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1-Hydroxypyrene as a Biomarker for Fish Exposure to Polycyclic Aromatic Hydrocarbons

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Polycyclic aromatic hydrocarbons (PAHs) represent an important group of ubiquitous lipophilic environmental pollutants, that are mostly generated by processes involving incomplete combustion of organic matter. Among many harmful effects observed in biota, the carcinogenic and/or mutagenic potential have been documented for some compounds of this group.

Contamination of the aquatic environment by PAHs has been shown to be causally associated with various gross and/or microscopic disorders including neoplasia in many shellfish and fish species (Malins et al. 1987). Aquatic vertebrates, including fish, take up these contaminants from polluted water, sediments and food web organisms. However, only limited accumulation of PAHs occurs in organs of aquatic biota, since parent compounds are rapidly converted to many metabolites. Biotransformation is mediated by hepatic microsomal mixed function oxidases (MFO). These Phase I enzymes convert lipophilic xenobiotics into oxygenated and/or hydroxylated (generally more polar) products. Some intermediates containing an epoxide group are responsible for PAHs toxicity in biota due to the formation of PAHs-DNA adducts, which are thought to be accountable for carcinogenic action. Generally, in Phase II metabolism, highly water-soluble excretable products, such as glucuronide/sulfate conjugates, are formed and successively released into gallbladder from which they are subsequently excreted (Varanasi et al. 1989).

In earlier studies employing radiolabeled PAHs, the highest concentrations were detected in the hepatobiliary system of exposed fish (Stein et al. 1984, Balk et al. 1984). Several other laboratory studies demonstrated that the presence of PAHs metabolites in bile was correlated with short-term exposure of respective biota to parental compounds (Britvic et al. 1993; Collier and Varanasi 1991; van der Oost et al. 1994; Yu et al 1995) and this trend has been confirmed in field studies (Krahn et al. 1992, 1993; McDonald et al. 1995).

For the purpose of water pollution monitoring, the above concept is quite attractive, because of the following reasons: fish bile is easy to collect, its analysis is relatively simple, unlike other tissues such as liver, muscle or gills. No specific

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pre-treatment of bile samples is needed as this biotic fluid is practically free of lipids, in some cases allowing its direct analysis (Lin et al. 1994; Krahn et al. 1993). Determination of the whole range of PAHs metabolites is laborious and time-consuming. Moreover, it is complicated by poor availability of relevant commercial standards. In practice, simple methods relying on determination of one or a few common metabolites have been employed for screening of fish after exposure.

The aim of the presented study was to assess the contamination of several aquatic ecosystems along Czech rivers (Elbe and Vltava) by PAHs. The use of 1-hydroxypyrene (1-OHPY) determined in bile of common fish species for this purpose has been evaluated. A simple HPLC/FLD method has been developed for the determination of this exposure biomarker in biological fluids.

The choice of 1-hydroxypyrene as a single target analyte for the assessing occurence of bioavailable PAHs in an aquatic ecosystem was based on the consideration of following aspects: (i) 1-OHPY was shown to be a good biomarker of human exposure to PAHs (Jongeneelen et al. 1987); (ii) in most biota, 1-OHPY represents the predominant product of biotransformation of pyrene (a wide range of metabolites is originated from other PAHs hence their analytical concentrations might be below LOQ) (Escartin and Porte 1999a), (iii) the bioavailability of pyrene for aquatic organisms is relatively high (Escartin and Porte 1999a); (iv) relatively high levels of pyrene are often detected in river sediments as well as in the other environmental compartments (Lin et al. 1994); (v) 1-OHPY exhibits a strong fluorescence which enables its sensitive detection (Lin et al. 1994); vi) 1-OHPY is one of the few analytical standards of PAH metabolites that is commercially available.

MATERIAL AND METHODS

Samples of fish bile and sediment collected at the respective sampling sites (see Fig. 1) since 1998 to 2000 were provided by the Research Institute of Fish Culture and Hydrobiology (VURH), University of South Bohemia. As shown in Fig. 1, sampling sites were located along two main Czech rivers. To get data needed for the identification of potential sources of pollution, fish samples were collected in the vicinity (upstream and downstream) of large urban areas represented by the cities of Prague and Pardubice.

The main characteristics of fish species examined within our study are summarised in Table 1. Chub *(Leuciscus cephalus)* and bream *(Abramis brama)* were chosen as biomonitors for our experiments not only because of their occurrence in Czech rivers; but also due to the diverse food habits (i.e. difference in ways/extent of dietary exposure to PAHs).

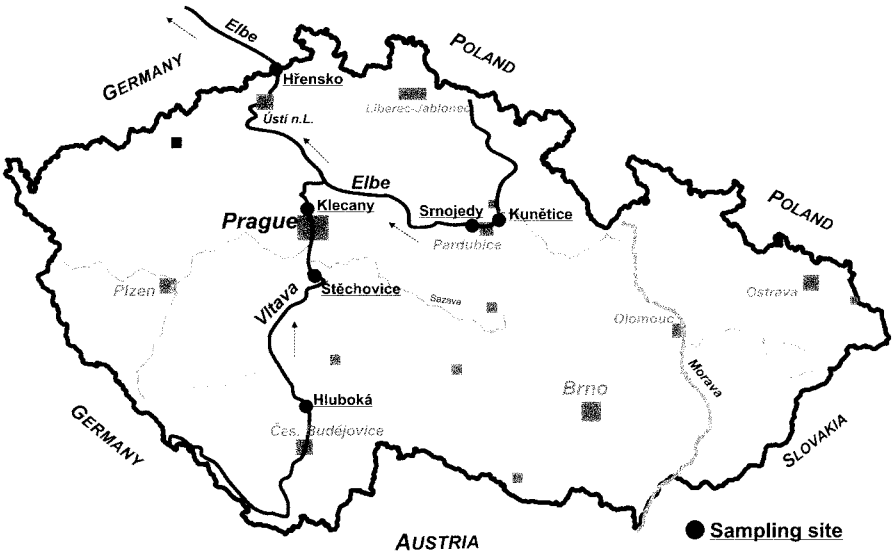

Figure 1 Map of Czech Republic, location of sampling sites within presented study

Bile samples were transferred from gallbladders to test tubes, frozen and stored at −18 °C until analysed. Sediments originating from the same localities as fish bile were frozen and stored in plastic bottles at -18 °C until analysed.

Table 1 The main characteristics of the fish population collected (number of individually analyzed bile samples is given in parentheses)

River	Species	1998 Weight (g)	1999 Weight (g)	2000 Weight (g)
Elbe	Chub *(Leuciscus cephalus)*	942 ± 363 (30)	579 ± 373 (57)	701 ± 395 (40)
	Bream *(Abramis brama)*	844 ± 460 (18)	834 ± 271 (34)	1103 ± 355 (20)
Vltava	Chub *(Leuciscus cephalus)*	333 ± 194 (20)	636 ± 404 (20)	669 ± 425 (25)
	Bream *(Abramis brama)*	1009 ± 739 (8)	1247 ± 378 (12)	1297 ± 324 (6)

Individual samples were analysed by reverse-phase HPLC with fluorescence detection after a release of 1-OHPY from conjugates by enzymatic hydrolysis: 25 μL of fish bile were dissolved in 9 mL of acetate buffer (0.4 M; pH = 5). After addition of 5 μL of β-glucuronidase/arylsulphatase solution, the mixture was shaken during incubation for 1 hr at 37 °C. Prior to sample loading, a LiChrolut®EN SPE cartridge (200 mg, Merck, Germany) was conditioned with 5 mL of acetone followed by 5 mL of acetate buffer (0.4 M, pH = 5). Nine mL of hydrolysed sample was loaded onto the cartridge at a flow rate of 2 mL/min, while a slight vacuum was applied at its outlet. The cartridge was subsequently washed

with 5 mL of distilled water and then dried 1 minute in an air stream. 1-OHPY was eluted with 8 mL of acetone. The solvent was evaporated at 40 °C on a rotary vacuum evaporator (RVO) to dryness. The residue was dissolved in 1 mL of methanol. HPLC determination of 1-OHPY was accomplished on a C_{18} reverse-phase LiChroCART 250-4 column (sorbent Purospher®, 5μm) with a LiChroCART 4-4 guard column (Merck, Germany). Column temperature was held at 45 °C. The flow rate of the mobile phase was 1 mL/min. The gradient of the mobile phase was as follows: 1 min of methanol-water (75 : 25 v/v); followed by a linear gradient in 8 min to 100 % methanol and eluted for another 4 min. Detection of 1-OHPY was performed by a programmable fluorescence detector (FLD), excitation/emission wavelengths setting was 248/397 nm.

The confirmation of its identity was made by the addition of a standard solution. Performance characteristics of this HPLC/FLD method obtained through the validation procedure were as follows: repeatability (as relative standard deviation; n=6): 3 %, recovery (spiked bile 25 ng/mL): 92.4 ± 4 %, limit of quantitation (LOQ): 4 ng/mL.

To determine dry weight, 200 g of sediment were dried at 105 °C for 16 h. 5 g of this sample was then mixed with anhydrous sodium sulphate and extracted with 50 mL of dichloromethane-methanol mixture (9:1, v/v). The slurry was sonicated for 15 minutes and then filtered through a layer of anhydrous sodium sulphate. This extraction step was repeated twice. Combined extracts were evaporated on the rotary vacuum evaporator to dryness and the residue was dissolved in 4 mL of chloroform. This concentrated crude extract was cleaned-up on the Bio-Beads S-X3 column. Flow-rate of the mobile phase (chloroform) was 0.6 mL/min and the collected fraction under these conditions was 15 – 29 mL. Elution solvent was evaporated using the rotary vacuum evaporator to dryness and the residue was dissolved in 1 mL of acetonitrile. Determination of PAHs in the purified sample was accomplished by reverse-phase HPLC with the column temperature at 35 °C and flow-rate at 1.2 mL/min. Gradient: 1 min of acetonitrile-water (55:45, v/v); linear increase to acetonitrile - water (65:35, v/v) in 9 min, then a linear gradient in next 7 min to 100 % acetonitrile. Detection of analyte was carried out by a programmable FLD. Recovery of PAHs from sediments exceeded 80 % for all compounds at concentration level of individual PAHs 100 ng/g d.w.

The following reference materials, reagents and other experimental materials were used: 1-hydroxypyrene (methanolic solution, 10 ng/μL) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); β-Glucuronidase/arylsulphatase isolated from *Helix pomatia* (100,000 Fishman U/mL and 800,000 Roy U/mL) was purchased from Boehringer-Mannheim (Germany); a standard mixture of 16 priority PAHs (PAHs - Mix 9) - acenaphthene (Ace), acenaphthylene (Acy), anthracene (Ant), benz[a]anthracene (B[a]A), benzo[a]pyrene (B[a]P), phenanthrene (Phe) benzo[b]fluoranthene (B[b]F), benzo[ghi]perylene (B[ghi]P), benzo[k]fluoranthene (B[k]F), chrysene (Chr), dibenz[a,h]anthracene (DB[ah]A),

fluoranthene (Flt), fluorene (Flu), indeno[1,2,3-cd]pyrene (I[1,2,3-cd]P), naphthalene (Nap), and pyrene (Pyr) dissolved in acetonitrile (10 μg/mL each) was supplied by Dr. Ehrenstorfer GmbH (Germany). Stock and working solutions of PAHs were prepared in acetonitrile by dilution.

Acetone, chloroform (p.a., Lachema, Czech Republic) were purified by distillation before use. Methanol, acetonitrile, dichloromethane (gradient grade for chromatography, Merck, Germany), acetic acid, and sodium hydroxide (p.a., Lachema, Czech Republic) were used as supplied. HPLC-grade water was generated from distilled water by a water purification system (Milli-Q RG Millipore, USA).

An automated GPC (gel permeation chromatography) system ASPEC XLI (GILSON, France) consisting of GILSON 305 master pump, 231 XL sampling injector, 401C dilutor, and stainless steel column (50 cm x 0.8 cm i.d.) packed with Bio-Beads S-X3, 200 - 400 mesh (Bio-Rad Laboratories, USA) was used for clean-up of crude extracts of sediments. The HPLC system used for the determination of target analytes was composed of a Hewlett-Packard 1050 pumping system, HP 1050 autosampler, and HP 1046 A fluorescence detector.

RESULTS AND DISCUSSION

In the first set of experiments, a simple method for determination of 1-OHPY in fish bile has been developed and validated. Similar to procedures routinely applied for examination of human urine, 1-OHPY bound in conjugates has to be released by enzymatic hydrolysis. Based on the evaluation of the chromatographic profile of analyte fraction obtained by various SPE cartridges (alumina, florisil etc.), Lichrolut EN was identified as the most suitable for removing of potentially interfering bile components prior to HPLC/FLD analysis. Routine screening of this biomarker was found as simple and feasible with sufficient accuracy (recovery 92 ± 4 %) and its utilisation for monitoring of fish exposure to bioavailable PAHs can be recommended.

The results obtained within the three monitoring years are summarised in Table 2. The range of 1-OHPY levels determined in fish bile was comparable with that reported by other authors (Escartin and Porte 1999a,b). Noticeable variability in 1-OHPY levels between individual fish collected from specific sampling sites (see standard deviation within group of individually analyzed fish biles shown in Table 2) was probably caused mostly by actual volume of bile in the gallbladder when removed from fish, a variable moisture content in bile corresponds to dilution of metabolites.

Contents of PAHs in sediments collected from sampling sites at the Elbe and Vltava Rivers are shown in Fig. 2. The highest levels of 1-OHPY in bile were found in all fish species from Srnojedy, situated downstream from the industrial

city of Pardubice. Its pollution might be attributed to intensive anthropogenic activities accompanied by PAHs emissions. It seems that atmospheric imissions related to burning processes are not responsible for contamination of the Elbe river, since significantly lower levels of 1-OHPY were determined in Kunětice, located upstream of Pardubice. Most probably, sewage and the other effluents from local industrial sources are responsible for the main PAHs input into the aquatic environment.

For the Vltava River, similar considerations might be drawn to explain the relatively high contamination at Klecany (downstream from Prague), compared to Štěchovice, located upstream from the Prague industrial area with many potential emission sources. On the other hand, we can not explain the serious pollution at Hluboká. Besides common activities typical for small urban areas, a significant pollution source is not known here. The levels of PAHs in sediments found 145 km downstream from Hluboká in Štěchovice were evidently lower, probably due to sedimentation of PAHs-bearing particles in the dam located between these two sampling sites.

Linear regression was applied for the description of the relationship between 1-OHPY levels in fish bile and the concentrations of PAHs in sediments. For both species, chub and bream, positive correlations were found: correlation coefficients were 0.81 and 0.76, respectively. Similar positive correlations were obtained when comparing 1-OHPY levels in fish bile with concentrations of pyrene in sediment. The values of correlation coefficients for chub and bream were 0.78 and 0.77, respectively. This finding indirectly confirms the assumption of a proportional relationship between pyrene levels and the sum of the PAHs occurring in particular environmental compartment. Comparison of mean levels of 1-OHPY in fish bile can provide evidence on PAHs input from industrial (and/or municipal) sources into an aquatic ecosystem.

Based on our results, chub and bream seem to be suitable indicator fish species which reflect the exposure by bioavailable PAHs. Since no correlations were found between 1-OHPY levels in fish bile and either weight or length of fish (i.e. no bioaccumulation occurs), the levels of this biomarker reflect the temporary pollution. The levels of 1-OHPY in bile of bream were generally higher than those in chub. This might be probably caused by different food habits of these two fish species; the other reason can be the higher metabolic activity of the chub than that of bream. Generally, chub and bream can be recommended for the biomonitoring of freshwater ecosystem as these fish species well reflect contamination of bottom sediments with PAHs.

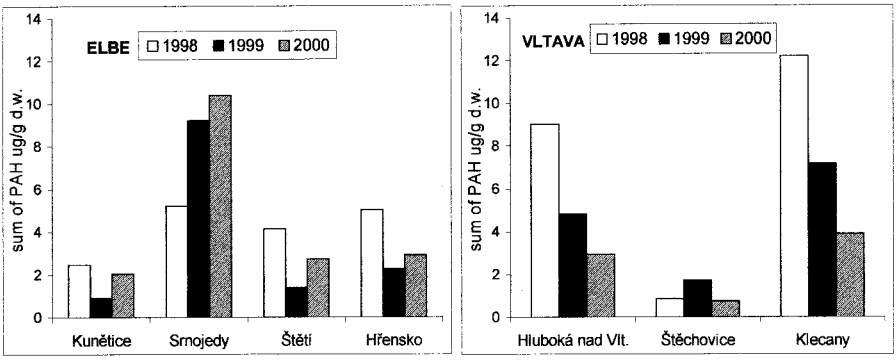

Figure 2 PAHs levels in sediments (μg/g dry weight) at monitored localities

Table 2 1-OHPY levels in bile from fish collected in various sampling localities

			1998	1999	2000
river	**locality**	**species**	**X ± Std (n)** ***ng/mL***	**X ± Std (n)** ***ng/mL***	**X ± Std (n)** ***ng/mL***
Elbe	Kunětice	chub	123 ± 40 (6)	58 ± 45 (16)	363 ± 194 (11)
↓		bream	275 ± 25 (4)	122 ± 136 (5)	553 ± 175 (5)
	Srnojedy	chub	336 ± 191 (4)	370 ± 259 (14)	566 ± 445 (10)
↓		bream	410 ± 137 (3)	678 ± 351 (15)	766 ± 156 (3)
	Štětí	chub	258 ± 94 (10)	95 ± 47 (18)	80 ± 39 (10)
↓		bream	371 ± 55 (3)	125 ± 35 (2)	180 ± 157 (6)
	Hřensko	chub	149 ± 24 (10)	109 ± 58 (9)	150 ± 98 (9)
		bream	299 ± 85 (8)	143 ± 38 (12)	294 ± 196 (6)
Vltava	Hluboká /Vl.	chub	601 ± 153 (8)	616 ± 138 (9)	301 ± 100 (9)
↓		bream	1073 ± 147 (3)	621 ± 138 (3)	445 ± 69 (2)
	Štěchovice	chub	26 ± 14 (3)	37 ± 19 (2)	49 ± 38 (8)
↓		bream	66 ± 47 (2)	65 ± 39 (2)	88 ± 35 (2)
	Klecany	chub	567 ± 248 (9)	285 ± 65 (9)	220 ± 130 (8)
		bream	688 ± 431 (3)	450 ± 190 (7)	237 ± 166 (2)

X - mean value; Std - standard deviation within each group of individually analyzed fishes;
(n) - number of fish examined

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