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Effect of polychlorinated biphenyls (Delor 103) on haematological and enzyme parameters of the rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Rainbow trout at a weight of 223 ± 12 g (mean ± SD) were experimentally injected with a technical mixture of Delor 103 to evaluate the red blood cell indices (red blood cell count, haematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration) and some biochemical and enzyme parameters of the blood plasma (total protein, glucose, inorganic phosphate, total calcium, sodium, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase). Delor 103, administered by the i.p. route at a concentration of 0.24 g kg^{-1} 120 h^{-1}, caused an increase in the red blood cell counts, haematocrit values, haemoglobin concentrations, inorganic phosphate, alanine aminotransferase and lactate dehydrogenase. The sodium level fell. The fish injected with Delor 103 showed a relative decrease in the lymphocyte count and a relative increase in the count of neutrophile band forms.

KEY WORDS: Trichlor biphenyl · Toxicity · Red blood cell indices · Biochemical indices · Differential leucocyte

INTRODUCTION

It is currently a problem that the biosphere is contaminated with a wide spectrum of pollutants. Exposure to pollutants is harmful to human health and affects both domestic and wild-ranging animals. Besides persistent pesticides, an important role among lipophilous xenobiotics is played by polychlorinated biphenyls (PCBs), which are very inert in biochemical terms and considerably resistant to metabolic transformation. Their production, which started in the 1930s, was gradually scaled down until it was totally stopped (Czechoslovakia stopped their production in 1984). However, PCBs can be expected to continue being released into the aquatic environment as the total quantity of PCBs produced in the world is estimated at 1.2 million tonnes; the former Czechoslovakia alone produced 20 000 tonnes. It is assumed that 31% of this quantity has already leaked to the environment, 65% is still used within closed systems and only 4% has been disposed of in incineration plants. Hence, at present, redistribution of the PCBs that penetrated into the environment in the past is the main source of pollution (Holoubek et al. 1996). The dominant PCB transport process in the aquatic medium is adsorption to sediment or to another organic phase. Experiments have shown that PCB concentrations are greater in sediments and suspended sediments than in the water column. Though adsorption, followed by sedimentation, may immobilise PCBs in the aquatic system for a relatively long period of time, PCBs have proved to be redissolved in the water column (ATSDR/TP-92/16 1993). Hence, PCBs in sediments act as a pool that can re-circle again (Lohse 1988, ATSDR/TP-92/16 1993). This is why great attention is paid to PCBs from the hygienic and toxicological points of view (Hajšlová et al. 1997, Řehulka 2001, 2002a). PCBs have adverse effects on the state of fish health (Hinton et al. 1978, Malins et al. 1987; Myers et al. 1987, 1994, Svobodová et al. 1994) and on the endocrine system mainly in the

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area of the reproductive function of fish (Nelson 1974, Janssen et al. 1995). Bio-concentration factors (BCFs) in aquatic animals ranging from 26 000 to 66 000 have been shown experimentally (ATSDR/TP-92/16 1993). PCB levels depend on depth and on certain other factors characterising the environment where the animal lives. Through sorption of atmospheric PCBs, a layer develops on the water level where the concentration is up to 500 times higher than deeper underneath. As a result, the bio-concentration in fish is higher by several orders (Holoubek et al. 1996). We believe that not only accidental PCB pollution, as a result of levels exceeding the maximum admissible concentrations (MAC) of 2 mg kg^{-1} in muscle (fresh weight), can affect fish health (Svobodová et al. 1994), but also long-term fish exposure (over several fish generations) to PCBs in the hydroecosystem, where the PCB levels in the fish flesh are well below the MAC. This is indicated by the results from our fish health monitoring surveys continued for several years in water-source reservoirs (Řehulka 2001, 2002a) with descriptions of cholangiofibrosis, cholangiocellular carcinoma and Sertoli cell adenoma. According to Sonstegard (1977), PCBs and/or DDT may be involved in the aetiology of the gonadal tumour. In addition, symptoms of genotoxic damage (micronucleus) and the erythrocyte nuclear lesions observed in the fish in the reservoirs also encouraged us to carry out trials aimed at evaluating the effect of intraperitoneal administration of Delor 103 on the state of rainbow trout health. Delor 103, a manmade product containing isomers with a smaller number of chlorine atoms in its structure, is a mixture of about 100 PCB congeners with an addition of polychlorinated dibenzofuranes (2.88 to 42.5 ppm). Delor 103 is equivalent to Aroclor 1254, in which the non-observed effect level (NOEL) for the embryonic and larval stage of rainbow trout after 22 d was determined at 0.01 g l^{-1} (Melicharčík & Velek 1994). Studies hitherto performed with this commercially produced mixture have been focused on the humoral immune response (Cleland et al. 1988), on the resistance to infectious haematopoietic necrosis virus (Spitzbergen et al. 1988), on hepatocarcinogenicity (Shelton et al. 1984), on hepatic enzymes (Voss et al. 1982), and on the pathological effects in the kidney, liver and spleen (Nestel & Budd 1975). The studies were based on the administration of Aroclor 1254 in the diet.

The objective of our study was to evaluate the haematological and biochemical response to intraperitoneally administered Delor 103 (product of Chemko Strážské) to rainbow trout for 5 d. The trials were conducted within the framework of the experiments organised by the T. G. Masaryk Water Research Institute in Ostrava with a view to preparing fish hepatic S9 for genotoxicity tests.

MATERIALS AND METHODS

Experimental environment and fish. The experiments were conducted on a trout hatchery where the fish had been kept on a long-term basis to acclimate to the chemical composition of the water and the oxygen saturation thereof. The fish were kept in fibre-glass tanks $4.0 \times 0.6 \times 0.6$ m in size with a continuous supply of new fresh water and with a photoperiod of 13 h light:11 h dark. We formed 2 test groups and 2 control groups, each comprised of 20 fish. For 14 d prior to the start of the trial, the fish were left to adapt to the environment and during the trial they were not fed. The water had the following physical and chemical characteristics during the trial: water temperature 14°C, dissolved O_2 13 mg l^{-1}, O_2 saturation of water 125%, pH 6.6, total hardness 4° N, COD_{Mn} (chemical oxygen demand) 5.8 mg l^{-1}, NH_4^+ 0.01 mg l^{-1}, NO_3^- 2.5 mg l^{-1}. The experimental fish was healthy rainbow trout of the Kamloops strain at an age of 9 mo. All the fish were of the same origin and all had about the same weight 223 ± 12 g (mean ± SD). Their standard length was 244 mm and their Fulton's condition factor (body weight in g × 100/standard length^3, in cm) was 1.4. Delor 103 was administered (upon anaesthesia of the fish) by the intraperitoneal route on the right side between the pectoral and pelvic fins. The dose was 0.24 g Delor 103 in sunflower oil per kg of fish (1 ml sunflower oil contained 100 mg of Delor 103). The control fish received injections of the same sunflower oil which was free of Delor 103.

Preparation of blood samples. The fish were anaesthetised with Menocaine (Spofa) (3-aminobenzoic acid ethylester natrium hydrogen sulphate) at a concentration of 0.06 g l^{-1} (Král 1988) and then samples were taken by puncturing the caudal vessels (from 08:00 to 09:00 h). EDTA (ethylene diamine tetra acetate) and sodium heparin (5000 IU in a 1 ml injection) were used as anticoagulants, the former for the haematological examination and the latter for the biochemical analyses of the blood plasma. Haematocrits (Hcts) were determined immediately after sampling, using a microhaematocrit centrifuge (15 250 × *g* for 3 min). Red blood cell counts (RBCcs) and haemoglobin concentrations (Hb) were determined afterwards. The blood plasma was obtained by centrifuging the blood at 4100 × *g* for 10 min at 4°C; then the blood was separated into plastic syringes and all determinations were performed within 12 h. Blood smears were air-dried and stained by the May Grünwald and Giemsa Romanowski methods.

Haematology and clinical chemistry. RBCcs (Tl^{-1}, T: tera, i.e. 10^{12}) were determined using a Bürker couting chamber and Hayem solution and the erythrocytes were counted in 2×20 rectangles per sample. Hct values were determined in duplicate by using microhaematocrit-heparinised capillary tubes. Haemoglobin concentra-

tions (Hb, in g l^{-1}) were determined by the cyanhaemoglobin method using a wavelength of 540 nm. The mean corpuscular volume (MCV, in fl), mean corpuscular haemoglobin (MCH, in pg) and mean corpuscular haemoglobin concentration (MCHC) were calculated from haematological figures. The leucocytes were differentiated according to Ivanova (1983) and the relative abundance of all cell types was determined by counting a total of 200 white blood cells.

A Hitachi 704 instrument was used for the following determinations: total protein (TP, in g l^{-1}), glucose (GL, in mmol l^{-1}), inorganic phosphate (P, in mmol l^{-1}), total calcium (Ca, in mmol l^{-1}), alanine aminotransferase (ALT, in µkat l^{-1}, Kat = Katal), aspartate aminotransferase (AST, in µkat l^{-1}), alkaline phosphatase (ALP, in µkat l^{-1}) and lactate dehydrogenase (LD, in µkat l^{-1}). The sodium cation (Na^+, in mmol l^{-1}) was determined by flame emission photometry. Kits, PLIVA-Lachema and DIALAB, were used for the determination of all indices. For controls, BIO-LA-TEST® LYONORM HUMAN A, DIACON A and DIALAB were used.

The haematological and biochemical parameters are expressed in international units (SI).

Statistical analysis. All the calculations were made using the UNISTAT® statistical package for MS Windows. Data from the control and experimental groups were compared using the *F*-test and *t*-test.

RESULTS

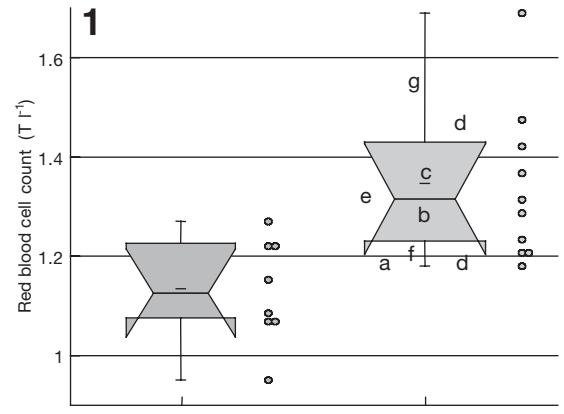
During the trial, the clinical state of the fish injected with Delor 103 did not show any signs of worsening. However, a comparison of the haematological examinations of the injected and control fish showed an increase in RBCcs, Hct (Figs. 1 & 2) and Hb (Table 1). For RBCcs, the values ranged from 1.18 to 1.69 vs 0.95 to 1.27 ($p = 0.005$), for Hct from 0.427 to 0.625 vs 0.378 to 0.452 ($p = 0.000$) and for Hb from 67.6 to 86.9 vs 56.8 to 83.9. The differential leucocyte count differed between the 2 groups in both its variability and levels, as shown in Table 1. Fish exposed to Delor 103, as distinct from the control fish, showed an evident relative decrease in the lymphocyte count (0.44 to 0.93 vs 0.7 to 0.96) and an increase in neutrophile band forms (0.05 to 0.23 vs 0.01 to 0.08). The values for MCV, MCH, MCHC and TP, GL, Ca, AST and ALP remained unaffected by Delor 103.

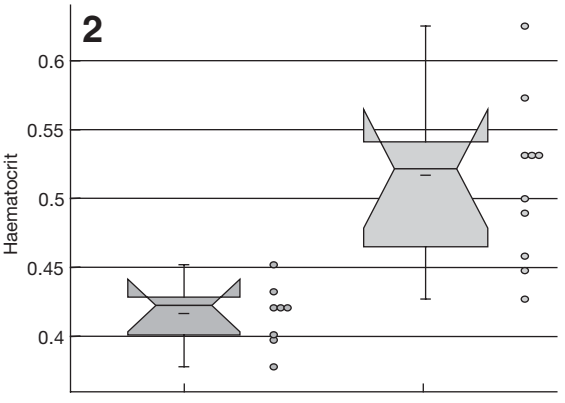
As for the biochemical parameters, we recorded an elevation of the concentration of P (6.45 to 8.25 vs 6.2 to 6.85, $p = 0.003$) and a decline in the level of Na^+ (136 to 162 vs 160 to 166, $p = 0.006$) (Figs. 3 & 4). As for the enzyme activities, which were evaluated using the ALT and AST aminotransferases, and also ALP and LD, there was an increase in the catalytic concentration of ALT (0.2 to 0.5 vs 0.17 to 0.33 (Table 1) and of LD (25.1 to 71.6 vs 13.1 to 55.7, $p = 0.002$) (Fig. 5).

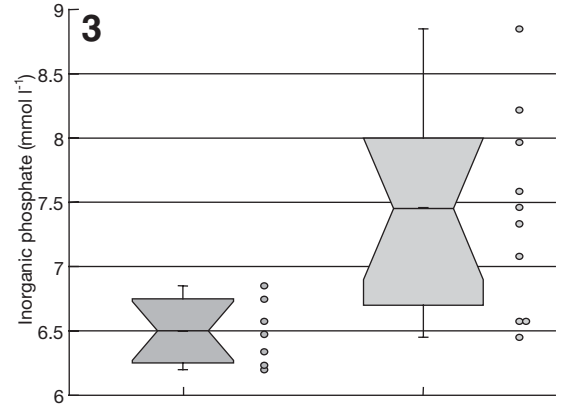
In the experimental group, as distinct from the control group, we recorded a correlation between ALP and TP ($r = 0.854$, $p = 0.004$) and between ALP and Na^+ ($r = -0.828$, $p = 0.006$). The dependences between these parameters are expressed by the following regression line equations: ALP = −3.2501 + 0.1447 TP and TP = 26.9308 + 5.0446 ALP, respectively, and Na^+ = 180.733 − 12.5663 ALP and ALP = 10.6032 − 0.0546 Na^+, respectively.

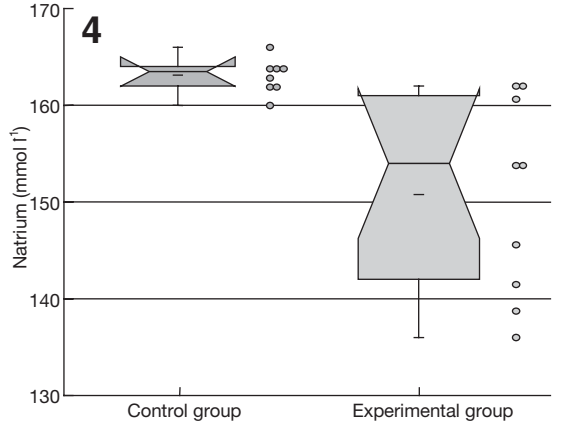
Table 1. *Oncorhynchus mykiss*. Comparison of haematological and biochemical parameters of control rainbow trout injected with sunflower oil and fish injected with 0.24 g kg^{-1} Delor 103 in sunflower oil sampled 5 d i.p. (n = 10). SEM: standard error of the mean; $^*p \le 0.05$; $^{**}p \le 0.01$. Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; TP: total protein; GL: glucose; Ca: total calcium; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase

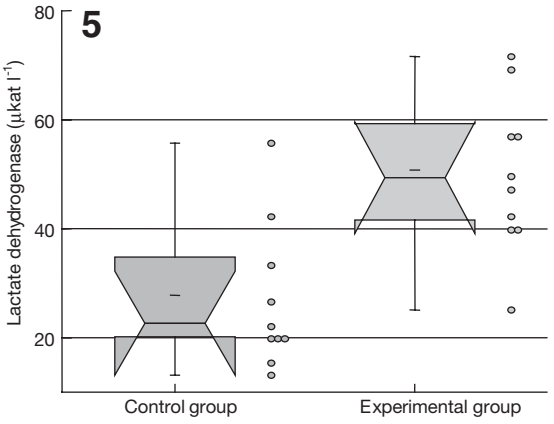
Indices	Control fish			Experimental fish			*F*-test		*t*-test	
	Mean	SD	SEM	Mean	SD	SEM	*F*-value	Probability	*t*-value	Probability
Hb g l^{-1}	68.6	9.09	2.88	76.4	6.68	2.11	1.854	0.186	−2.204*	0.041
MCV fl	372	33.2	11.1	386	41.2	13	1.54	0.277	−0.78	0.446
MCH pg	56	4.2	1.3	57	6.1	1.9	2.087	0.144	−0.256	0.801
MCHC	0.151	0.004	0.001	0.149	0.011	0.003	6.976**	0.009	0.687	0.505
Differential										
Lymphocytes	0.88	0.06	0.02	0.75	0.134	0.044	5.025*	0.017	2.688*	0.021
Neutrophils:										
Bands	0.04	0.017	0.006	0.11	0.062	0.021	13.113**	0.0007	−3.413**	0.007
Segments	0.08	0.048	0.016	0.13	0.095	0.032	3.926*	0.035	−1.62	0.132
TP g l^{-1}	36.3	6.68	2.11	40.4	5.52	1.84	1.464	0.301	−1.463	0.162
GL mmol l^{-1}	4.05	1.15	0.364	4.01	1.4	0.44	1.491	0.281	0.07	0.945
Ca mmol l^{-1}	3.52	0.274	0.087	3.57	0.271	0.086	1.017	0.49	−0.418	0.681
AST µkat l^{-1}	9.86	3.459	1.094	11.02	3.171	1.121	1.19	0.419	−0.733	0.474
ALT µkat l^{-1}	0.25	0.061	0.021	0.36	0.11	0.039	3.307	0.069	−2.559*	0.023
ALP µkat l^{-1}	1.79	0.637	0.202	2.28	0.678	0.226	1.133	0.425	−1.615	0.125











Figs. 1–5. *Oncorhynchus mykiss*. Haematological and biochemical indices in experimental and control rainbow trout shown by notch box graphs with filaments: a = width of the box, indicating the size of the set; b = mid-diagonal of the box, representing the position of the median in relation to the *y*-axis; c = mark inside the box showing the position of the arithmetic mean; d = lower and upper edge of the box, indicating successively the position of the lower and upper quartiles; the outgrowths on the upper and lower edges of the box mean that the confidence interval has exceeded the value of the upper and lower quartiles; e = width of the notch, corresponding to the confidence interval around the median; f = the lower filament, with a length corresponding to the value of the lower quartile reduced by 1.5 × the span of the quartiles. If this value is lower than the minimum value in the set, the length of the filament corresponds to this minimum value. If values lower than those corresponding to the coordinate of the end point of the lower filament do occur in the set, then these values are signalled as remote; g = the upper filament, with a length corresponding to the value of the upper quartile enlarged by 1.5 × the span of the quartiles. If this value is higher than the maximum value in the set, the length of the filament corresponds to this maximum value. If values higher than those corresponding to the coordinate of the end point of the upper filament do occur in the set, then these values are signalled as remote

DISCUSSION

Owing to the high stability of PCBs in the environment, pollution of hydroecosystems through the release of PCBs from sediments can be expected to continue for many years; in addition, fish may be exposed for short periods to high PCB concentrations, as there are considerable quantities of waste containing PCBs on the Czech Republic at present. Taking this into account, as well as the experience from monitoring the state of fish health from PCB-contaminated environments (Svobodová et al. 1994, Řehulka 2000), our laboratory prepared an experiment focused on the evaluation of the action of the technical mixture of Delor 103 on rainbow trout, which belongs to the group of model species used in examining the toxicity of pollutants in acute and chronic toxicity tests.

Fifteen haematological and biochemical tests were performed, focused on haematopoiesis, nitrogen, carbohydrate and mineral metabolism, and on the activities of certain enzymes. A dose of 0.24 g kg^{-1}, administered by the intraperitoneal route for 5 d, was found to influence erythropoiesis, P and Na^+ concentrations, as well as the activities of ALT and LD. The formation of erythrocytes or their release from haemopoietic tissues can also be stimulated by certain metals such as copper and chromium (McKim et al. 1970, van der Putte et al. 1983), detergents (Dhillon & Gupta 1983) and pesticides. Aldrin caused a dose-dependent increase in RBCc and Hb concentration, but the MCH actually decreased (Dhillon & Gupta 1983). An increase in the Hct and MCV, together with a reduction of the MCH, was described by Svobodová (1975) who examined carp exposed to 3 organophosphate pesticides containing fenitrothion, dichlorvos and imidan as their active ingredients at an LD_{50} concentration. The mechanism of this stimulatory effect of chlorinated hydrocarbons has not yet been explained. A reverse finding was obtained by Svobodová et al. (1994), who tested exposure of carp (average weight of 67.9 ± 4.3 g) to Delor 103 (a dose of 0.1 to 0.2 ml), but divided into 6 doses, for a period of 42 d. They reported a significantly reduced Hct in the injected fish. These results suggest that investigation of Delor 103 should continue, focusing on the issue of whether the stimulation of erythropoiesis is transient or permanent. We believe that a similar effect is occurring to that described by Rous & Jelínek (2000), who studied the action of cadmium, lead, mercury and chromium on haematopoiesis in rabbits.

The RBCcs and Hct levels in the fish exposed to an injection of Delor 103 exceeded the upper 97.5% quantile reference values (1.42 for RBCcs and 0.502 per Hct), as calculated for farmed rainbow trout under our conditions (Řehulka et al. 2004) in a normoxic (8.8 to 10.2 mg O_2 l^{-1}) and hyperoxic (11.9 to 13.3 mg O_2 l^{-1}) environment (Caldwell & Hinshaw 1994). In the evaluation of the action of Delor 103 on the metabolism of phosphorus, we confirmed the results published by Lopuchovský (1986), who, on the basis of oral administration of Delor 103 to chickens (5, 50 and 100 mg kg^{-1}), recorded an increase in the level of P in the blood serum. In contrast, Ruprich & Piskač (1990) recorded a significant reduction of P concentration in chickens exposed to Delor 103 at a concentration of 150 mg kg^{-1}. A significant increase of the level of P in the blood plasma was also found in rainbow trout intraperitoneally injected with TCDD (2,3,7,8-tetrachlordibenzo-p-dioxin) and DEHP (di-2-ethylhexyl phthalate) (J. Řehulka unpubl. data). Whether this P is released from the destroyed skeleton is not known so far. In our trials, the Delor 103 concentration examined had no effect on the concentration of another important macro-element, namely Ca. Determination of Na^+ and Ca concentrations allowed us to evaluate the osmotic state of the organism. In general, a lower Na^+ concentration might signal a trend leading to a reduction of the osmotic pressure of the blood. The values established in our trials fit within our laboratory's reference range (146.6 to 163 mmol l^{-1}).

Enzymes are increasingly used not only as indicators of pathological processes (Bell 1968, Racicot et al. 1975, Shieh 1978, Harbell et al. 1979, Waagbø et al. 1988, Řehulka 2002b) but they also play an important role in toxicology (Lusková et al. 2002) and as indicators of stress. LD, creatine-kinase, and the ALT and AST aminotransferases are primarily used for the last-mentioned purpose. An increase of the catalytic concentration of these enzymes is a better indicator than hormones for the indication of tissue damage due to stress (Cairns & Christian 1978, Navrátil et al. 1998). The increase of the ALT and LD levels in our trials, with an unchanged AST activity and with a negative histological finding in the liver, may be ascribed to an increased permeability of the cell membrane without major toxic damage, which is, as Racek (1999) believes, accompanied by a higher level of AST than ALT. Lopuchovský (1986) found that the catalytic concentration of ALT increased by up to 311%. However, in contrast to our findings, Lopuchovský (1986) also recorded an increase in AST.

Changes in the differential leucocyte count included, first of all, a relative increase of neutrophile band forms, yet without evidence of a leukaemoid reaction. Similar results were recorded by Własow (1985) in rainbow trout after a 2 mo exposure to phenol at a concentration of 2 mg l^{-1} and also by Svobodová & Pečená (1988), who examined carp subjected to acute intoxication with pesticides at a concentration of LD_{50}/48 h. The above changes in the composition of white blood cells are probably due to exposure to stress, caused by the PCBs, which weaken the specific and non-specific immunity mechanisms (Wedemeyer 1997) and increase the susceptibility of the fish to diseases (the immunosuppression effect). The decreased level of circulating lymphocytes and macrophages leads to reduced resistance of the fish to opportunistic pathogens (Barton & Iwama 1991).

What is important from the practical point of view is that the Delor 103 concentration, which was almost equal in our tests to the concentration found in the muscle of the European eel (243 mg kg^{-1}) (Svobodová et al. 1994) from a PCB-polluted river, elicited a significant response in the peripheral blood of rainbow trout. Experiments need to be performed to check whether the described changes are Delor 103-specific or PCB-specific and, as such, applicable to identifying the

causes behind accidental intoxications. However, the elevated RBC indices and the increase of the catalytic concentrations of enzymes can also be considered as a response to acute stress. In this context, the acceleration of haematopoiesis can be simply explained as a stress response by releasing stored cells from the spleen. It was an interesting finding that there was no hyperglycaemia, which is otherwise a frequent response of fish to the action of a number of pollutants (Srivastava 1981, Singh & Srivastava 1982, Natarajan 1989, Gill et al. 1990, Balint et al. 1995, Sancho et al. 1997, Lusková et al. 2002).

The experiment contributed to extending the knowledge of the action of PCBs (exposure to a technical mixture of Delor 103) on the state of health of rainbow trout from the viewpoint of clinical haematology and biochemistry. Considering the partial results, we should point out the effect on erythropoiesis, phosphorus metabolism, Na^+ cation as one of the indicators of the state of the internal environment, and the ALT and LD activities as indicators of tissue damage. The results suggest that further studies are needed to examine, in particular, the changes in erythropoiesis and phosphorus mechanism and also to explain and confirm the identified dependences.

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