isomers compared to the fresh oil, indicating less degradation for these more branched constituents[2]. This weathering is in agreement with the known biodegradation of aliphatic hydrocarbons relative to more branched hydrocarbons[16,17]. The biodegradation and partitioning (between water and sediments) of linear alkylbenzene sulfonates (LABS, synthetic surfactants) has also been investigated[18–20]. Different rates of degradation and solubility have been related to the position of the benzene ring and size of the alkyl side chain.

Using uv/f analysis of individual bile samples obtained from the higher exposure, the coefficient of variation ([standard deviation/mean] × 100) to be expected for the bile metabolites was determined to be 25%. A high coefficient of variation has been associated with higher exposure, in the case of the analysis of free PAH in feral populations (>100%)[2,21], while the opposite has been observed in laboratory experiments (<100%)[2]. This difference is explained by the confined environment in the laboratory, with standardised variables compared to sampling where the past history of the biota is not as well defined.

Results of the synchronous fluorescence (SF) performed on hydrolysed, non-acetylated metabolites are presented in Figure 2, where an increase is observed in the uv/f absorbance with exposure. The control and E-5 fish displayed similar profiles

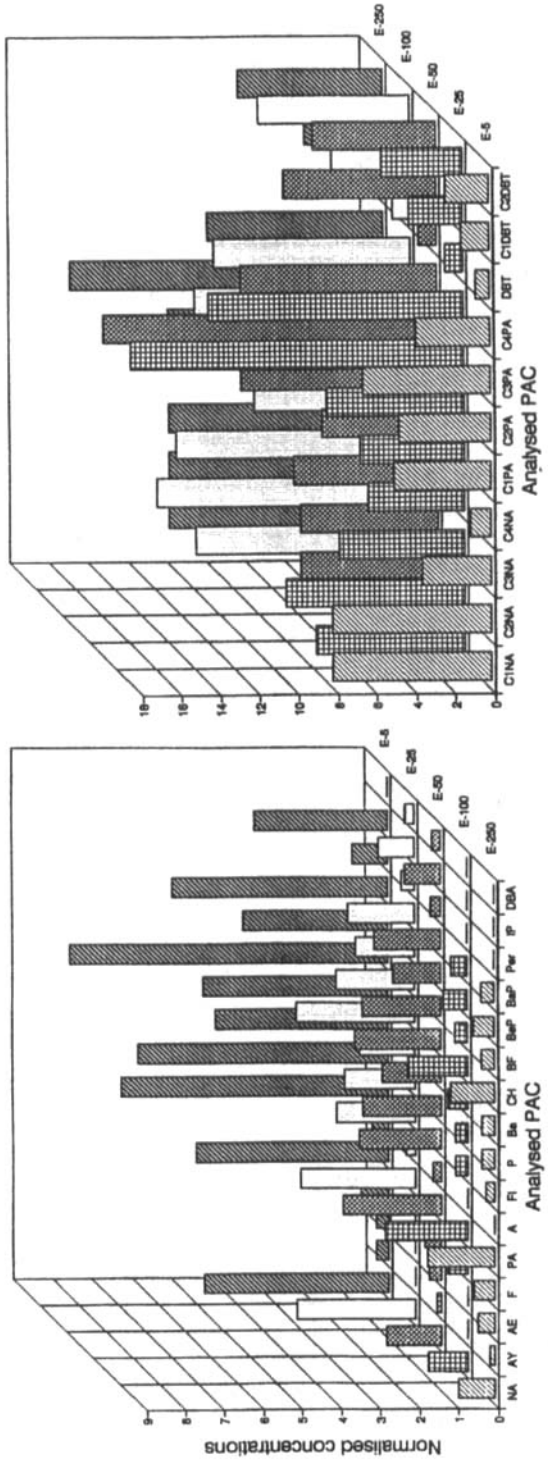

Figure 1 Normalised concentrations of 27 *parental and alkylated* polycyclic aromatic compounds analysed from sediments collected at the end of the exposures. Results of E-5 sediments from the end of the exposure were not previously published, since they were not available at the time[2]. NA: naphthalene, AY: acenaphthylene, AE: acenaphthene, F: fluorene, PA: phenanthrene, A: anthracene, DBT: dibenzothiophene, FL: fluoranthene, P: pyrene, Ba: benz(a)anthracene, CH: chrysene, BF: benzofluoranthene, BeP: benzo(e)pyrene, BaP: benzo(a)pyrene, Per: perylene, DBA: dibenzanthracene, IP: indeno(1, 2, 3 cd)pyrene. Numbers refer to alkylation of the ring (number of carbons).

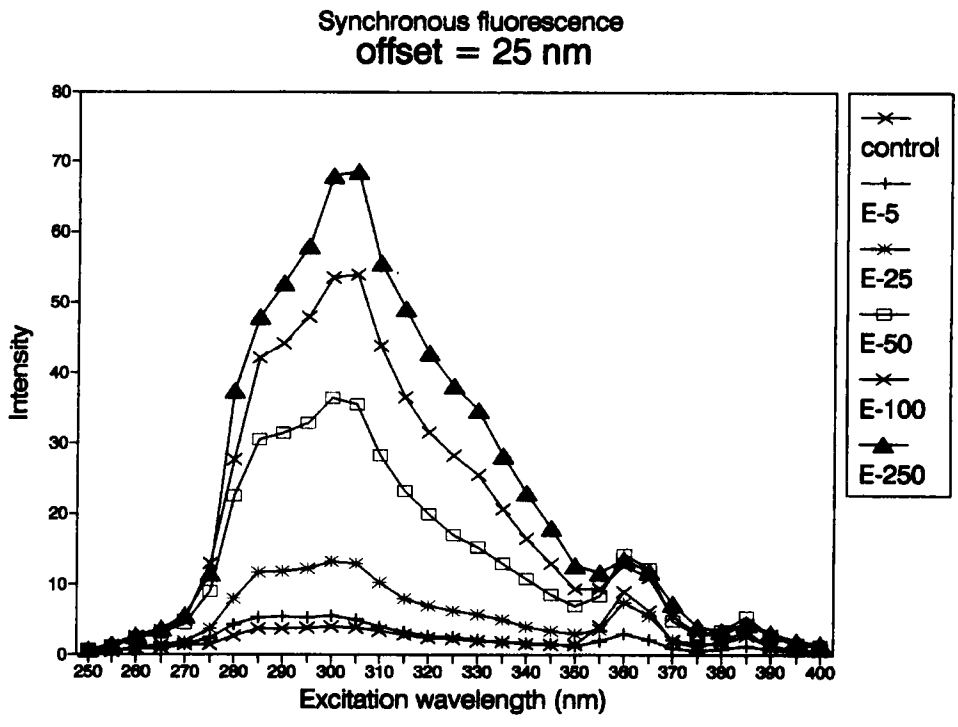

Figure 2 Synchronous fluorescence results obtained on bile metabolites of fish exposed to different levels of oil in sediments. Intensity is expressed in terms of µl of bile.

(values obtained by SF, up to an excitation wavelength of 350 nm, differed by less than 25%), while an increase and different fingerprint was observed for the higher treatments. Several maxima of decreasing intensity were apparent at: 305/330, 285/305, 330/355 (a shoulder), 360/385 and 385/410 nm. The first two more intense maxima appear in an increasing relative ratio (4:5), in contrast to the decreasing ratio observed in muscle (3:2), but similar to that in liver (5:6)[2]. These first two wavelength pairs would correspond to benzenoid and biphenyl type structures (monocyclic aromatic), while the next three can be assigned to bicyclic, tricyclic and tetracyclic structures (PAH)[22–24]. These last three maxima were not detectable in muscle tissue, indicating the predominance of monocyclic aromatic compounds, also confirmed by GC-MS-TIC analysis[2].

Ultraviolet/fluorescence is an analytical technique that has been widely used for the analysis of free PAH in a variety of matrices[25–29] and also for the analysis of bile metabolites[30–33]. Although uv/f represents a semi-quantitative technique, where a mixture of chemicals is analyzed and concentrations reported in terms of chosen standard(s), it is a highly sensitive technique. Chrysene has been recommended by the World Health Organization (WHO) for the analysis of free PAH and was used here for the analysis of bile metabolites. Adopting this analysis as a first step in an investigation of aromatic compounds permits the acquisition of preliminary results, which may justify spending more resources for further in depth analyses.

The method adopted in the present study followed the investigation of the effect caused by natural components present in bile extracts, at different stages of the protocol

and correlating absorbance at various wavelength pairs with exposure level and GC-MS analysis of hydrolysed, acetylated derivatives. It was found that the crude bile, pre-hydrolysis contained uv/f interferences. However post-hydrolysis, cholesterol and the sex hormones predominated by testosterone, the major biogenic components of the extract, displayed a consistently low background. Although the maximum absorbance of these steroids is at 280/305 nm, in SF (Figure 3), according to GC-MS analysis, the biogenic components were present at the same concentration, in all the exposures. This would tend to indicate that the observed maxima at the higher exposures is due to the presence of the anthropogenic components accumulating in the bile. Since the intensity of the uv/f maxima (SF) due to the oxidised PAH present in the bile, significantly correlated with the chrysene wavelength (better than 1% level, using Spearman rank correlations), metabolites were quantified in terms of chrysene units. This standard is easily available and was also used to quantify free PAH in muscle tissue and therefore allows a degree of comparison.

The muscle and gall bladder bile results obtained in terms of chrysene units are summarised in Table 1, while correlation with the level of aromatics in sediments is presented in Table 2. The volume of bile in each fish was approximately 200 µl, while the weight of muscle was 200 g (wet), regardless of exposure. Therefore the ratio of the reported values, bile/muscle, broadly reflects the degree of short-term bioelimination to longer-term bioaccumulation of aromatics in fish. However, since the whole content of the gall bladder can be analyzed, while only a small fraction of muscle is generally processed, this comparison demonstrates the importance and usefulness of the gall bladder in determining exposure[4,30-33]

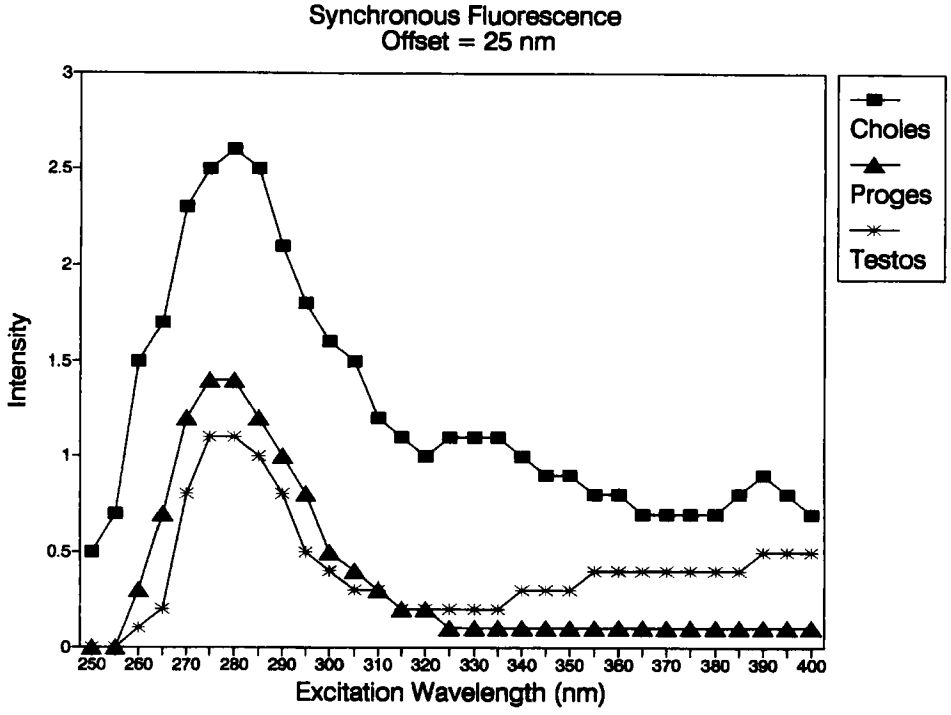

Figure 3 Synchronous fluorescence results obtained on predominant biogenic compounds present in bile: choles (terol), proges (terone) and testos (terone).

Table 1 Comparison of concentrations of PAH in fish tissues*, expressed in chrysene units (uv/f, 310/360 nm)

Exposure	*Bile (μg/μl)*	*Muscle (μg/g, dry weight)*
Control	0.13	ND
E-5	0.22	0.01
E-25	0.62	0.05
E-50	1.3	0.09
E-100	2.4	0.12
E-250	3.3	0.17

Muscle tissue weighed 200 g/per fish and contained 75-80% water and 2–5% lipids. Gall bladder contained approximately 200 μl of bile.

Table 2 Threshold level of aromatic hydrocarbons in sediments

Detectable as:	*Free hydrocarbons*	*Metabolites*
Weathered oil: end of experiment	E-25	E-5
Gravimetry (mg/g)	0.22	0.10
Σ 27 PAC (μg/g)	0.24	0.12
Σ C-1 to C-4 NA (ng/g)	63	35
Σ C-1 to C-4 PA (ng/g)	108	27

* Free hydrocarbons in muscle and metabolites in the gall bladder bile become detectable when the concentration of hydrocarbons in sediments (dry weight) is above the levels indicated in terms of different hydrocarbon components. Detailed concentrations of hydrocarbons in sediments can be found in Hellou *et al*[2].

The above comparison neglects the actual chemical composition of these two sets of extracts and represents a shortcoming. Higher molecular weight PAH were detectable in the bile (SF), whereas none were apparent in the muscle. Since it has been shown that the extinction coefficient, which is directly related to absorbance, will increase with the number of linear rings in a PAH, it follows that the maxima will vary with the specific structure and alkylation of a compound, as well as the position of hydroxylation[22–24,34] (in the ring moiety or alkylated portion, producing a batochromic shift compared to the PAH starting material in the first case and none in the case of oxidation of the side chain). Thus, the present comparison has limitations. However, the higher sensitivity of uv/f and the wider analytical target makes this spectroscopic technique an excellent first step choice in determining exposure, in terms of free and conjugated aromatics.

A more specific insight into the structures of the hydrolysed metabolites is provided by the GC-MS analysis of the acetylated bile samples. In the case of the control and E-5 groups, no peaks other than those due to steroids (cholesterol and testosterone, predominantly) could be detected. However, the E-25 to E-250 samples displayed the presence of non-biogenic peaks between RI = 0.1942 and 0.9709, with an identical fingerprint of resolved peaks, for the three higher exposures (e.g. Figure 4). A large variety of acetylated PAH derivatives could be identified from the MS obtained for peaks detectable in the TIC (Table 3). Further information was also obtained by extracting ions representative of a variety of PAH: M^+ and M^+–42 (CH_2CO) and confirmed by examination of the whole mass spectrum and comparison to published data[33,35].

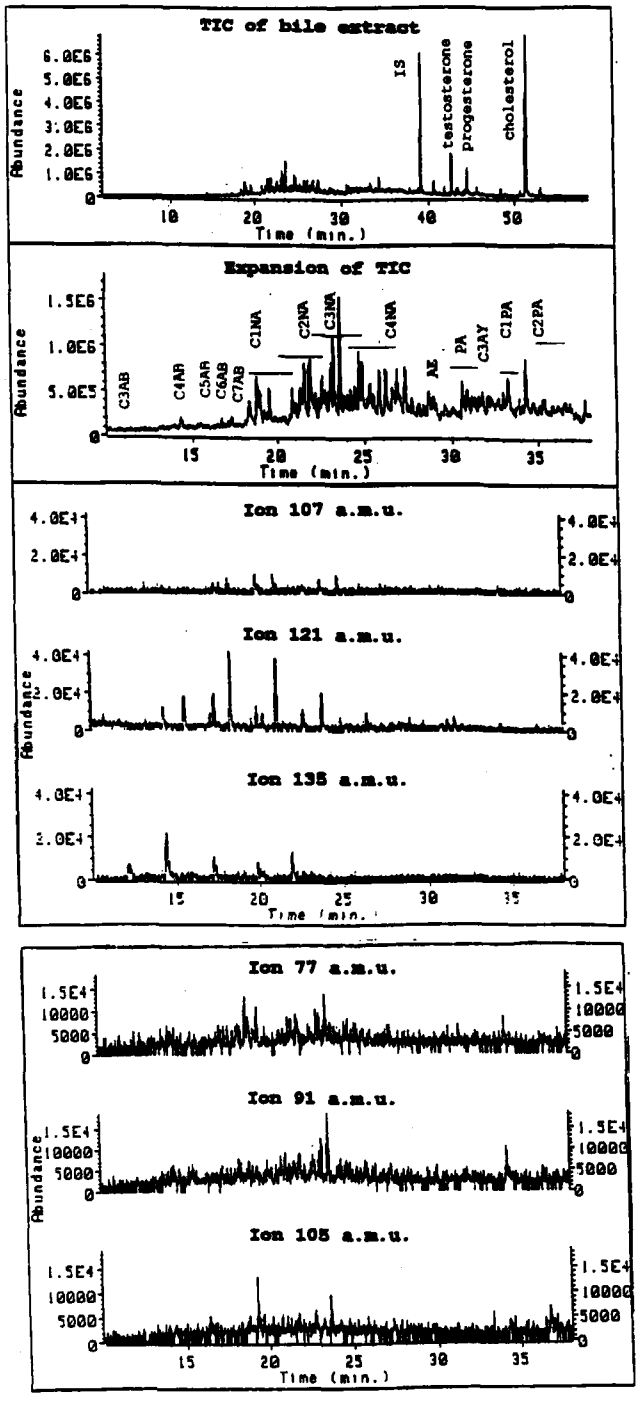

Figure 4 GC-MS-TIC representing the components present in bile of fish collected from the highest exposure and extracted ion chromatograms used to identify the presence of alkylbenzenes. No reliable full spectra could be obtained for peaks represented by ions of m/z 91, 105 and 119 a.m.u.

Table 3 Metabolites identified according to MS fragmentatiion

Compound	*Molecular formula (MW)*[1]	*Relative index (#)*[2]	*Total*
C-3AB	$C_9H_{12}O$ (136)	0.2058	3
C-4AB	$C_{10}H_{14}O$ (150)	0.23–0.28 (3)	
C-5AB	$C_{11}H_{16}O$ (164)	0.3010 (1)	
C-7AB	$C_{13}H_{20}O$ (192)	0.3359 (1)	
other AB		0.25–0.60 (5)	
C-1NA	$C_{11}H_{10}O$ (158)	0.35–0.46 (6)	29
C-2NA	$C_{12}H_{12}O$ (172)	0.38–0.43 (5)	19
C-3NA	$C_{13}H_{14}O$ (186)	0.42–0.48 (9)	12
C-4NA	$C_{14}H_{16}O$ (200)	0.46–0.58 (8)	18
AE or BP	$C_{12}H_{10}O$ (170)	0.5631 (1)	2
C-3AY or C-2F	$C_{15}H_{14}O$ (210)	0.6194 (1)	2
PA	$C_{14}H_{10}O$ (194)	0.59–0.66 (3)	7
C-1PA	$C_{15}H_{12}O$ (208)	0.64–0.66 (4)	4
C-2PA	$C_{16}H_{14}O$ (222)	0.68–0.72 (series)	3
C-3PA	$C_{17}H_{16}O$ (236)	0.73–0.78 (series)	1

1-MW: molecular weight after loss of CH_2CO (m/z = 42 a.m.u.)
2-Relative index taking cholesterol acetate as 1, followed by minimum number of resolved peaks.
AB: alkylbenzene, NA: naphthalene, AY: acenaphthylene, AE: acenaphthe-ne, F: fluorene, BP: biphenyl.

Monocyclic aromatic derivatives were identified in the gall bladder bile. These hydrocarbons have side chains ranging from C-3 to over C-8 and displayed base peaks in the MS at m/z = 107, 121 or 135 a.m.u., with fragment ion m/z = 121 a.m.u. being more common (Figure 4). Unlike the free benzenoid derivatives, the fragmentation of the metabolites can generate a variety of ions, depending on the number of metabolites produced and the position of hydroxylation. Previous studies indicate that enzymatic attack during metabolism of aromatics will take place preferentially on the terminal position of the side chain instead of on the ring of an alkylated benzene[13,35]. Depending on the structure of the substituent, more than one product can be formed, with more branching of the chain appearing to favour the formation of phenols. Although minor in quantity, phenols and larger aromatic alcohols can be formed, especially in the case of parental PAH and would be expected to affect the uv/f. It is these oxidized aromatics, which are of concern, since they are more reactive, more nucleophilic than aliphatic alcohols and can lead to detrimental biological effects[e.g. 36,37].

Unlike the free PAH in muscle[2], naphthalene and phenanthrene derivatives were predominant in the resolved components of the TIC. As well, acenaphthene or biphenyl and C-3 acenaphthalene or C-2 fluorene were well resolved in the TIC. Higher molecular weight metabolites (4, 5 and 6 rings) were not abundant enough to enable detection and characterization. The superior sensitivity of uv/f methods relative to GS-MS for the detection of larger versus smaller aromatic compounds has been previously discussed for free PAH[38,39].

Water temperature, feeding state, sex and fish species are some of the variables that can play a role in the production of metabolites and these have been standardised in the present experiment. The water temperature was typical of that found year-round in many areas of the Northwest Atlantic (-1 to +3°C)[40]. Fish were not feeding, because the experiment took place in the winter and flounder enter into a dormant state during that time[11,12]. It has been shown that biota will be less affected by contaminants, if

feeding[e.g. 41]. Fish were all males, since these were available in larger number and enabled the elimination of the sex variable. The fish species relates to the activity of mixed-function oxygenase enzymes and lipid content of tissues that can play a role in the distribution of contaminants in tissues[28,42].

Varanassi and Stein[42,43] have previously pointed out that larger (4, 5 and 6 rings, also less water soluble) PAH appear to be metabolised more readily than the smaller (2 and 3 rings, more water soluble) PAH. According to the present exposure it would appear that the more branched benzenoid hydrocarbons and other hydrocarbons belonging to the unresolved complex mixture (UCM) characteristic of petroleum products accumulate more as the free form (in muscle) compared to the less branched and higher molecular weight aromatics that appear to be more readily metabolized.

Bile metabolites were easily characterised as naphthalene and phenanthrene derivatives, while monocyclic aromatic derivatives were not resolved within that mixture. The higher sensitivity of bile metabolites in determining exposure to hydrocarbons, over that of free PAH in tissues was confirmed. The higher sensitivity was in the order of two or three folds, in terms of various concentrations of hydrocarbons in the weathered petroleum extracts. A major difference between these two approaches is due to the analysis of nearly the whole gall bladder compared to a fraction of muscle tissue.

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