

Organic Pollutant Contamination of the River Tichá Orlice as Assessed by Biochemical Markers

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Abstract

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This study used biochemical markers to assess contamination at two contaminated sites (Králíky and Lichkov) and one control site (Červená Voda) on the River Tichá Orlice, a left-side tributary of the River Elbe. The brown trout (*Salmo trutta fario*) was selected as an indicator species. Enzymes of the first stage of xenobiotic conversion, namely cytochrome P450 (CYP 450) and ethoxyresorufin-O-deethylase (EROD) in the liver were selected as biochemical markers. Blood plasma vitellogenin concentrations were used to evaluate xenoestrogenic effects of contamination. Results were compared with the most important inducers of these markers, i.e. with organic pollutants (PCB, HCH, HCB, OCS and DDT and their metabolites in fish muscle and with PAH concentrations in bottom sediments). The highest contamination with organic pollutants was at Králíky, and this was reflected in increased cytochrome P450, EROD activity and vitellogenin concentrations. Significant differences were demonstrated in EROD activity and vitellogenin concentrations between Králíky and Červená Voda ($P < 0.001$). At the most contaminated site (Králíky), a significant negative correlation ($r_s = -0.964$) between EROD activity and vitellogenin concentrations was demonstrated. This relationship was discussed from the point of view of a possible induction or inhibition of the assessed biomarkers at persistently highly contaminated sites.

Cytochrome P450, EROD, vitellogenin, Salmo trutta fario, PCB, river contamination

The River Tichá Orlice (Czech Republic) is a tributary of the River Elbe. The River Tichá Orlice is 107.5 km long and its basin covers 755.4 km². Broodstock of brown trout (*Salmo trutta fario*) and European grayling (*Thymallus thymallus*) from the upper reaches of the river and its tributary, the Kralický brook, have been used for artificial reproduction. Fish reproductive problems were detected for the first time in the early 1990s, including low effectiveness of spawning. The results of reproduction were not satisfactory and high losses of spawners were noticed. Monitoring the River Tichá Orlice and its tributary Kralický Brook has been proceeding since 1989. The presence of organic pollutants (PCB; DDT and its metabolites, HCB and HCH) and heavy metals in brown trout muscle have been investigated (Kredl et al. 1989; Svobodová et al. 2004; Kolářová et al. 2005). The Kralický brook has been an important source of organic pollutants (Kolářová et al. 2005) and heavy metals (mercury and copper) released by “Tesla Králíky” which manufactures electronics and is located on the Kralický brook (Svobodová et al. 2004).

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To assess the importance of the pollution, it was desirable to evaluate the impact of the pollutants contaminating the aquatic environment on fish. One possible approach was to use biochemical markers, biochemical indicators responding to substances with a similar mode of toxic action.

Cytochrome P450 is an important biochemical marker and an indicator of aquatic contamination by industrial and agricultural pollutants. These pollutants induce the expression of CYP1A (CYP1A is the most important indicator of aquatic environmental contamination) through a ligand that binds to the aryl hydrocarbon receptor (AhR) (Billiard et al. 2002). Following its interaction with xenobiotic substances, it is carried to the nucleus where it causes enhanced expression of genes for CYP1A and, subsequently, an increasing synthesis of cytochrome proteins. Its most important inducers include the so-called persistent organic pollutants (POPs) i.e. polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), nitrated polycyclic aromatic hydrocarbons (NPAH) and polychlorinated dibenzo-*p*-dioxins and dibenzo-*p*-furans (PCDD/Fs) (White et al. 1997; Van der Oost et al. 2003; Šíroková and Drastichová 2004). These substances accumulate in large quantities in river bottom sediments (Malins et al. 1984), and from there they transfer to aquatic organisms.

Functionally linked to CYP 450 is another enzyme, ethoxyresorufin-O-deethylase (EROD). This enzyme is believed to be catalyzed principally by CYP1A1 in fish (Klotz et al. 1983). EROD is more sensitive for contamination monitoring of aquatic environment than CYP 450.

Vitellogenin is an important biochemical marker for the assessment of ground water contamination by substances exhibiting xenoestrogenic effects (Sumpter and Jobling 1995). The primary reason for monitoring vitellogenin concentrations in trout was the discovery of reproductive disturbances of fish. It has been known that some environmentally persistent man-made chemicals can act as weak estrogens. A further finding is that a broad range of widely-used chemicals, and sometimes their major degradation products, can act as weak estrogens (Soto et al. 1991; White et al. 1994).

The aim of the study presented here was to use the assessment of biochemical markers (CYP 450, EROD and vitellogenin) in the liver of the indicator fish species (*Salmo trutta fario*) to evaluate contamination levels at selected sites along the Tichá Orlice, and its tributary the Kralický brook. Results of biochemical monitoring were correlated to results of chemical monitoring (POPs).

Materials and Methods

Animals and Sampling

Fish were examined from each of the three sites along the river Tichá Orlice (Červená Voda, Králíky and Lichkov). The main characteristics of fish captured in individual locations are summarized in Table 1. The sites of Červená Voda, Králíky and Lichkov are 103, 100 and 93 km, respectively, east of the junction of the Tichá Orlice with the Elbe (Fig. 1). Individual sites were separated by cross barriers. Červená Voda was chosen as a control site because it showed minimum loads for almost all indicators (Kolářová et al. 2005). Samples of muscle, liver, and blood from male brown trout (*Salmo trutta fario*) were obtained in June 2003. Bottom sediment samples were collected at the same time. Separate blood and liver samples were collected and processed for each fish, while individual muscle samples were pooled on site to create a combined sample for chemical analysis. Tissue and blood plasma samples were immersed in liquid nitrogen immediately after collection and taken to the laboratory, where they were stored at -80 °C until they were analyzed.

Liver sample processing

Liver samples were homogenized in buffer (pH = 7.4), poured into centrifugation tubes and centrifuged at 10 000 g for 20 min at 4 °C. The supernatant was carefully pipetted into ultracentrifugation tubes and centrifuged again at 100 000 g for 1 h at 4 °C. The supernatant was drained; pellets were washed with buffer and re-suspended in buffer. The suspension was put into separate Eppendorf tubes and stored in a freezer at -80 °C for later enzymatic assay. Microsomal protein concentrations were determined by the method of Lowry (Lowry et al. 1951) before assay.

Table 1. The main characteristics of sampled brown trout males (*Salmo trutta fario*) population

Locality	N	Weight (g) (mean \pm SD)	Mean age (years) (min-max)
Červená Voda	7	91 \pm 12	2.1 (2-3)
Králíky	8	118 \pm 66	2.4 (2-4)
Lichkov	6	119 \pm 43	2.5 (2-4)

dithionite and after the complex with CO was formed. The method is described in the study of Šíroková et al. (2005). Repeatability of cytochrome P450 could not be performed because of the small amount of samples.

Quantitative determination of cytochrome P450

Total quantities of cytochrome P450 content were determined by visible light spectrophotometry at 400-490 nm, on the basis of the difference between absorbance readings at 450 and 490 nm. Measurements were made after cytochrome reduction by sodium

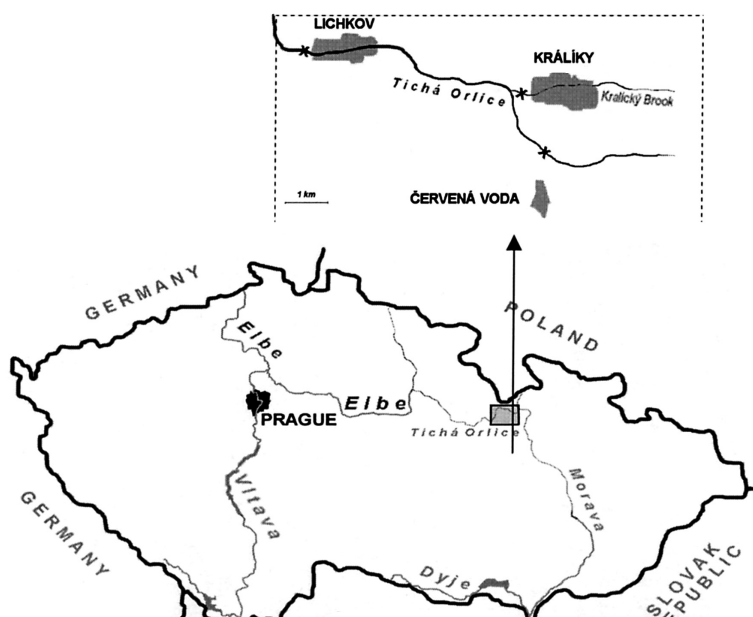


Fig. 1. Map of the Czech Republic and location of sampling area

EROD activity determination

Activity of EROD was measured by spectrofluorometry. In the presence of the enzyme, the substrate ethoxyresorufin is transformed into resorufin in the presence of NADPH. Measurements were made with a fluorescence spectrometer (excitation: 535 nm, emission: 585 nm). EROD activity was subsequently calculated based on a comparison with fluorescence of the standard (resorufin) of known concentration (Šíroková et al. 2005). All but four samples were measured 2 - 5 times with the aim to analyse the repeatability of EROD values. EROD values were homogenous in more than 90% of measured samples (coefficient of variation less than 15%).

Determination of blood plasma vitellogenin

Blood samples were taken by caudal venipuncture, and centrifugation and deep freezing of blood plasma were performed *in situ* at the sample sites. Vitellogenin concentrations in fish blood plasma were assessed using the ELISA-Rainbow trout vitellogenin EIA kit (Biosense Laboratories, Norway). The limit detection for vitellogenin in blood plasma is 0.05 $\mu\text{g}\cdot\text{ml}^{-1}$.

Determination of POPs in muscle samples

Polychlorinated biphenyl (PCB) indicator congeners - IUPAC numbers 28, 52, 101, 118, 138, 153 and 180, hexachlorbenzene (HCB), α -, β -, γ -isomers of hexachlorocyclohexane (HCH), octachlorostyrene (OCS), and 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 dimensions, differing in selectivity (DB-5 and DB-17), and two electron-capture detectors (ECD). Isolation of target analytes from fish muscle was carried out by Soxhlet extraction into a 1 : 1 v/v hexane : dichloromethane solvent mixture.

Clean-up of the extracts was performed, similarly to alkylphenols, by GPC on a Bio-Beads S-X3 column and mobile phase ethylacetate : cyclohexane (1 : 1, v/v) extraction. The method is described in detail in the study of Hajšlová et al. (1995).

Determination of PAH in sediments

Polycyclic aromatic hydrocarbon isolation from pre-dried sediment was performed by Soxhlet extraction, using dichloromethane, and gel permeation chromatography to separate PAH from co-extractors. Polycyclic aromatic hydrocarbons were determined using reversible high performance liquid chromatography and fluorescence detection (HPLC/FLD). Individual analytes were identified and quantified using appropriate standards and the external standard method. The following 15 PAHs were found in bottom sediments: fluorene, naphthalene, acenaphthene, fenantrene, anthracene, fluoranthene, chrysene, pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1, 2, 3-c, d)pyrene, benzo(g, h, i)perylene and dibenzo(a, h)anthracene.

Statistical methods

Ethoxyresorufin-O-deethylase, cytochrome P450 and vitellogenin values were not normally distributed; therefore non-parametric Kruskal-Wallis test was used to compare values from individual sites. This test was followed by a multiple comparison to detect significant differences between sites. Relationships among EROD, cytochrome P450 and vitellogenin parameters were assessed by means of the non-parametric Spearman correlation.

Results

Values of biochemical markers

The lowest values of cytochrome P450 content, EROD activity, and vitellogenin concentration in the brown trout liver, at assessed localities were found at the site of Červená Voda, whereas the highest values of these markers were determined at Králíky.

The highest mean levels of cytochrome P450 were found at Králíky ($0.114 \text{ nM}\cdot\text{mg}^{-1}$ protein), lower levels at Lichkov ($0.085 \text{ nM}\cdot\text{mg}^{-1}$ protein), and the lowest at the control site Červená Voda ($0.077 \text{ nM}\cdot\text{mg}^{-1}$ protein). However, these differences in cytochrome P450 values between sites were not significant (Kruskal-Wallis $N = 30$; $Q = 1.269$; $P = 0.530$).

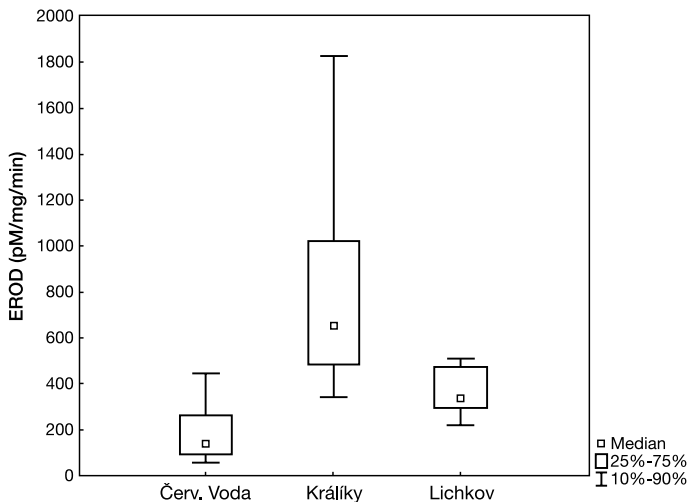


Fig. 2. EROD activity (pM/mg/min) in brown trout at sampling sites along the River Tichá Orlice

The values of EROD activity in brown trout liver samples from assessed localities are given in Fig. 2. The differences in EROD values between sites were significant (Kruskal-Wallis $N = 30$; $Q = 16.304$; $P < 0.001$). Multiple comparisons showed a significant difference in EROD values between Králíky and Červená Voda ($P < 0.001$). A positive significant

correlation between EROD activity and cytochrome P450 content was found at Červená Voda ($r_s = 0.787$, $P = 0.007$). This relationship was not significant at either Králíky ($r_s = -0.049$, $P = 0.894$) or Lichkov ($r_s = -0.159$, $P = 0.662$).

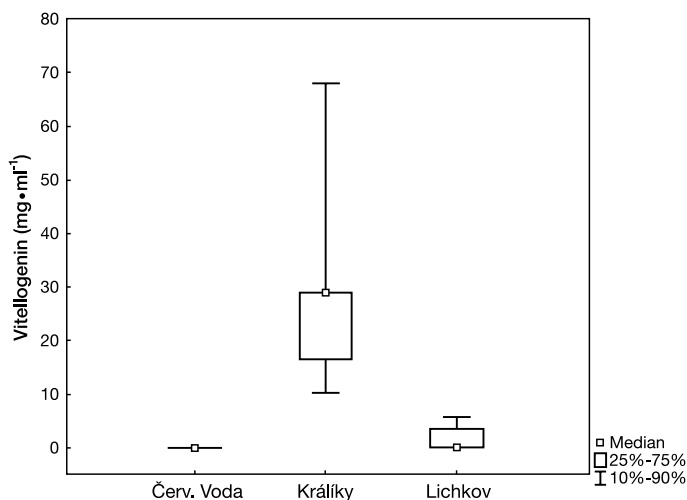


Fig. 3. Vitellogenin concentrations ($\mu\text{g}\cdot\text{ml}^{-1}$) in brown trout at sample sites along the River Tichá Orlice

The values of vitellogenin concentrations in brown trout liver samples from monitored localities are given in Fig. 3. The differences in vitellogenin concentrations between sites were significant (Kruskal-Wallis $N = 20$; $Q = 16.009$; $P < 0.001$). Multiple comparison showed a significant difference in vitellogenin values between Králíky and Červená Voda ($P < 0.001$) and between Lichkov and Králíky ($P = 0.037$). It was not possible to determine EROD and cytochrome P450 correlations with vitellogenin at Červená Voda because vitellogenin levels in all samples from that site were below the limit of detection. At Králíky, which was the most contaminated site, a significant negative correlation between EROD and vitellogenin ($r_s = -0.964$, $P < 0.001$) was demonstrated. No such correlation was found at Lichkov ($r_s = 0.030$, $P = 0.954$). At Králíky and Lichkov there was no significant correlation between cytochrome P450 and vitellogenin (Králíky: $r_s = -0.561$, $P = 0.190$; Lichkov: $r_s = 0.091$, $P = 0.864$).

Results of chemical analysis

The pollutants found in muscle of brown trout caught at the monitoring sites included seven PCB congeners; HCH (as a sum of three congeners), HCB, OCS, and DDT including its metabolites are presented in Table 2. The differences among localities could not be tested because of the low number of samples (just two samples from each locality). The presence of PAH (as a sum of 15 selected PAHs) in the sediment is also demonstrated. PCB congener 153 was determined at the highest levels ($18.6 \text{ ng}\cdot\text{g}^{-1}$ muscle) at Králíky. No correlation between PCB and biochemical markers from individual sites was observed.

Discussion

It is evident from the elevated values of biochemical markers at Králíky that exposure to organic pollutants at that site is higher than at the other monitored sites. Lichkov is situated downstream of Králíky and it is adversely influenced by contamination from Králíky. Concentrations of cytochrome P450 and EROD activity in the liver can be used

Table 2. Content of persistent organic pollutants in brown trout (*Salmo trutta fario*) male population ($\mu\text{g}\cdot\text{kg}^{-1}$ of muscle, w.w.)

Locality	PCB ^{a)}	HCH ^{b)}	HCB	OCS	DDT ^{c)}	PAH ^{d)} *
Červená Voda	9.84 ± 0.17	0.32 ± 0.01	0.71 ± 0.06	0.03 ± 0.01	39.22 ± 2.17	2800
Králíky	47.86 ± 3.62	0.22 ± 0.10	2.59 ± 0.14	0.05 ± 0.01	48.12 ± 20.56	16360
Lichkov	27.47 ± 6.09	0.14 ± 0.03	1.21 ± 0.03	0.05 ± 0.01	27.35 ± 5.10	1380

^{a)} sum of 7 indicator congeners (28, 52, 101, 118, 138, 153, 180)

^{b)} sum of HCH isomers (α , β , γ)

^{c)} sum of DDT and its metabolites (*o,p'*-DDE; *p,p'*-DDE; *o,p'*-DDD; *p,p'*-DDD; *o,p'*-DDT; *p,p'*-DDT)

^{d)} ($\text{ng}\cdot\text{g}^{-1}$ dry matter)

* PAHs were determined in river sediment

for determination of the contamination degree at a site. The different levels of EROD activity observed in fish caught in the River Tichá Orlice could be related to the typical cytochrome P4501A induction in fish, where small differences in the level of exposure to cytochrome P4501A inducers may give rise to great differences in enzymatic activity. In natural fish populations, the combined influence of biotic and abiotic factors is known to cause background variations in cytochrome P450 levels and activities (Goksøyr and Förlin 1992).

Because fish in the upper reaches of the River Tichá Orlice exhibited reproductive disorders, vitellogenin was selected as a biochemical marker of contamination. The highest vitellogenin concentrations were found at Králíky, and the lowest vitellogenin concentrations were found at Červená Voda. A similar situation was reported for the Tichá Orlice (Kolářová et al. 2005). Vitellogenin concentrations in brown trout in running water were also monitored by Kavanagh et al. (2004) or Bjerregaard et al. (2006). Increased vitellogenin levels in brown trout males have been directly responsible for disruptions of spermiogenesis and reproductive dysfunction (intersexual males).

This study demonstrated the connection between values of biochemical markers and the ambient level of organochlorine pollutants. PAH and PCB have been considered as the most important inductors of monitored biochemical markers. The highest PAH concentrations in sediments were found at Králíky (together with the highest EROD activities, and cytochrome P450 and vitellogenin levels). PAHs concentrations in the sediments of Czech rivers (River Elbe and River Vltava) were also measured by Hosnedl et al. (2003), who chose 1-hydroxypyrene as a suitable biochemical marker. The reported polycyclic aromatic hydrocarbon contamination is comparable to that in the River Tichá Orlice. The ability of CYP1A to indicate exposure to PAH has been demonstrated in several studies where fish were exposed to increased concentrations of AhR ligands (Goksøyr et al. 1994; Stagg et al. 2000).

The highest fish muscle PCB concentrations were found in brown trout from Králíky, and the lowest concentrations at Červená Voda. Of all PCB congeners the most abundant was the congener 153 which may be an evidence of the use of high-chlorinated congeners of PCB, e.g. Delor, in the manufacturing process. Similar results were reported for shorthorn sculpin (*Myoxocephalus scorpius*) from the Saglek Bay (Kuzyk et al. 2005) and for dab (*Limanda limanda*) from the North Sea (Sleiderink and Boon 1995).

Biochemical markers have been widely used to evaluate the level of contamination of water environment by organochlorine pollutants (Van der Weiden et al. 1993; Al-Arabi et al. 2005; Behrens and Segner 2005). Increased levels of organic pollutants result in an increase in values of corresponding biochemical markers. Monitoring biochemical markers showed differences in contamination levels between individual sites, and the levels of organic pollutants in sediments confirmed these differences.

On the other hand, a decrease of EROD activity and cytochrome P450 induction in the aquatic environment may be attributable to high concentrations of heavy metals (Cu, Zn, Pb, Cd, Hg or Ni) and to nonplanar PCB congeners, which may act as specific cytochrome

P450 inhibitors (Förlin et al. 1986; Boon et al. 1992; Stien et al. 1997; Bozcaarmutlu and Arinc 2004; Brammell et al. 2004; Henczova et al. 2006). At the site with high long-term contamination downstream of Králíky on the Kralický brook, a significant negative correlation between EROD and vitellogenin levels was demonstrated. Chronic exposure to persistent organic pollutants (PCB, PAH, DDT and its metabolites and other pollutants) may reduce the response of an organism to contamination, instead of inducing total cytochrome P450 (Stegeman et al. 1997; Schlezinger and Stegeman 2001), i.e., it may cause an insufficient response in relation to the synthesis of new cytochrome P450 (Brammell et al. 2004). EROD activity may be suppressed by the presence of estradiol or its synthetic homologues of suppression (Arukwe and Goksøyr 1997; Solé et al. 2000). The same substances, on the other hand, seem to induce vitellogenin in the male fish liver (Baldigo et al. 2006).

At the Králíky site, increased mercury and copper contamination was found (Svobodová et al. 2004), and, according to Keith (1997), mercury is one of the environmental endocrine disruptors. There is an inverse relation between concentrations of estrogenic substances and biotransformation enzyme activity. Although estradiol significantly suppresses CYP1A catalytic activity in fish, little is known about the effects of xenoestrogens on CYP1A. The biochemical mechanism of this interaction is not clear (Solé et al. 2003). High vitellogenin concentrations in the liver of male brown trout determined in this study and previously (Kolářová et al. 2005), as well as reproductive problems in salmonids here confirm the highly xenoestrogenic condition of this site.

Využití biochemických markerů k posouzení kontaminace řeky Tiché Orlice organickými polutanty

Cílem práce bylo pomocí vybraných biochemických markerů posoudit kontaminaci vybraných lokalit řeky Tiché Orlice - levostranný přítok Labe. Hodnoceny byly dvě kontaminované lokality (Králíky a Lichkov) a jedna kontrolní lokalita (Červená Voda). Jako indikátorový druh byl zvolen pstruh obecný (*Salmo trutta fario*). Jako biochemické markery byly použity enzymy první fáze přeměny xenobiotik, cytochrom P450 (CYP 450) a ethoxyresorufin-O-deethyláza (EROD) v játrech. Pro hodnocení xenoestrogenního účinku zatížení prostředí byl zvolen biomarker vitellogenin v krevní plazmě. Výsledky byly porovnány s nejdůležitějšími induktory těchto markerů - s organickými polutanty (PCB, HCH, HCB, OCS a DDT a jeho metabolity ve svalovině ryb, a s hodnotami PAH v sedimentech dna). Lokalita Králíky vykazuje největší znečištění organickými polutanty, které se projevilo ve zvýšených hodnotách cytochromu P450, v aktivitě EROD i vitellogeninu. Statistická významnost rozdílu byla prokázána v aktivitě EROD a koncentraci vitellogeninu mezi lokalitami Králíky a Červená Voda ($P < 0.001$). Na nejvíce kontaminované lokalitě (Králíky) byla prokázána statisticky významná negativní korelace mezi hodnotami EROD a vitellogeninu ($r_s = -0.964$). Tento vztah byl diskutován s ohledem na možnost indukce nebo inhibice sledovaných biomarkerů na dlouhodobě vysoce kontaminované lokalitě.

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