Bull. Environ. Contam. Toxicol. (2006) 76:233–241

DOI: 10.1007/s00128-006-0912-3

Bulletin of Environmental Contamination and Toxicology

Biomarkers Detected in Chub (*Leuciscus cephalus* L.) to Evaluate Contamination of the Elbe and Vltava Rivers, Czech Republic

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Received: 20 May 2005/Accepted: 12 December 2005

In the Czech Republic, the middle reach of the Elbe river and the lower reach of the Vltava river downstream from Prague are the most polluted aquatic ecosystems. Heavy metals and organic coumpounds have been deposited here.

A whole series of contaminants may negatively affect the endocrine systems of aquatic animals. Endocrine disrupting chemicals (EDCs) are synthetic or naturally occurring chemicals (e.g. PCBs, PAH, phthalates, styrenes, alkylphenols, pharmaceuticals, Hg and others) which affect the balance of normal animal hormonal functions. Related to their activity, they may be characterized as estrogenic- or androgenic modulators (Keith 1997).

Consequences of fish exposure to environmental chemicals can be studied using suitable biomarkers. Biomarkers usually react with groups of substances with the same mechanism of toxic effect. With exceptions, they are not specific to individual xenobiotics. Their advantage is in the capacity to report on the effect of pollution in a complex way (Tyler et al. 1998). Vitellogenin (VTG) is an important biochemical marker to assess the loading of surface waters with substances having estrogenic effects. The presence of vitellogenin in blood plasma of males indicates the presence of EDCs in the aquatic environment (Schwaiger and Negele 1998). Some studies showed that exposure of fish to various substances with the effect of endocrine disruptors decreased the concentration of sex steroid hormones (e.g. 11-ketotestosterone – 11-KT) (Mills et al. 2001; Hecker et al. 2002). Cytochrome P450 (isoform CYP1A) and the associated enzymatic activity EROD (7-ethoxyresorufin-O-deethylase) detected in fish liver are important biochemical markers of contamination of the aquatic environment with PAHs, nitro-PAHs, PCBs, dioxins and some pesticides (Široká and Drastichová 2005). 1-hydroxypyrene (1-OHPY), a PAH metabolite detected in fish bile, is another biochemical marker of PAH contamination of aquatic environments (Hosnedl et al. 2003).

The chub (*Leuciscus cephalus* L.) was selected as a suitable bioindicator for the field study. It is an omnivorous fish abundant in all monitored localities. Blood plasma and tissue samples were collected from wild chub from localities of the Elbe, Vltava and Blanice rivers, Czech Republic, in 2003.

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MATERIALS AND METHODS

Samples of fish tissues were collected from 7 localities of the Elbe River, 2 localities of the Vltava River and from 1 locality of the Blanice River (control locality), Czech Republic. Localities were chosen as follows:

Verdek - Locality on the upper reaches of the Elbe River. Minor engineering- and textile factories are the potential sources of pollution. **Němčice** - Locality downstream from Hradec Králové urban agglomeration. **Valy** - Locality downstream from the Synthesia Pardubice chemical enterprise. Territory of this enterprise is an example of a very large complex ecological load. The territory is partly located in an inundation zone of the Elbe river. A whole series of substances may be present: toxic metals, oil products, PCBs, PAHs, chlorinated aliphatic hydrocarbons, etc. **Lysá** - Locality on the middle reaches of the Elbe river, potentially contaminated by industrial activity in municipalities situated upstream. **Obříství** - Locality downstream of the Spolana Neratovice chemical enterprise. Parts of the territory are contaminated with dioxins, mercury, chlorinated aliphatic hydrocarbons, organochlorine pesticides, etc. **Podolí** - Locality upstream from Prague, downstream from the confluence of Vltava and Berounka rivers. **Zelčín** - Locality on the lower reaches of the Vltava river, affected by Prague agglomeration. **Děčín** - Locality downstream from the Czech part of the Elbe river, affected by agricultural and industrial activities in the particular region. **Hřensko** - Locality close to the border with Germany. **Blanice** - Control locality on the upper reaches of Blanice river upstream from the Husinec drinking water reservoir, without any known sources of pollution. It was not possible to obtain an unpolluted site of the Elbe River due to present pollution of its entire reach. Therefore, chubs were sampled from the Blanice River with minimal anthropogenic pollution.

Wild populations of chub of both sexes were sampled at the end of July 2003. The fish were caught by electrofishing and were blood-sampled within 1 h of capture in order to maintain capture and handling stress at a minimum level. Ten individual samples of chub were analyzed from most localities. Altogether 90 fish were analysed (Table 1). The sex was determined macroscopically and confirmed by light microscopic evaluation of hematoxylin–eosin stained sections.

Blood samples were collected from the caudal vein into heparinized tubes. Samples were centrifuged *in situ* on monitored sites and blood plasma samples were frozen in liquid nitrogen. Length and body weight were measured, and scales were collected to age the fish. The gonads and livers were dissected from the fish. Bile was drained from the gallbladder using a needle and syringe. The samples of liver and bile were put into tubes and also kept frozen in liquid nitrogen for subsequent analysis of EROD activity and 1-hydroxypyrene respectively. Muscle tissue samples were put into polyethylene bags, labelled and stored in a freezer at -18 °C.

A carp vitellogenin ELISA kit (Biosense Laboratories, Norway) was used to

determine the concentration of vitellogenin (VTG) in blood plasma. The use of carp vitellogenin ELISA for determination of vitellogenin in chub was validated by Flammarion et al. (2000). The 11-ketotestosterone ELISA kit (Biosense Laboratories, Norway) was used to determine the concentration of 11-ketotestosterone (11-KT). 1-hydroxypyrene (1-OHPY) occurring in fish bile was determined by a reversed phase HPLC/FLD method described in detail earlier (Hosnedl et al. 2003). 1-OHPY was released from conjugates by β-glucuronidase/arylsulphatase enzymes mixture. LiChrolut® EN solid phase extraction (SPE) cartridges were used for purification of bile hydrolysate. Within the validation study, spiked samples were analysed (n = 6). The recovery (spiking level 25 $ng.ml^{-1}$) was 92.5 % ± 4% and the limit of quantification (LOQ) was 0.6 $mg.ml^{-1}$.

Table 1. The main characteristics of sampled chub (*Leuciscus cephalus* L.)

Locality	Sex (F/M)	(n)	body weight (g) *M ± SD*	total length (mm) *M ± SD*	age (years) *M ± SD*
Blanice	F	1	295	300	4.0
	M	9	192 ± 37	274 ± 17	3.2 ± 0.4
Podolí	F	3	245 ± 90	275 ± 30	3.7 ± 0.5
	M	7	242 ± 82	281 ± 35	4.0 ± 0.8
Zelčín	F	3	1008 ± 314	447 ± 59	7.0 ± 1.7
	M	7	413 ± 227	324 ± 52	4.3 ± 1.7
Verdek	F	1	615	320	5.0
	M	8	336 ± 138	267 ± 34	3.8 ± 0.7
Němčice	F	5	293 ± 308	401 ± 57	6.8 ± 0.8
	M	5	463 ± 323	326 ± 76	5.4 ± 1.7
Valy	F	3	435 ± 27	343 ± 6	4.0 ± 0.0
	M	7	264 ± 41	292 ± 11	3.1 ± 0.4
Lysá n. L.	F	1	245	255	4.0
	M	2	440 ± 389	310 ± 120	4.5 ± 2.1
Obříství	F	6	1045 ± 333	440 ± 62	8.0 ± 1.1
	M	2	435 ± 170	330 ± 42	4.5 ± 0.7
Děčín	F	2	703 ± 25	393 ± 11	6.5 ± 0.7
	M	8	476 ± 222	338 ± 53	5.1 ± 1.5
Hřensko	F	2	503 ± 244	350 ± 57	4.5 ± 0.7
	M	8	368 ± 108	317 ± 32	4.5 ± 1.1

n = number of fish examined, M ± SD = mean ± standard deviation

For the determination of alkylphenols, the fish sample was desiccated with anhydrous sodium sulphate. The powder material was transferred into an Erlenmayer flask. After addition of surrogate standards (4-*n*-nonylphenol and 4-*n*-octylphenol), repeated sonication was used for enhancement of extraction efficiency by *n*-hexane/dichlormethane (1:1, v/v). The combined extracts were dried over a layer of anhydrous sodium sulphate. Gel permeation chromatography on Bio-Beads S-X3 [mobile phase cyclohexane/ethyl acetate (1:1, v/v)] was used for separation of analytes from most matrix co-extracts. Sample extracts were

analysed by gas chromatography (HP 6890 gas chromatograph) employing mass selective detector HP 5973 operated in selected ion monitoring for data acquisition. Isotopically labelled d_8-4-*n*-nonylphenol was used as an internal standard for correction of matrix effects. Separation of sample components was carried out on a DB-5ms column (60 m × 0.25 mm × 0.25 μm). An aliquot of 1 μl of the purified extract was injected (pulsed splitless mode) at 250°C. The oven temperature was programmed from 60°C (2 min) to 180°C at 45°C.min^{-1}, to 209°C at 2°C.min^{-1} and to 290°C at 60°C.min^{-1}. The final temperature was maintained for 10 min. Helium was used as a carrier gas (1 ml.min^{-1} flow). The recovery obtained by repeated analyses (n = 6) of fortified fish samples was 85±12% for 4-*tert*-octylphenol (spiking level 1 ng.g^{-1} muscle) and 77±16% for 4-*tert*-nonylphenols (spiking level 10 ng.g^{-1} muscle). The limits of quantification (LOQs) were 0.2 ng.g^{-1} muscle and 2 ng.g^{-1} muscle for 4-*tert*-octylphenol and 4-*tert*-nonylphenol, respectively.

Persistent organochlorine pollutants (PCB indicator congeners - IUPAC numbers 28, 52, 101, 118, 138, 153 and 180; hexachlorobenzene; α-, β-, γ-isomers of hexachlorocyclohexane, octachlorostyrene - OCS; DDT and its degradation products DDE and DDD) were determined in pooled muscle samples by means of two-dimensional capillary gas chromatography (2D/HRGC) employing two parallel columns of equal dimension differing in selectivity (DB-5 and DB-17) and two electron capture detectors (ECD). Isolation of target analytes from fish muscle tissue was carried out by Soxhlet extraction into hexane:dichloromethane (1:1, v/v) solvent mixture. The clean-up of obtained extracts was performed, similarly to alkylphenols, by GPC on column Bio-Beads S-X3 and mobile phase ethylacetate:cyclohexane (1:1, v/v). The method is described in detail in our previous study (Hajšlová et al. 1995).

The determination of EROD enzyme activity was performed spectrofluorometrically (detection limit 2 pmol.mg^{-1}.min^{-1}). In the presence of NADPH, EROD enzyme activity converts the substrate ethoxyresorufin, which is a fluorescent product. Standard phosphate buffer, NADPH and suspension adequate for 0.2 mg.ml^{-1} protein were put in a cell. Ethoxyresorufin was then added and the increase in fluorescence was recorded for 5 min (excitation/emission wavelengths setting was 535/585nm). EROD activity was subsequently calculated based on a comparision with fluorescence of the standard (resorufin) of known concentration (Široká et al. 2005).

The determination of total mercury content in fish tissues was performed by means of cold vapour atomic absorption spectrometry on AMA-254 (Altec Ltd.,Czech Republic) single-purpose mercury analyser (detection limit 0.001 mg.kg^{-1}; recovery 82 ± 6 %). Accuracy of the results was validated using standard reference material BCR-CRM 463 (Tuna fish).

The data obtained did not meet the requirements of parametric statistical tests (normality etc.) and so these data were analysed non-parametrically. The descriptive statistics were expressed as the median and percentiles.

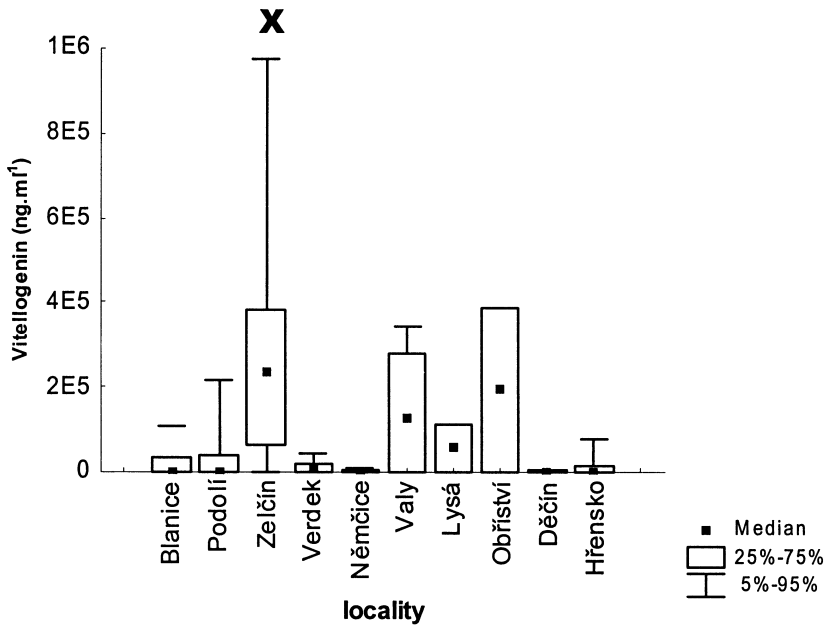

Figure 1. Comparison of vitellogenin concentrations in blood plasma (ng.ml^{-1}) of chub males (*Leuciscus cephalus* L.) from the monitored sites.
[x – significantly different from control locality (Blanice)]

The results were subjected to a Mann-Whitney test to analyse regional differences in the parameters measured. Significance was accepted when $p<0.05$. The Spearman rank correlation coefficient was used for analysis of relationship between analysed parameters. The analyses were performed in Statistica for Windows 7.1 (Statsoft Inc., 2005).

RESULTS AND DISCUSSION

Along the Elbe river and its tributaries significant differences in environmental pollution of the studied sites were observed. Fish from Obříství showed the worst health status. Fins of most specimens examined had uneven edges with mechanically damaged surface. All chubs exhibited large alterations on the skin (open lesions and abscesses) and on the eyes (exophthalmus and large keratoleukomas).

The highest vitellogenin levels in blood plasma of chub males were found at Zelčín and Valy (Fig. 1). The lowest value of 11-ketotestosterone content (Fig. 2) was found at Zelčín. The loads at Obříství and Lysá nad Labem could not be determined unequivocally due to insufficient number of males caught. A significant negative correlation (r^2= -0.28, p = 0.028) was found between the concentration of vitellogenin and 11-ketotestosterone in blood plasma of males, assessed as a total sample from all localities.

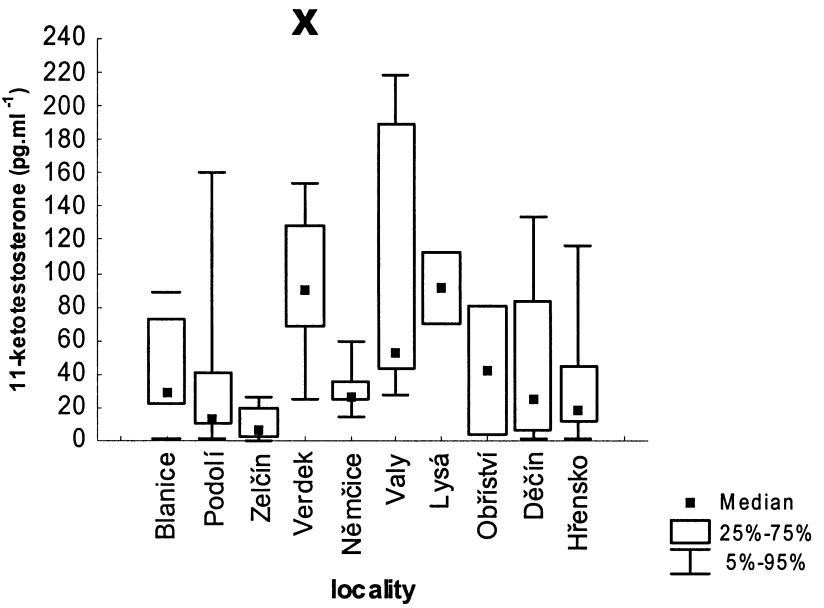

Figure 2. Comparison of 11-ketotestosterone concentrations in blood plasma ($pg.ml^{-1}$) of chub males (*Leuciscus cephalus* L.) from the monitored sites.
[x – significantly different from control locality (Blanice)]

This negative correlation of the concentrations of vitellogenin and 11-ketotestosterone in blood plasma of males confirmed results of laboratory studies reporting that exposure of fish to various substances with effects of endocrine disruptors decreased the concentration of sex steroid hormones, thus also of 11 – ketotestosterone (Mills et al. 2001). It could be concluded from the presented results that the highest load of endocrine disruptors was at Zelčín. No significant relations were found between biomarker values (VTG, 11-KT) and the pollutants analysed.

The highest EROD values were found at Zelčín, Valy, Obříství and Lysá (Fig. 3). The EROD level in fish from the control locality was significantly lower than those from all other localities studied. It can be therefore stated that all localities studied on the Elbe and Vltava rivers showed presence of substances causing the activation of EROD. Results of chemical monitoring also showed that localities with the highest EROD value were also the most contaminated with xenobiotics, mainly with persistent organochlorinated pollutants (Table 2).

The highest findings of 1-OHPY were at Zelčín (1117 ± 1044.0 $ng.ml^{-1}$ bile) and Děčín (1125 ± 782.2 $ng.ml^{-1}$ bile). Increased values were also detected at the control locality (817 ± 247.0 $ng.ml^{-1}$ bile). Average concentration of 1-OHPY in bile of chub from all other monitored sites ranged from 357 to 649 $ng.ml^{-1}$ bile. Particularly Prague and its surroundings may be considered as an important source of contamination of the aquatic environment with PAHs.

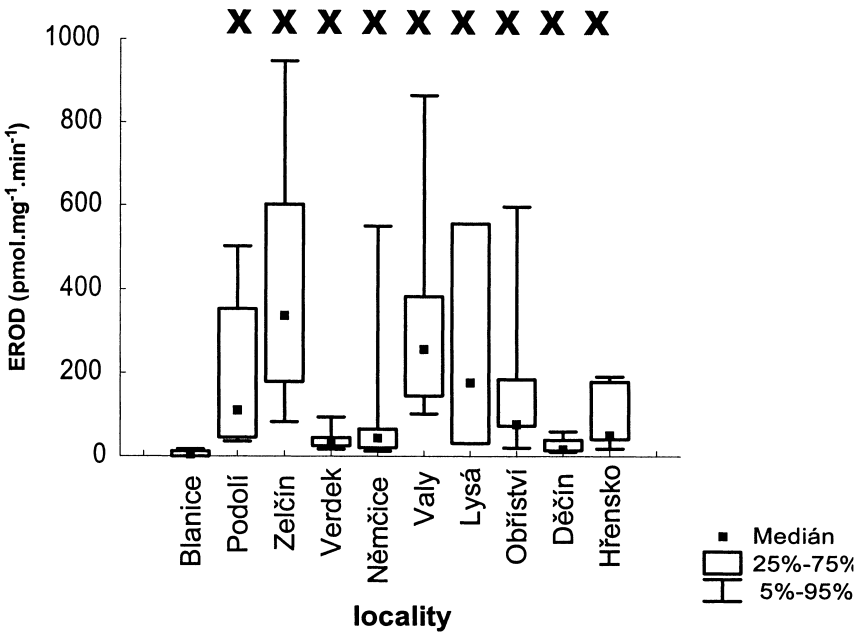

Figure 3. Comparison of (EROD) ethoxyresorufin-O-deethylase activity in liver samples (pmol.mg^{-1}.min^{-1}) of chub (*Leuciscus cephalus* L.) from the monitored sites. *[x – significantly different from control locality (Blanice)]*

The highest values of mercury in fish muscle were found at Obříství (1.631 ± 0.781 mg.kg^{-1} wet weight) and Lysá (0.920 ± 0.663 mg.kg^{-1} w.w.).

Average concentration of mercury in muscle of chub from all other monitored sites (including control site) ranged from 0.141 to 0.335 mg.kg^{-1} w.w. The highest contamination by PCB was detected in indicator fish caught at Němčice, Obříství, Zelčín, Valy and Lysá (Table 2). The PCB contamination in muscle of chub was one order of magnitude higher at all localities studied on Elbe and Vltava rivers than at the control locality on Blanice river. In case of HCB, the highest extent of contamination was found at Děčín, Hřensko, Obříství and Valy. Similarly, the concentration of this pollutant in muscle of chub was higher at all localities studied on the Elbe and Vltava rivers than at the control locality. The most HCH - contaminated locality was Obříství. Concentrations of this pollutant in indicator fish at other localities were quite similar. Rather surprisingly, the contamination of chub from the control locality differed little (except for Obříství) from fish caught at other sampling sites. Regarding DDT, the highest value was found at Obříství. The p,p-DDE, its persistent metabolite, was dominant at all localities. Relatively high values of p,p-DDE were found at the control locality as well. Considering relatively high levels of octachlorostyrene (OCS) in fish from Valy, the specific pollution source apparently exists in this locality. Its negative impact on the environment was demonstrated by increased contamination of fish from localities situated in the longitudinal profile of the Elbe river downstream of Valy.

The study concerned with the occurrence of alkylphenols in aquatic biota represented by fish was the very first one conducted in the Czech republic. The highest average values of alkylphenols studied (sum of 4-*tert*-nonylphenol and 4-*tert*-oktylphenol) were registered at Valy (2.94 ± 0.83 $\mu g.kg^{-1}$ w.w.), Obříství (2.93 ± 0.73 $\mu g.kg^{-1}$ w.w.) and Zelčín (2.77 ± 0.85 $\mu g.kg^{-1}$ w.w.). Values of alkylphenols registered in fish in the Czech Republic were comparable with levels in fish tissues from the Kalamazoo River, Michigan (Kannan et al. 2003).

Table 2. Content of persistent organic pollutants in chub (*Leuciscus cephalus* L.), pooled samples ($mg.kg^{-1}$ of lipids).

Locality	Lipid content (%)	PCB*	HCH**	DDT***	HCB	OCS
Blanice	1.6	0.20	0.042	1.63	0.040	0.001
Podolí	1.6	4.36	0.025	3.17	0.100	0.007
Zelčín	2.0	6.83	0.014	3.03	0.117	0.011
Verdek	2.0	1.87	0.010	1.38	0.105	0.004
Němčice	1.8	6.15	0.030	4.54	0.154	0.023
Valy	1.2	4.84	0.023	2.45	0.290	0.414
Lysá	0.7	9.65	0.038	5.20	0.209	0.135
Obříství	1.9	8.76	0.442	8.3	0.374	0.044
Děčín	5.0	2.17	0.048	1.48	0.317	0.097
Hřensko	2.1	2.72	0.060	2.76	0.576	0.116

* sum of 7 indicator congeners (28, 52, 101, 118, 138, 153, 180)
** sum of HCH isomers (α, β, γ)
*** sum of DDT (*o,p´*-DDE; *p,p´*-DDE; *o,p´*-DDD; *p,p´*-DDD; *o,p´*- DDT; *p,p´*-DDT)

The results presented in this study indicate the highest contamination of localities downstream from the major chemical factories (Obříství, Valy) and agglomerations (Zelčín). The highest values of vitellogenin content and the lowest ones of 11-ketotestosterone were determined in blood plasma of chub males from Zelčín. This provides evidence of a strong contamination of this locality with EDCs. Increased values of biomarkers mostly corresponded with increased values of pollutants monitored though significant relationships were not found. The EROD parameter appears to be a parameter with good reporting value for the assessment of aquatic environmental contamination. Biomarkers studied provide a sensitive, physiologically based method of determining contaminant presence and bioavailability to aquatic organisms in field studies. Chub (*Leuciscus cephalus*) proved to be a good indicator organism for contamination assessment of running waters.

Acknowledgments. This study was supported by the USB RIFCH no. MSM6007665809 and the Ministry of the Environment CR VaV/650/5/03.

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