Biochemical markers for the assessment of pollution of selected small streams in the Czech Republic

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Abstract

OBJECTIVES: The objective of the presented study was to investigate levels of the most important biochemical markers in fish which were caught upstream and downstream from sites near waste water treatment plants situated at 3 selected small streams of the Czech Republic. Organic pollutants and mercury were determined in muscle tissue of fish for complex assessment.
DESIGN: Levels of biochemical markers (cytochrome P450 (CYP450), ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase (GST), vitellogenin (VTG)) were measured in selected tissues (liver, plasma, brain, gill and kidney) samples of brown trout. The concentrations of persistent organic pollutants (POPs) and mercury were determined in muscle samples.
RESULTS: Significantly higher levels of biochemical markers (EROD, GST, VTG) were measured downstream from waste water treatment plant (WWTP) in Vlachovo Březí (the small Libotýňský brook). Values of biochemical markers at the other localities Černý brook and Moravice River were measured downstream from WWTP consistently or slightly lower with values of upstream. The values of POPs and mercury were in all monitored sites higher upstream compared with downstream from WWTP. The highest values of POPs and mercury were found on Černý brook in Bruntál (more industrial and agricultural activity).
CONCLUSION: The highest occurrence of selected POPs and mercury was generally upstream suggesting that small WWTP are able to partially remove these substances in those locations. But finding higher values of biochemical markers in downstream shows that there are many other substances endocrine disrupting chemicals (EDCs) that WWTP are unable to remove.

ORIGINAL ARTICLE

Abbreviations:

CYP450	- Cytochrome P450
DDT	- Dichlordifenyltrichlorethan
EDCs	- Endocrine disrupting chemicals
EROD	- Ethoxyresorufin-O-deethylase
GST	- Glutathione S-transferase
HCB	- Hexachlorobenzene
HCH	- Hexachlorocyclohexane
PCBs	- Polychlorinated biphenyls
POPs	- Persistent organic pollutants
SEM	- Standard error of the mean
VTG	- Vitellogenin
w.w.	- Wet weight
WWTP	- Waste water treatment plant

INTRODUCTION

The aquatic environment is contaminated with a lot of types of organic and non-organic pollutants including agricultural, industrial or domestic pollutants (Bernet *et al.* 1999; Fernandes *et al.* 2008; Van Ael *et al.* 2012). Contaminants have negative effects on the flora and fauna of affected localities, resulting in the intake and accumulation of those toxic compounds in food chains. They can interfere with the endocrine system of aquatic organisms (Kohler *et al.* 2007; Randak *et al.* 2008) and cause severe health problems or genetic defects not only in humans but in general in all non-target organisms (Han *et al.* 2013).

Fish can be used as one of the most significant indicator species in freshwater systems (Lana *et al.* 2010; Adedeji *et al.* 2012; Lasheen *et al.* 2012). They have been used for the assessment of the quality of aquatic ecosystems for several reasons. Fish play increasingly important role in the monitoring of water pollution because they respond to changes in the aquatic environment very well (Siroka & Drastichova 2004). They produce many eggs and sperm and the effects of potential endocrine disruptors can be easily examined in both sexes (Kime *et al.* 1999). Muscle of fish is commonly analysed to determine contaminant concentrations for assessment of the health risks because it is consumed by humans (Lasheen *et al.* 2012).

Biochemical markers were defined as measurable responses at the molecular, cellular, physiological or behavioural levels to assess the importance of the pollution (Van der Oost *et al.* 1996; Siroka & Drastichova 2004; Mayon *et al.* 2006; Modra *et al.* 2008; Havelkova *et al.* 2009). The biomarker approach is used for *in vivo* and *in vitro* studies to evaluate the combined effects of many thousands of xenobiotics in the environment such as polychlorinated biphenyls (PCBs), organochlorine pesticides, polycyclic aromatic hydrocarbons, phthalates, styrenes, alkylphenols, mercury and others (Flammarion *et al.* 2002; Van der Oost *et al.* 2003; Shirani *et al.* 2012).

Detoxification enzymes (phase I and II) in fish have been widely used in field of biochemical monitoring studies as well as in experimental studies (Mayon *et al.* 2006; Haluzova *et al.* 2010; Mikulikova *et al.* 2011; Hostovsky *et al.* 2012). Majority of xenobiotics is metabolized by oxidative reactions in phase I of biotransformation which are catalysed by the cytochrome P450 (CYP450) (Shirani *et al.* 2012). The most important indicator of aquatic environment contamination seems to be subfamily 1A of CYP450 (Siroka & Drastichova 2004) with the consequent effector enzyme ethoxyresorufin-*O*-deethylase (EROD) activity which is considered the most reliable used enzyme marker (Shirani *et al.* 2012) for the assessment of organic pollutants´ impact on fish health status (Gagnon & Hodson 2012). The conjugation of endogenous and exogenous substrates with several highly hydrophilic compounds is catalyzed by enzymes of detoxification phase II. In phase II, enzyme glutathione *S*-transferase (GST) and tripeptide glutathione in its reduced form are the most important liver biomarkers which are commonly assessed during environmental monitoring (Jedamski-Grymlas at el. 1995; Havelkova *et al.* 2008; Sole *et al.* 2009). Phospholipoglycoprotein vitellogenin (VTG) is an important biochemical marker for the evaluation of aquatic environment contamination by substances having estrogenic effects (Kelly *et al.* 2010). The presence of VTG in blood plasma of females plays a key role in the reproduction of oviparous fishes and its changed levels or its occurrence in males indicates the presence of endocrine disrupting chemicals (EDCs) in the aquatic environment (Penaz *et al.* 2005; Pettersson *et al.* 2006; Blahova *et al.* 2009; Hinfray *et al.* 2010).

This study aimed at the use of selected biochemical markers (VTG, CYP450, EROD, GST) in fish to assess the contamination levels of selected small streams of the Czech Republic. The concentrations of persistent organic pollutants (PCBs, DDT and their metabolites, hexachlorobenzene (HCB), hexachlorocyclohexane (HCH)) and mercury were measured in muscle samples as the chemical evidence of the fish exposure. Brown trout was selected as the environmental indicator species to assess the potential risk in the Czech waters.

MATERIAL AND METHODS

Study area

Monitoring of aquatic contamination was performed at three selected sites, which are situated on small streams in the Czech Republic. One in the south Bohemia (Libotýňský brook) and two in the north Moravia (Černý brook and Moravice River). The localities studied on the Libotýňský brook (flow length 13.5 km, river kilometres 7.5–8.5 and the flow rate $0.066\,m^3.s^{-1}$) are upstream and downstream from waste water treatment plant (WWTP) in Vlachovo Březí with approximately 1,700 inhabitants. The localities studied on the Černý brook (flow length 24.3 km, river kilometres 4.0–7.0 km and the flow rate $0.88\,m^3.s^{-1}$) are upstream and downstream from WWTP in Bruntál which is the city with 17,000 inhabitants and the localities studied on the Moravice River (flow length 100.5 km, river kilometres 75.5–78 km and the flow rate $3.45\,m^3.s^{-1}$) are upstream and downstream from WWTP in community Břidličná with approximately 3,500 inhabitants. Map of the Czech Republic and locations of sampling sites is presented in Figure 1.

Sample collection

Brown trout (*Salmo trutta m. fario*) was selected as the most suitable indicator species, due to its relative abundance at the sampling sites as well as being sensitive bioindicators of freshwater pollution. At each location, twelve to twenty fish were caught by electrofishing in September 2012. Immediately after capture, the blood samples were taken from the caudal vein using heparinized syringe. The blood samples were centrifuged (800 × g, 10 min, 4 °C) and plasma samples were stored at −85 °C until the analysis of VTG. Immediately after the blood collection, the fish were killed, weighed and measured. Sex was determined macroscopically. The liver tissue was quickly dissected, put in Eppendorf tubes and stored at −85 °C for later analyses of CYP450, EROD and GST. Activity of GST was determined also in samples of brain, gill and kidney. Individual muscle samples for later analyses of pollutants were placed in polyethylene bags, labelled and stored at −20 °C.

Biochemical analyses. Plasma VTG concentration was measured using commercial enzyme-linked immunosorbent assay kit. Total content of CYP450 was determined by visible light spectrophotometry (390 to 490 nm) and activity of EROD was measured by spectrofluorometry (excitation: 535 nm, emission: 585 nm) (Siroka *et al.* 2005). The activity of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione (Habig *et al.* 1974).

Chemical analyses

Levels of selected persistent organic pollutants were determined in samples of muscle. The analyses were carried out in mixed samples. A 50 g of muscle tissue was homogenized and extracted into diethyl ether. The extract was dried using anhydrous sodium sulphate and evaporated with a rotary vacuum evaporator. An aliquot of obtained lipid (0.2±0.05 g) was dissolved in n-hexane and cleaned up on Florisil packed column. The cleaned solution was concentrated by rotary vacuum evaporator and 2 ml were used for analysis. Hexachlorobenzene; α-, β-, γ-, δ-isomers of HCH; PCBs (indicator congeners – IUPAC number 28, 52, 101, 118, 138, 153, 180); DDT, and its degradation products DDE and DDD, assessments were carried out by Varian 450-GC gas chromatograph with a Varian 220-MS ion trap mass spectrometer and VF-5ms (30 m×0.25 mm) column (Varian, Inc. USA) (Magnusson *et al.* 2006). Mercury concentrations in individual muscle tissue were determined by the direct method of cold vapor atomic absorption spectrometry on an Advanced Mercury Analyzer 254 (Altec).

Statistical analysis

Statistical analysis was performed using Unistat 5.6. software. At first, data were tested for normal distribution using Shapiro-Wilk test. Data of CYP450 and EROD were logtransformed to improve the normal distribution. One-way analysis of variance was applied to the differences of biochemical markers among sampling locations. Individual differences among the means were tested using Tukey-HSD test. Since the non-normal distribution of VTG was identified, non parametric Kruskal-Wallis test, followed by multiple comparison, was used for determination differences among localities. Significance was accepted at $p<0.05$.

RESULTS

Biometric parameters

The main characteristics of fish captured at the individual localities are summarized in Table 1. The highest

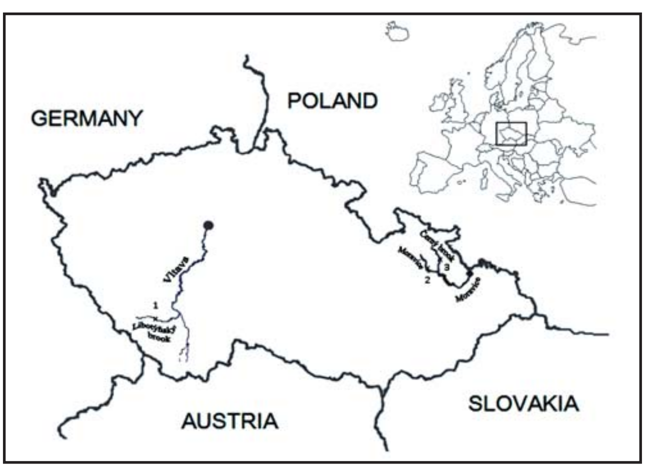

Fig. 1. Map of the Czech Republic and locations of sampling sites (1. Vlachovo Březí (Libotýňský brook), 2. Břidličná (Moravice River), 3. Bruntál (Černý brook)).

Tab. 1. Characteristics of fish captured at the individual localities.

Locality	Fish (n)	Total lenght (mean±SEM) (cm)	Weight (mean±SEM) (g)
Libotýňský brook			
Vlachovo Březí – upstream	20	19.0±0.6	69.2±7.8
Vlachovo Březí – downstream	20	16.1±4.4	50.3±8.3
Černý brook			
Bruntál – upstream	14	26.65±0.5	162.7±10.3
Bruntál – downstream	14	21.4±0.7	93.5±10.7
Moravice River			
Břidličná – upstream	12	22.9±0.7	77.2±10.2
Břidličná – downstream	13	19.2±0.7	131.2±16.0

SEM – standard error of mean

average body weight and total length was observed in the locality of Černý brook – especially upstream from Bruntál and the lowest in the locality Libotýňský brook – especially downstream from Vlachovo Březí.

Tab. 2. Content of vitellogenin in positive samples of blood plasma. Significant differences ($p<0.05$) are indicated by alphabetic superscript.

Locality	Number of samples / positive samples	Range of detected concentrations (ng.mL^{-1})
Vlachovo Březí – upstream	10/2	584.1–1520.6[a]
Vlachovo Březí – downstream	7/5	5.4–2978.1[a]
Bruntál – upstream	8/4	362.4–2882.9[a]
Bruntál – downstream	12/5	2.8–2304.4[a]
Břidličná – upstream	10/2	69.1–449.4[a]
Břidličná – downstream	11/1	10.2[*a]

* only one possitive sample

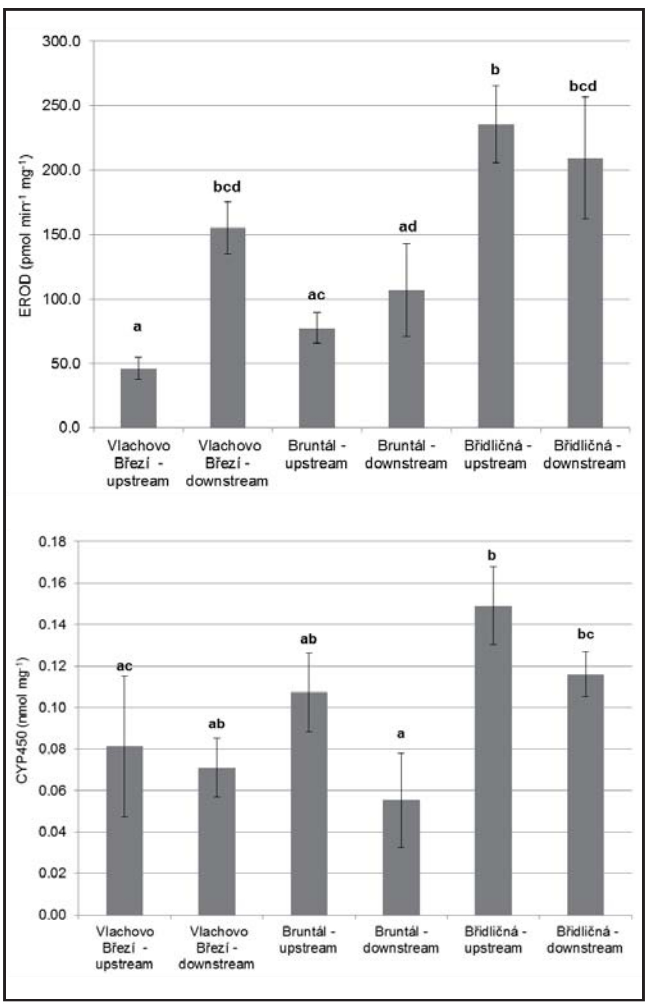

Fig. 2. Results of detoxifying enzymes activities of the I. phase detoxification in the liver tissue (mean ± standard error of mean). Significant differences ($p<0.05$) are indicated by alphabetic superscript.

Biochemical analyses

Brown trout plasma VTG concentration is summarized in Table 2. Vitellogenin was only detected in some of the samples; in the cases where VTG was not detected, half of the detection limit (0.5 ng.ml^{-1}) was applied for statistical analysis and there is also demonstrated how many positive samples were found among the samples analyzed. The highest VTG concentration (2978.1 ng.ml^{-1}) and the biggest percentage of positive samples were found on Libotýňský brook – especially downstream from Vlachovo Březí. Vitellogenin levels did not differ significantly among between locations.

The results of EROD activity and CYP450 content in brown trout liver are presented in Figure 2. The highest average of EROD activity was found in liver samples on Moravice River – especially upstream from Břidličná (235.7±30.0 nmol.min^{-1}.mg^{-1} protein), and the lowest average value on Libotýňský brook – especially upstream from Vlachovo Březí (46.2±8.6 nmol.min^{-1}.mg^{-1} protein). Significant differences ($p<0.05$) within one locality were found between localities situated upstream and downstream from Vlachovo Březí. The highest average of CYP450 was obtained on Moravice River – especially upstream from Břidličná (0.15±0.02 nmol.mg^{-1} protein), and the lowest on Černý brook – especially downstream from Bruntál (0.06±0.02 nmol.mg^{-1} protein). Content of CYP450 did not differ significantly among locations within one city.

The results of GST acitivities in different tissues are shown in Table 3. Significant differences were also found in other organs, but the most significant are results in liver. In the other organs were found the same or similar trend as in the liver.

Chemical analyses

Results of organic pollutants content in muscle tissues are presented in Table 4. Concentrations of PCB ranged from 7.0 to 57.4 µg.kg^{-1} w.w. Concentrations of DDT ranged from 14.5 to 107.7 µg.kg^{-1} w.w. and in the analysis of DDT, its metabolite *p,p´*-DDE exhibited the highest concentration of all monitored metabolites at all locations. The highest level of DDT and its metabolites was 107.7 µg.kg^{-1} w.w. and this value was seven times higher than those obtained from the location with the lowest level of the monitored pollutant (on Moravice River – especially downstream from Břidličná). Concentrations of HCH and HCB ranged from 0.1 to 2.6 µg.kg^{-1} w.w. and the highest levels of HCB and HCH concentrations were found on Černý brook – especially upstream from Bruntál (2.3 and 2.6 µg.kg^{-1} w.w., respectively). The lowest levels of HCB and HCH concentrations were on Libotýňský brook – especially downstream from Vlachovo Březí (0.1 and 0.1 µg.kg^{-1} w.w., respectively). The analysis of HCH isomers showed that isomers of β and δ were represented the most abundantly. The obtained results show that the highest levels of all pollutants were detected on Černý brook – especially upstream from Bruntál. The most abundantly represented PCB

Tab. 3. The results of GST activities (nmol.min^{-1}.mg^{-1}) in different tissues (mean ± standard error of mean). Significant differences (p<0.05) are indicated by alphabetic superscript.

Locality	liver	kidney	gill	brain
Vlachovo Březí – upstream	915.0±47.3^{b}	385.2±37.6^{a}	631.0±32.8^{a}	310.4±21.4^{ab}
Vlachovo Březí – downstream	1173.4±56.8^{c}	503.3±39.0^{ac}	917.1±63.3^{bc}	298.1±22.4^{ab}
Bruntál – upstream	1025.1±46.8^{bc}	569.8±35.8^{bc}	685.1±43.7^{a}	367.2±20.0^{b}
Bruntál – downstream	624.6±39.1^{a}	477.6±56.1^{ac}	696.2±45.5^{a}	225.3±23.6^{a}
Břidličná – upstream	1159.5±108.6^{bc}	759.8±53.8^{d}	1057.5±59.5^{b}	346.7±21.5^{b}
Břidličná – downstream	931.3±141.8^{bc}	792.0±24.0^{d}	757.2±39.7^{ac}	331.0±9.7^{b}

Tab. 4. Average contents of persistent organic pollutants in muscle tissue at monitored locations.

Locality	Σ PCBs (μg.kg^{-1} w.w.)	Σ HCH (μg.kg^{-1} w.w.)	Σ HCB (μg.kg^{-1} w.w.)	Σ DDT (μg.kg^{-1} w.w.)
Vlachovo Březí – upstream	7.29	0.35	0.21	30.53
Vlachovo Březí – downstream	7.01	0.05	0.13	31.15
Bruntál – upstream	57.36	2.59	2.33	107.70
Bruntál – downstream	20.30	0.72	0.85	32.94
Břidličná – upstream	22.24	1.72	1.12	69.89
Břidličná – downstream	7.65	0.20	0.86	14.47

PCB – sum of congeners; HCH – sum of isomers; HCB; DDT and its metabolites

congeners 28 and 153 were evaluated as dominant PCB congeners.

The results of total mercury content in muscle tissue of brown trout are presented in Figure 3. The highest average concentration of mercury was found on Černý brook – especially upstream of Bruntál (0.14±0.02 mg.kg^{-1}) and the lowest average of detected concentrations was observed on Moravice River – especially downstream from Břidličná (0.07±0.004 mg.kg^{-1}). Significant differences (p<0.05) were found between localities situated upstream and downstream from Bruntál. Mercury content in Bruntál was significantly different from all other locations.

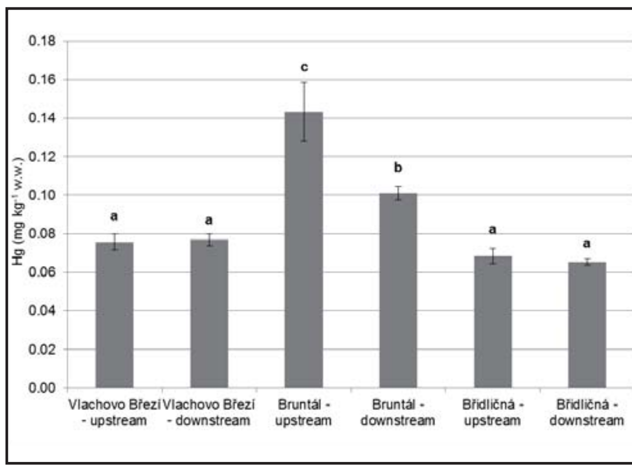

Fig. 3. Results of total mercury content in muscle tissue (mean ± standard error of mean). Significant differences (p<0.05) are indicated by alphabetic superscript.

DISCUSSION

Methods of chemical and biochemical monitoring are one of the best potential posibility for assessing of environmental contaminants. An environmental species for the assessment of pollution are used fish. Brown trout are being generally used on the upper reaches and a chub is used in the lower reaches (Kolarova *et al.* 2005; Randak *et al.* 2008).

Our monitoring has shown that WWTP improves the situation in the case of POPs and the situation in the case of mercury is the same or improves. The results of POPs in fish muscle show that the highest levels of organic pollutants and mercury were detected in fish from Bruntál – Černý brook, especially upstream WWTP. In most sites, the most abundantly represented PCB congeners 28 and 153 were evaluated as dominant PCB congeners. In the analysis of HCH isomers, isomers of β and δ were represented the most abundantly. In the analysis of DDT, *p,p´*-DDE exhibited the highest concentration of all monitored metabolites at all locations. Lana *et al.* (2010) demonstrated the determination of PCB congeners in muscle of chub caught on the Svratka River (Czech Republic) upstream from Modřice and downstream from Rajhradice. They found that there were no significant differences between both sampling localities ($p > 0.05$). Dominating congeners of PCB were 138, 153 and 180, the concentration of HCHs were very low with dominating β and γ isomers in their study. The study by Havelkova *et al.* (2007) documented that *p,p´*-DDE showed the highest concentration of the total DDT amount in the muscle of chub (*Leuciscus cephalus* L.) caught in the rivers of the Czech Republic. It may be associated with the use in agriculture and the persistence of this substance. Leaños-Castañeda *et al.* (2007) documented that *o,p´*-DDT may induce disruption of the endocrine system. Endocrine disrupting chemicals were investigated worldwide and have been identified as eligible substances that are not actually included in routine monitoring programmes at the European level (Kelly *et al.* 2010). Dusek *et al.* (2005) found that a significant part of the pollution is obtained from closely located towns, industrial and agricultural pollutants are the most important contributors and considering the overall situation in the Czech Republic. One reason of the high occurrence of pollutants in our study is up to 5 times the number of people living in this area and the associated intensive industrial and agricultural activities, as well as domestic waste water.

On the other hand, WWTP was on the downgrade in the case of biochemical markers (EROD, VTG and GST). Libotýňský brook is a small stream that has a small tributary of water. The increased number of positive samples of VTG in fish, the increased activity of EROD and GST indicated the presence of substances with estrogenic effects and may be caused by the degradation products of surfactants, musk compounds, contraceptives or pharmaceuticals (Siroka & Drastichova 2004; Celander 2011). The high incidence of positive samples VTG and high EROD activity were found downstream from Vlachovo Březí, Bruntál and Břidličná are similar. Kelly *et al.* (2010) detected VTG in male brown trout at 8 of the 11 Shannon International River with significant levels. The higher percentage of positive samples of fish was detected also from downstream sampling sites. The study by Penaz *et al.* (2005) showed that VTG concentration in a barbel, *Barbus barbus* were significantly lower on Jihlava River upstream from Třebíč than downstream. There were present intersex speciments (upstream 2.0%, downstream 14.8%). Blahova *et al.* (2009) found that EROD activity increased at downstream from Svratka River.

Organic pollutants does not provide adequate monitoring of environmental contamination while biochemical markers include the effect of all present substances. Havelkova *et al.* (2008) showed that the highest values of CYP450 content and EROD activity in the liver of brown trout caught in the Tichá Orlice River, VTG concentration in the plasma of brown trout and chemical pollutants in muscle of brown trout were determined at Králíky site. However, several studies report that determination of the total cytochrome P450 is not too specific to fish which was confirmed in our study (Siroka & Drastichova 2004). Kolarova *et al.* (2005) recorded high VTG concentration in plasma of brown trout also on the polluted Králíky site which resulted in significant differences in comparison with the control site Červená voda and also the site downstream of the source of pollution in Lichkov. That may confirm the requirement that problems with reproduction of fish in the Kraliky site are result of the surface water loading by endocrine disruptors.

The highest occurrence of selected POPs and mercury in our study was generally always upstream suggesting although small WWTP are able to partially remove these substances in those locations. For the evaluation of the total effect of the monitored sites it is necessary to use the identification of biochemical markers – especially EROD, VTG and GST.

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