Biliary 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons in fish

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Abstract

OBJECTIVES: The aim of this study was the assessment of the Svitava and Svratka rivers contamination by polycyclic aromatic hydrocarbons (PAH) using 1-hydroxypyrene (1-OHP) concentrations in fish bile as a biomarker.

DESIGN: Levels of 1-OHP were determined by reverse phase HPLC with fluorescence detection. For valid assessment of bile accumulation levels, the 1-OHP concentration was normalized to the biliary protein content. The content of 1-OHP was correlated with the PAH content of river bottom sediments.

RESULTS: The highest level of 1-OHP in fish bile (136.1 ng.mg–1 protein) was found at the locality Rajhradice, which is situated downstream of Brno. Also the greatest level of sum of PAHs was found at this locality (17.1 μg.g–1 dry mass). Significant positive correlation (p≤0.05) between the level of 1-OHP and sum of PAHs in sediment was found only in case of sediments collected in the same month as fish samples (in June).

CONCLUSION: Our results document that 1-OHP in fish bile is a suitable biochemical marker for the assessment of aquatic ecosystem contamination by PAHs.

Abbreviations

EPA - Environmental Protection Agency
GC/MS - Gas chromatography/mass spectrometry
HPLC - High performance liquid chromatography
1-OHP - 1-hydroxypyrene
PAH - Polycyclic aromatic hydrocarbon
WWTP - Waste water treatment plant
INTRODUCTION

Polycyclic aromatic hydrocarbons represent an unique class of persistent organic pollutants comprising hundreds of substances. They have been observed to be most concentrated in estuaries and coastal environments near urban centres, where inputs from watersheds and airsheds are most localized. The major sources of PAHs in the aquatic environment include urban runoff, wastewater effluents, industrial output, atmospheric deposition, and spills and leaks during the transport and production of fossil fuels (Douben, 2003). Depending on the chemical structure and level of exposure, PAHs and their metabolites have the potential to produce toxic, mutagenic and/or carcinogenic effects in fish and other vertebrates including humans (MacRae & Hall 1998; Douben, 2003). Numerous studies have shown a link between PAHs level in the aquatic environment and liver tumour incidence in fish populations (Baumann & Harshbarger, 1995; Vethaak et al. 1996), as well as the impairment of some reproductive functions in fish (among many organisms). These impairments can occur at different stages of vitellogenesis (Nicolas, 1999). The evaluation of xenoestrogenic potential of these compounds can be assessed by determination of common biomarker – vitellogenin (Mikula et al. 2006). The US Environmental Protection Agency identified 16 PAHs as particularly important due to their toxicity to mammals and aquatic organisms (EPA 1987).

1-hydroxypyrene is the main metabolite of pyrene, a widespread and common PAH that is generated by many pyrolytic and petrogenic industrial processes (Ariese et al. 1993; Hosnedl et al. 2003). Urinary 1-OHP has been used frequently as a biomarker of human exposure to PAHs, especially for workers in places of high potential PAH exposure, such as oil refineries, tar plants, aluminium plants, and coal-burning facilities (Zhang et al. 2007). It has been identified as one of the most abundant compounds present in fish bile, and this metabolite is regarded the best general indicator of PAHs exposure in aquatic organisms (Van der Oost et al. 1994; McDonald et al. 1995; Hosnedl et al. 2003).

Bile volume increases between meals and the gall bladder is emptied after feeding. In order to correct for differences in bile accumulation levels, normalization of the 1-OHP content is necessary. Vuorinen et al. (2006) described in their study the possibility of using biliverdin or protein content for normalization of 1-OHP contents.

The aim of the present study was to assess the contamination of selected localities on the Svitava and Svratka rivers around the industrial city of Brno by PAHs. Two sites were located upstream of the Brno agglomeration and other localities were downstream of this city, where their pollution might be attributed to intensive anthropogenic activities involving PAH emissions. Most probably, domestic waste, sewage and other effluents from local industrial sources are responsible for the main PAH input into the aquatic ecosystem.
MATERIAL AND METHODS

Animals and sampling

The chub (Leuciscus cephalus L.) was selected as the most suitable indicator species, because it is a common freshwater cyprinid that inhabits both clean and polluted rivers. Male fish were captured by electrofishing at 7 localities in the Svitava and Svratka Rivers (Figure 1) at the end of June and beginning of July 2007. The locations studied in the Svitava River were Bílovice above the city of Brno (Bílovice nad Svitavou) and another site downstream of Brno before the confluence with Svratka (Svitava before junction). The sites studied on the Svratka River were Kníničky above the city of Brno, a site downstream of Brno before the confluence with Svitava (Svratka before junction) and Modřice, Rajhradice, Židlochovice after the confluence of the two rivers. Fish were weighed and their scales collected for age determination, the main characteristics are summarized in Table 1. Bile was drawn by needle through the exposed gall bladder and emptied into an Eppendorf tube. Samples were immersed in dry ice and taken to the laboratory, where they were stored at –80 °C until analysis. At each locality, the composite bottom sediment samples were collected for determination of PAH content (at the beginning of May and the end of June 2007).

Analysis of 1-OHP in bile

1-OHP levels in bile were determined according to the method described by Hosnedl et al. (2003). Bile samples were deconjugated with an enzyme mixture of glucuronidase and arylsulphatase and purified on LiChrolut® EN column (Merck). The samples were eluted from the cartridges with acetone, desiccated and resuspended in methanol. Five μl of the extract was injected onto Polaris C18–A, 3 μ, 150×4.6 mm (Varian Inc.) in a HPLC system with fluorescence detector (Waters, USA). The excitation wavelength was 364 nm and emission wavelength was 384 nm. Separation was performed in 12 min using an acetonitrile:water mobile phase. The linear gradient was as follows: t = 0 min: 65% acetonitrile, t = 5 min: 70% acetonitrile, t = 10 min: 80% acetonitrile, t = 12 min: 65% acetonitrile. Recovery and reproducibility of the analyte were improved by the addition of ascorbic acid (1 mg.ml⁻¹) to the water eluent. The detection limit was 0.118 ng.ml⁻¹.

Analysis of bile protein

Total biliary protein was measured by a modified spectrophotometric method (570 nm) of Smith et al. (1985), using bicinchoninic acid and bovine serum albumin as a standard. The 1-OHP content of bile protein was used for normalisation.

Analysis of PAHs in sediment

The combination of HPLC with fluorescence detection and GC/MS was used for analysis of 16 PAHs according to EPA 610. Polycyclic aromatic hydrocarbons were quantified using the external standard method. Linear range and limits of quantification were calculated for PAHs, using a fluorescence detector. Due to low selectivity of the fluorescence detector for 2 and 3 ring PAHs, the GC/MS method was used for identification and quantification of low MW PAHs.

Statistical methods

Statistica for Windows 8.0. (StatSoft, Inc. USA) was used for data analysis. After testing for normality of 1-OHP content (Kolmogorov–Smirnov test) and homogenity of variances across groups (Levene test), an analysis of variance (One-way ANOVA) was used. The differences among localities were assessed with LSD tests (Fisher Least Significant Difference method) and p<0.05 was chosen as the level of significance. Spearman correlation coefficient was used to indicate the relationship of 1-OHP content in fish bile and PAHs content in sediment.

RESULTS

The 1-OHP concentrations in bile samples are shown in Figure 2. The highest median value (in ng.mg⁻¹ protein) was found in fish from Rajhradice (136.1). This

Table 1. Characteristics of chub captured at the individual localities.

<table>
<thead>
<tr>
<th>Locality (River km)</th>
<th>Fish n</th>
<th>Age (years) (min–max)</th>
<th>Weight ± SD (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Svitava River -- Bílovice nad Svitavou (18.0)</td>
<td>18</td>
<td>5 (4–7)</td>
<td>129 ± 33</td>
</tr>
<tr>
<td>Svitava River before junction (0.6)</td>
<td>11</td>
<td>4 (3–7)</td>
<td>166 ± 67</td>
</tr>
<tr>
<td>Svratka River -- Kníničky (56.2)</td>
<td>6</td>
<td>6 (3–9)</td>
<td>330 ± 234</td>
</tr>
<tr>
<td>Svratka River before junction (40.9)</td>
<td>12</td>
<td>5.5 (3–7)</td>
<td>235 ± 96</td>
</tr>
<tr>
<td>Svratka River -- Modřice (38.7)</td>
<td>10</td>
<td>5.5 (4–7)</td>
<td>266 ± 66</td>
</tr>
<tr>
<td>Svratka River -- Rajhradice (35.0)</td>
<td>10</td>
<td>5 (4–6)</td>
<td>248 ± 80</td>
</tr>
<tr>
<td>Svratka River -- Židlochovice (30.0)</td>
<td>11</td>
<td>4 (2–6)</td>
<td>238 ± 83</td>
</tr>
</tbody>
</table>
value was significantly higher than those obtained from Svratka before junction (119.0), Židlochovice (112.1), Bílovice nad Svitavou (97.3), Kníničky (94.2) and Svitava before junction (67.7). There was no significant difference between the sites at Rajhradice and Modřice (129.3). The lowest median value of 1-OHP content of bile samples was found at the Svitava before junction. This value was significantly lower than those obtained at all other localities with the exception of Kníničky.

At each location, one composite sample of bottom sediment was collected in May and June for determination of PAH content. Total PAH levels in bottom sediments are shown in Figure 3. Comparison of 1-OHP in bile and total PAH in sediment samples collected in June showed a significant Spearman’s correlation at $p \leq 0.05$ of significance. Similar positive correlations were obtained for 1-OHP content with concentration of pyrene and other individual PAHs in June. The values of correlation coefficients for total PAHs and individual PAHs ranged from 0.75 to 0.86. In June the highest values of total PAHs (in $\mu$g.g$^{-1}$ dry mass) were found at Rajhradice (17.1), Svratka before junction (13.2) and Modřice (9.5). The lowest values were found at Kníničky (1.3) and Bílovice nad Svitavou (3.4).

**DISCUSSION**

The state of the aquatic ecosystem is monitored in long-term studies by measuring concentrations of some persistent organic pollutants in a number of matrices, i.e. in
PAHs represent an important group of ubiquitous lipophilic environmental pollutants that are generated mainly by anthropogenic activities. Fish exposed to PAHs accumulate only a small amount of these pollutants in their tissues. Hydrophobic PAHs are readily transformed into hydrophilic metabolites and excreted in bile; consequently bile PAH metabolite analysis provides information about the actual exposure of fish to PAHs. For complex evaluations it is more efficient to assess the content of PAH metabolites in fish together with the content of PAHs in bottom sediments (Hosnedl et al. 2003).

The highest pollution by PAHs was found at Rajhradice (the Svratka River), which is situated below the confluence of the rivers and the effluent of municipal waste water treatment plant (WWTP) downstream of Brno. It is adversely influenced by contamination from domestic wastes, the WWTP in Modřice (about 4 km upstream of Rajhradice) and possible pollution from the Bobrava River (a tributary about 2 km upstream of Rajhradice). The highest level of total PAHs in bottom sediment in July was found at this locality along with the greatest concentrations of 1-OHP in fish bile. On the other hand, results obtained from the Svratka River in Židlochovice, located about 5 km downstream of Rajhradice, suggested that there are probably no further significant sources of PAHs along this river. The values of 1-OHP at Židlochovice were lower than those obtained at Rajhradice, Modřice and Svratka before junction, but significantly ($p<0.05$) different only from those at Rajhradice.

The second highest value of 1-OHP in bile was observed in samples obtained at Modřice (the Svratka River), which is located below the WWTP. This value was not significantly different from that obtained at Rajhradice. The WWTP in Modřice treats wastewater conveyed by a system of sanitary sewers from the city of Brno and, increasingly, by a system of pumping stations that connects other municipalities to the WWTP. The high level of 1-OHP at this location is evidence of the negative effect of WWTP on surface water contamination and its incomplete efficiency in elimination of these compounds.

Strikingly, the lowest content of 1-OHP was found at the location on the Svitava before junction where some impact of the Brno city and its industry were regularly recorded in the past. Recently, both chemical and biological determinants of the Svitava River pollution indicate the absence of any pollution sources during the river course through the Brno city. Extensive pollution of the Svratka River is also reflected in higher levels of 1-OHP and total PAH obtained at the site in the Svratka before junction and in the other localities of the Svratka River.

Locality Bílovice nad Svitavou reflects the state of surface water before entering the Brno city agglomeration. Although this site was chosen as a control locality, the content of 1-OHP is slightly higher, but this value is significantly ($p<0.05$) lower than the highest value obtained from the site at Rajhradice. It can be influenced by WWTP in Bílovice nad Svitavou (about 150 m upstream of the monitored site) and by chemical and engineering industries in nearby cities Adamov and Blansko.

Kniničky, on the Svratka River, is situated upstream of Brno bellow the Brno Reservoir dam. The analysis of bile samples showed the level of 1-OHP in fish bile to be the second lowest among the tested sites but not statistically different from the location in the Svitava before junction, where the lowest value of 1-OHP was found. The lowest value of total PAHs in sediments was also found at Kniničky. The relatively low levels of both markers indicate that PAHs are likely adsorbed onto sediment particles in the reservoir above the dam; there is minimal presence of these compounds downstream of the dam.

The positive correlation between 1-OHP contents in fish bile and total PAHs in sediments (in June) indirectly confirms the assumption of a proportional relationship between pyrene levels and total PAHs at the tested sites. These results are in agreement with the data reported by other authors (Van der Oost et al. 1994; Hosnedl et al. 2003). Hosnedl et al. (2003) calculated linear regression for the description of the relationship between 1-OHP levels in fish bile and the concentration of PAHs in sediments collected from sites along the Elbe and Vltava rivers in the Czech Republic. Positive correlations were found for chub and bream; coefficients were 0.81 and 0.76, respectively. On the other hand, these findings differ from reports of Siroka et al. (2005) who did not find a correlation between 1-OHP and PAH content of sediments collected from ten locations in the Elbe River basin. In particular, they obtained non-standard results at the control site Blanice (upstream of the Husinec water reservoir), where PAH values in sediment were below detectible limits, although levels of 1-OHP was relatively high. This can be ascribed to the characteristics of the Blanice river bottom, gravel and sand with a low proportion of organic component capable of binding PAHs.

The present study confirms the presence of PAH metabolites in fish bile correlated with their short-term exposure to parental compounds, and demonstrates that it is necessary to collect the organisms and bottom sediment at the same time in order to achieve valid results (Van der Oost et al. 1994). In conclusion, the study reinforces the suitability of using bile 1-OHP for monitoring of aquatic ecosystem contamination by polycyclic aromatic hydrocarbons.

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