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Effects of exposure to three environmental chemicals on the selected biochemical parameters of the blood plasma of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract: Rainbow trout *Oncorhynchus mykiss* at a weight of 115 ± 24 g (mean ± SD) were experimentally injected with di(2-ethylhexyl)phthalate (DEHP), 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) and gamma isomer of hexachlorocyclohexane (γ-HCH) to evaluate nitrogen metabolism (total protein [TP], blood urea nitrogen, uric acid [UA], creatinine), carbohydrate metabolism (glucose), mineral metabolism (inorganic phosphate[P], total calcium [Ca_t]), and catalytic activity of enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP], lactate dehydrogenase [LD]). After 21 days, the tested xenobiotics, administered by the intraperitoneally route, caused the following effect: in comparison with negative controls, fish injected with DEHP at concentrations of 200 or 50 mg kg^{-1} of body weight were found to have a higher level of P and lower level of Ca_t and decreased catalytic concentrations of ALT, AST, ALP and LD in the plasma of the peripheral blood. A higher level of TP and P and a decreased catalytic concentration of ALT were found when TCDD was administered at a dose of 2 μg kg^{-1}. A decrease in the catalytic concentrations of ALT and ALP occurred in fish injected with γ-HCH in a dose of 50 mg kg^{-1}. UA and P levels were decreased in fish injected with γ-HCH in a dose of 5 mg kg^{-1}. DEHP and TCDD caused neutrophilic leucocytosis with a marked left shift. Both concentrations of γ-HCH led to an increase in polychromatophilic erythrocytes. The frequency of micronuclei varied in all experimental groups, including the controls, ranging between 0.8 and 2.1 ‰. Histological examination in fish injected with DEHP at a concentration of 200 mg kg^{-1} revealed eosinophilic droplets in the epithelium of renal tubules and histological examination in fish injected with γ-HCH at a higher concentration revealed an increased activation of sinusoidal cells in the liver, a fibrous thickening of bile duct walls, bile duct hyperplasia and a periductal inflammatory.

Key words: 2,3,7,8-tetrachlordibenzo-*p*-dioxine (TCDD), di(2-ethylhexyl)phthalate(DEHP), hexachlorocyclohexane (γ-HCH), Blood plasma, Biochemical parameters, Haematology, Histopathology, Genotoxicity

Introduction

Ecotoxicologicl monitoring is conducted to gather information about the level of contamination of aquatic environment by pollutants and its purpose is also to assess the risks of the impact of contaminants on the health of fish populations. Fish and other aquatic species are exposed to a wide range of pollutants in water, food and sediments, which can affect the biotransformation reactions by inhibiting biotransformation enzymes or induce the levels of the P450 cytochromes (Machala 1993; Machala *et al.* 1997; Petřivalský *et al.* 1997), with the biotransformation capacity varying among different fish species (Lech & Bend 1980; Buhler & Williams 1988) and in dependence on their degree of development (Binder & Stegeman 1984). The essential biomarkers of the impact of environmental stress include histological methods describing liver lesions (Malins *et al.* 1987; Myers *et al.* 1987, 1991,1992, 1994; Myers & Rhodes 1988) and also the cytogenetic action of certain mutagenic and carcinogenic substances on the structure of the fish genome (Carrasco *et al.* 1990). The results of our experiment, focused on modulation of the 7-ethoxyresorufin-O-deethylase (EROD) activity of cytochrome P4501A (CYP1A) and glutathion-dependent enzymes in the liver tissue of rainbow trout after

21-day exposure to three major pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxine, hexachlorocyclohexane and di(2-ethylhexyl)phthalate, have motivated us to use more biochemical tests focused mainly on identifying disorders in the intermediary metabolism.

The objective of the study was to describe changes in the peripheral blood of rainbow trout through selected biochemical parameters of the blood plasma. The biochemical tests were complemented by examination of the profile of protein, nitrogen, carbohydrate and mineral metabolism, activities of the major enzymes, non-specific immunity and genomic damage.

Material and methods

Test chemicals

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), belongs to lipophilic and highly persistent organic pollutants (POPs) capable of bioaccumulation through food chains. Being linked to the Ah-receptor, most of its negative impacts are connected with a number of dioxin-type toxic effects (increased level of bioactivating enzymes, particularly the CYP1A, a risky increase in cell proliferation, immunotoxic effects, oxidative stress).The natural sources of TCDD include the biosynthesis of certain precursors, manufacturing processes involving chlorination of phenols, production of 2,4,5-trichlorophenol, production of chlorophenoxyacetic acid-based pesticides, production of PCB, copper, nickel, iron and steel smelting, waste-fired power generation, residential and industrial fires, pulp bleaching, automobile traffic and large-scale application of chlorophenoxyacetic acid-based pesticides containing polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/Fs) etc. Chemical effects include carcinogenicity and teratogenicity (Lenga 1988). According to Hutz *et al.* (2006) rainbow trout exposed to TCDD *in vivo* showed inhibition of oestrogen synthesis, potentially causing fertility defects. Giesy *et al.* (2002) reported a reduced survival of rainbow trout fry from females fed as little as 1.8 ng TCDD per kg feed. These authors observed no effect on growth or condition for adult rainbow trout exposed to TCDD concentrations as high as 90 ng TCDD/kg ww in food for up to 300 days. Biochemical, histological and behavioural aspects of visual function during the early development of rainbow trout were studied by Carvalho (2004). Hepatocellular glycogen depletion and a number of other pathological changes (increased mitosis, nuclear and cellular pleomorphism, single cell necrosis, margination and clumping of chromatin) are described by Walter *et al.* (2000) in adult female rainbow trout exposed dietary for 320 d to environmentally relevant doses of TCDD.

Di(2-ethylhexyl)phthalates (DEHP), are used as plasticisers in the manufacture of plastics (PVC, cellulose esters, synthetic elastomers) and to improve the mechanical properties of plastics (improve ductility, reduce brittleness). They enter the environment during production and by emanation from DEHP-containing products. In mammals it acts through what is called peroxisomal proliferation, involving increased proliferation of peroxisomes, oxidative stress and hepatocarcinogenicity (Lake 1995). Van Wezel *et al.* (2000) indicate environmental risk limits (ERLs) for di(*n*-butyl)phthalate (DBD) and DEHP. The ERLs are derived using data on ecotoxicology and environmental chemistry. The endpoints used are survival, growth, and reproduction. The resulting ERLs in water are 10 and 0.19 $\mu g\ l^{-1}$ for DBD and DEHP, respectively; in fresh soil and sediment with 10% organic matter the derived ERLs are, respectively, 0.7 and 1 mg per kg fresh nt. The chronic toxicity of six phthalate esters to rainbow trout was studied by Rhodes *et al.* (1995). The results of ELS studies (early life stage) indicated that chronic effects were observed for dimethyl phthalate (survival) and diethyl phthalate (growth) at 24 and 0.19 mg l^{-1}, respectively. This pattern of observed toxicity with the lower-molecular-weight phthalate esters and not the higher-molecular-weight phthalate esters is consistent with previously reported acute toxicity studies for several aquatic species.

Hexachlorocyclohexane (γ-HCH), belongs to insecticides containing a number of hexachloro-cyclohexane isomers, among which only γ-HCH is active. It is isolated during the manufacturing process. A sufficiently pure γ-HCH is called lindane. It is a contact, fumigant and ingestion insecticide, which enters the environment with the products made on its basis. Lindane is known as a carcinogenesis promoter. According to Tierney *et al.* (2014), the insecticidal properties of lindane were first discovered in the 1940s. It has been widely used in agriculture for disease vector control and as a pharmaceutical treatment for lice and scabies. The acute and chronic toxicity of lindane was studied by many authors. Some of the studies were focused on the histopathological effects of pesticides and related chemicals on the livers of fishes. Early necrotic, coagulative lesions were associated with the portal triads of rainbow trout exposed to critical levels of lindane (Couch 1975). Effects of lindane on the gills of young rainbow trout were described by Nenadic & Springer (1991). In the gills the higher concentrations (75 and 90 mg l^{-1}) produces necrosis, oedema and separation of the epithelium from the underlying tissue. Epithelial hypertrophy and hyperplasia occurred with the lower concentrations. Histopathological changes in the gills are also described in other fish species. Nandan & Nimila (2012) observed proliferation of the lamellar epithelium and lamellar fusion in *Etroplus maculatus*. Similar changes were

described in *Sparus aurata* by Gonzáles de Canales *et al.* (2009), and fusion of the secondary lamella, increased raising of the branchial epithelium and intraepithelial oedema were described in the same fish by Ortiz *et al.* (2003). Erosion of the tips of the gill filaments in *Colisa fasciatus* was described by Verma *et al.* (1975).

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 3.6 × 0.7 × 0.7 m in size with a continuous supply of new fresh water and with a photoperiod of 16.00 h light: 08.00 h dark. Two experimental groups and two control groups were formed, each comprised of 10 fish. The experimental fish was rainbow trout, all of the same origin and weight of 115 ± 24 g (mean ± SD) and their Fulton's condition factor (body weight in g × 100 / standard length^3, in cm) was 1.44 to 1.96. With respect to the results of our previous works and the studies referred to above, where differences in metabolite levels were caused by sexual dimorphism, we used in our trials female fish, whose number prevailed in the biomass of farmed rainbow trout (Řehulka *et al.* 2004, 2005; Řehulka & Minařík 2008, 2012). It was important in this study to ensure a steady state of health of the fish. Accordingly, the conditions specified below had to be met to ensure the absence of clinical signs of disease and of pathological and anatomic changes in the post-mortem examination (Roberts 2012) and /or bacterial examination (Austin & Austin 2012) of the fish, and in significant parasite infections (Ergens 1992; Lom & Dyková 1992). 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 15 – 17°C, dissolved O_2 8 – 9.5 mg l^{-1}, oxygen saturation 85 – 106 %, pH 6.6 – 6.8, COD_{Mn} (chemical oxygen demand) 9.7 – 9.8 mg l^{-1}, ammonium (NH_4^+) 0.12 – 0.44 mg l^{-1}, nitrites (NO_2^-) 0.014 – 0.075 mg l^{-1}, nitrates (NO_3^-) 3.9 – 21.5 mg l^{-1}. DEHP (experimental groups EG1 and EG2), TCDD (experimental group EG3) and γ-HCH (experimental groups EG4 and EG5), dissolved in 200 µl of dimethylsulphoxide (DMSO), were administered (upon anaesthesia of the fish) by the intraperitoneal route (i.p.) on the right side between the pectoral and pelvic fins, as shown in the scheme in Table 1. Fish in the control group (CG) were injected in the same manner with 200 µl of DMSO. Exposure time and the concentration of the test material were determined according to the results of the experiment (referred to above) focused on modulation of the 7-ethoxyresorufin-O-deethylase (EROD) activity of cytochrome P4501A (CYP1A) and glutathion-dependent enzymes in the liver tissue of rainbow trout. Concentrations exceeding those occurring under natural conditions were purposefully used in our study in order to learn the effects of higher concentrations that may occur in the event of uncontrolled leakage to water courses, accidental pollution or flood. Concentration of the test material were either injected once or sequentially at intervals.

Tab 1: *Oncorhynchus mykiss*. Time schedule of pollutant injecting (■) by the intraperitoneal route (□ marks the end of testing).

Experimental group	Test substance	The amount of test substance per kg of fish	1 day	4 day	8 day	10 day	16 day	21 day	28 day
EG1	DEHP	50 mg	■	■	■	■	■	□	
EG2	DEHP	200 mg	■	■	■	■	■	□	
EG3	TCDD	2 µg	■					□	
EG4	γ-HCH	50 mg	■					□	
EG5	γ-HCH	5 mg	■				■		□

Preparation of blood samples

In all experiments, blood samples were collected between 08.00 and 11.00 hours. The fish were anaesthetised with Menocain (Spofa, Prague, Czech Republic) (3-amino benzoic acid sodium hydrogen sulphate ethyl ester) at a concentration of 0.06 g l^{-1} (Král 1988) and then samples were taken by puncturing the caudal vessels. Sodium heparin (5000 IU in a 1ml injection) drawn through the syringe was used as anticoagulant. The blood plasma was obtained by centrifuging the blood at 4100 *g* for 10 min at 4^0C; then the blood was separated into plastic syringes and was transported at a temperature of 4°C to laboratory for analysis.

Clinical chemistry

A Hitachi 717 multiparametric analyser (Tokyo, Japan) was used for the determinations: total protein (TP, in g l^{-1}), blood urea nitrogen (BUN, in mmol l^{-1}), uric acid (UA, in µmol l^{-1}), creatinine (CREA, in µmol l^{-1}), glucose (GL, in mmol l^{-1}), inorganic phosphate (P, in mmol l^{-1}), total calcium (Ca_t , in mmol l^{-1}), alanine

aminotransferase (ALT, in μkat l^{-1}), aspartate aminotransferase (AST, in μkat l^{-1}), alkaline phosphatase (ALP, in μkat l^{-1}) and lactate dehydrogenase (LD, in μkat l^{-1}). Kits produced by PLIVA-Lachema, a.s. Brno, Czech Republic, and DIALAB Wien, Austria, and Prague, Czech Republic, were used for the determination of all indices.

Haematology

Blood smears were air-dried and stained by the May-Grünwald and Giemsa Romanowski methods. The leucocytes were differentiated according to Ivanova (1983). Next the same smears were stained according to the periodic acid – Shiff method and Sudan Black B method to identify granulocytes. The relative abundance of all cell types was determined by counting a total of 200 white blood cells per smear.

Genotoxicity

Piscine micronucleus test (MNT) (Hoftman & de Raat 1982) determining the frequencies of micronucleated red blood cells per 1000 cells was used to prove genomic damage.

Sampling procedure for histopathology

For histological examination, tissue samples of liver and kidney were fixed in 10% neutral formalin and the paraffin slices were stained with haematoxylin and eosin by the PAS method (periodic acid Schiff´s reagent) and were tested for demonstration of bile pigments (Stein).

Statistical analysis

Data from the injected and control fish were compared, using the *F* test and *t* test. For graphic processing we preferred to use the notch box graphs with filaments, as they complement statistical characteristics in Table 2 and, in particular, give a graphic idea of how the values for the analyses under study are positioned in the experimental and control fish. All the calculations were made using the UNISTAT® (2011) statistical package for MS Windows™

Results

Clinical symptoms, biochemistry and histopathology

DEHP

An increase in the level of P and decrease in Ca_t, and lower catalytic concentrations of ALT and ALP, were recorded in both experimental groups.

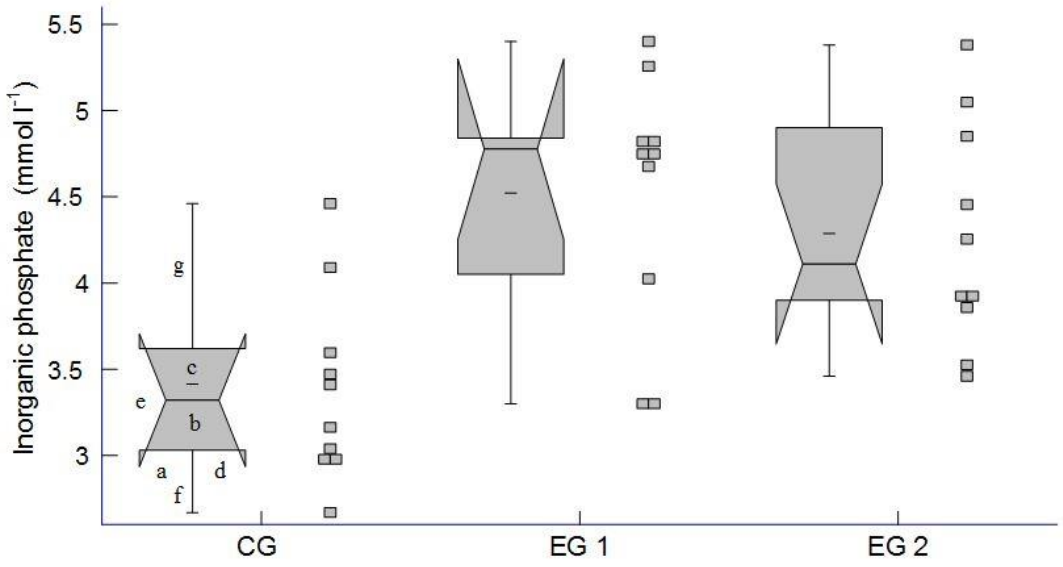


Fig 1.

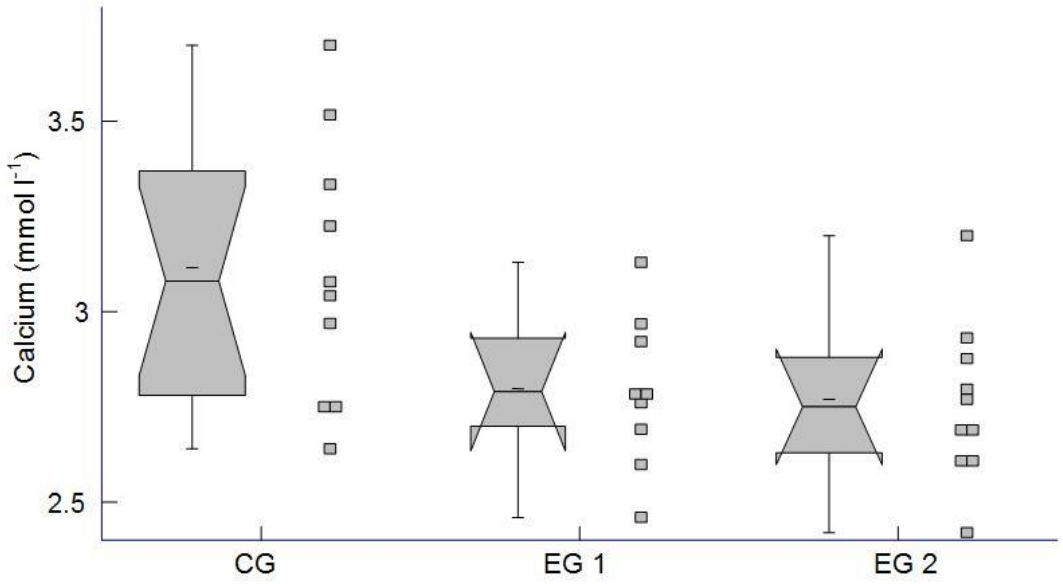

Calcium (mmol l-1)
3.5
3
2.5
CG
EG 1
EG 2

Fig 2.

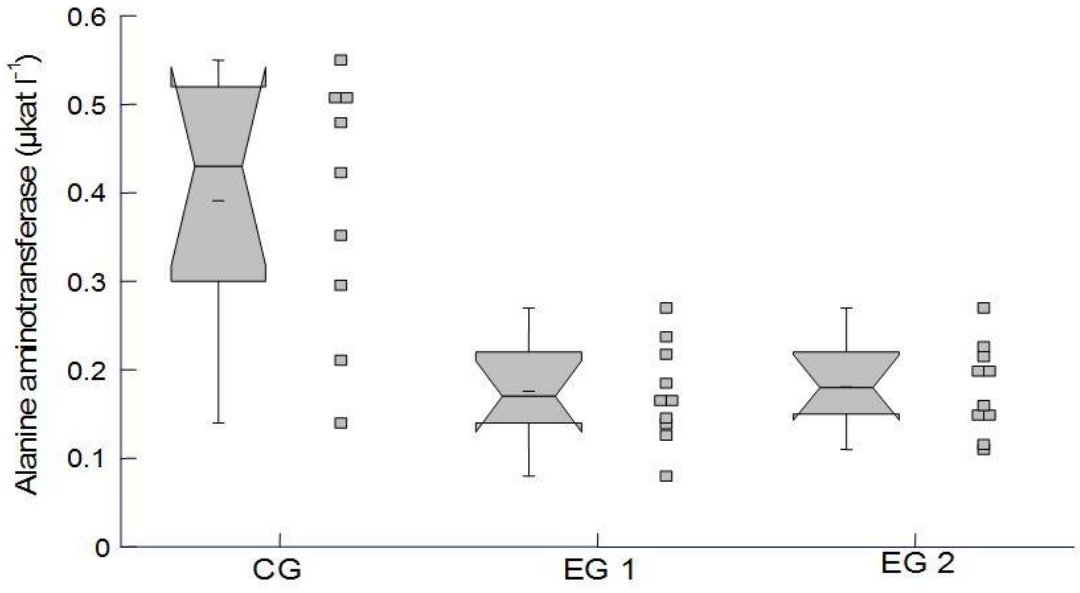

Alanine aminotransferase (µkat l-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
CG
EG 1
EG 2

Fig 3.

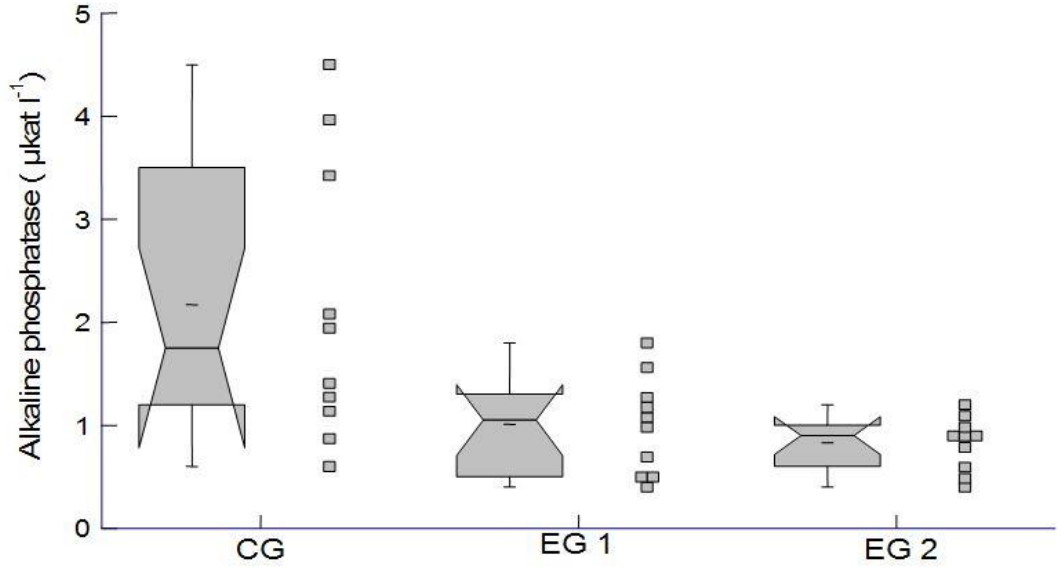

Alkaline phosphatase (µkat l-1)
5
4
3
2
1
0
CG
EG 1
EG 2

Fig 4.

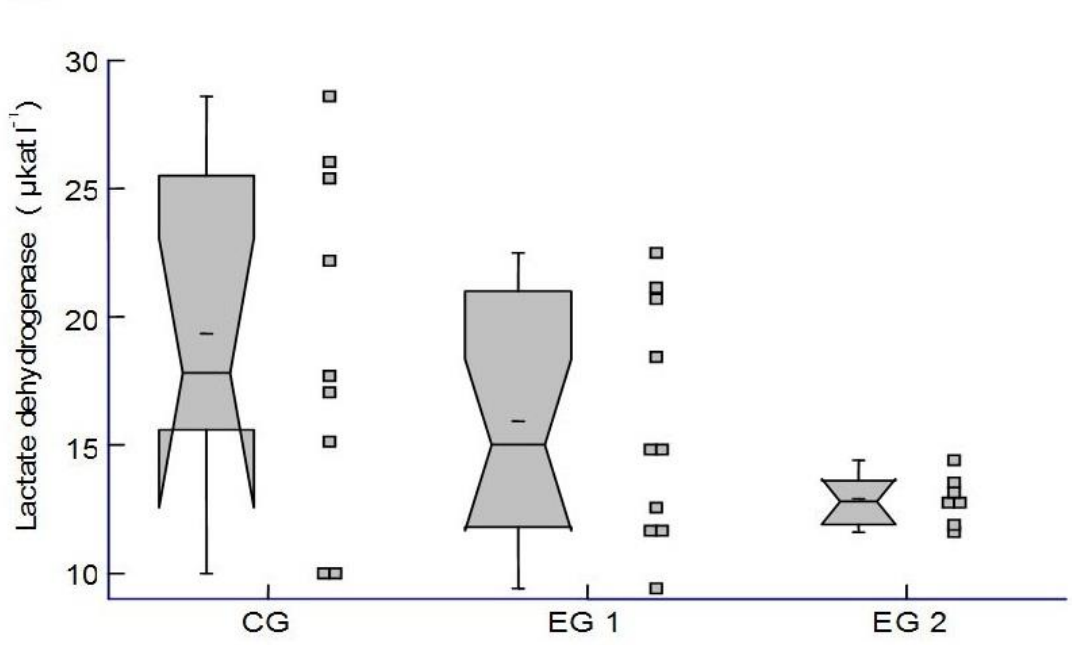

Lactate dehydrogenase (µkat l-1)
30
25
20
15
10
CG
EG 1
EG 2

Fig 5.

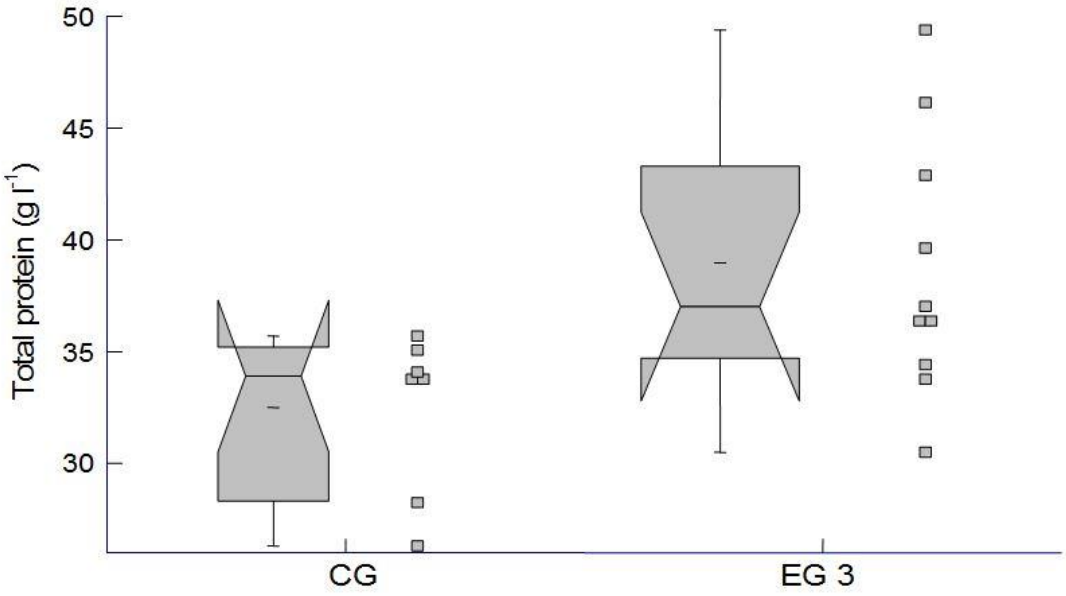

Total protein (g l-1)
50
45
40
35
30
CG
EG 3

Fig 6.

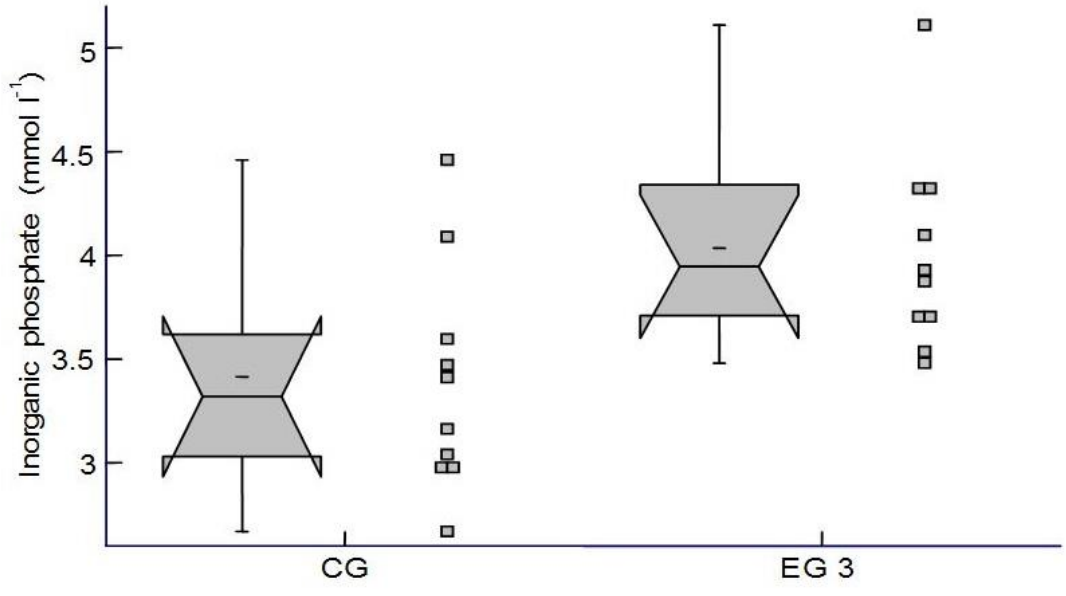

Inorganic phosphate (mmol l-1)
5
4.5
4
3.5
3
CG
EG 3

Fig 7.

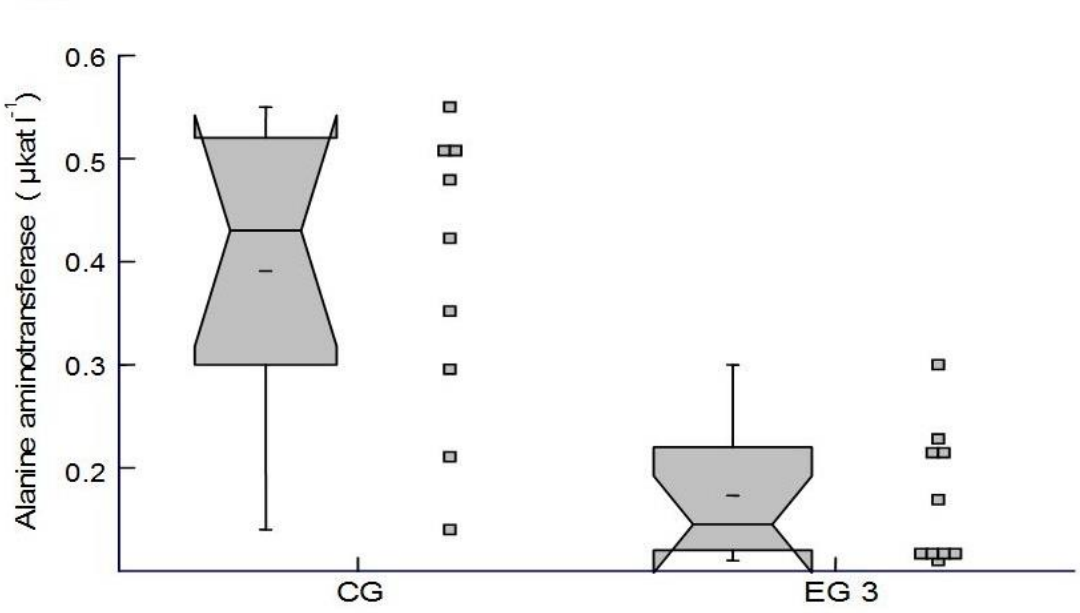

Alanine aminotransferase (µkat l-1)
0.6
0.5
0.4
0.3
0.2
CG
EG 3

Fig 8.

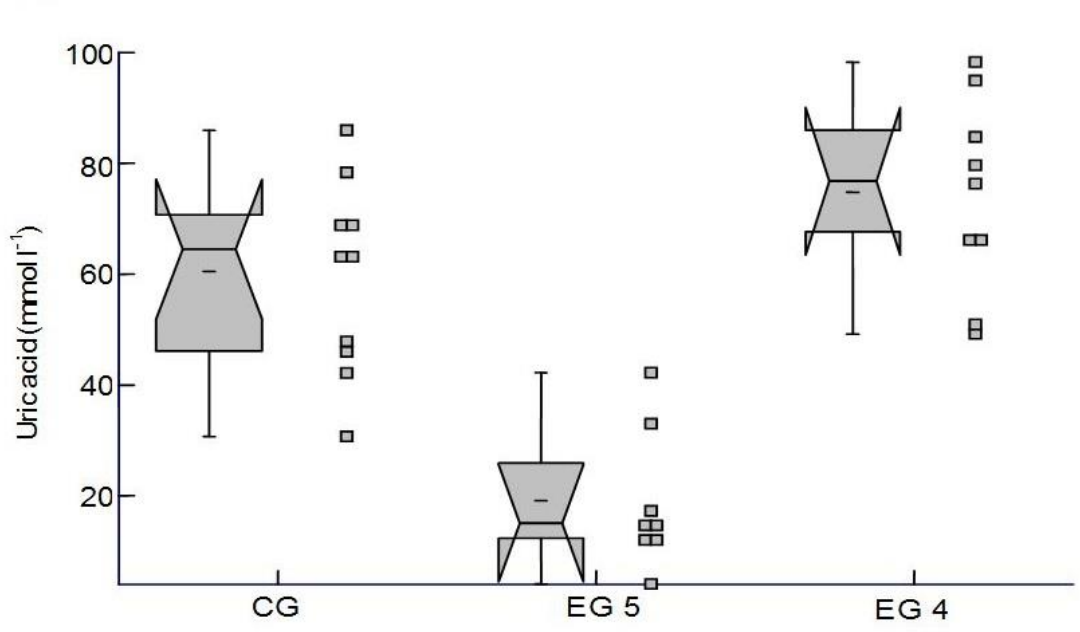

Uric acid (mmol l-1)
100
80
60
40
20
CG
EG 5
EG 4

Fig 9.

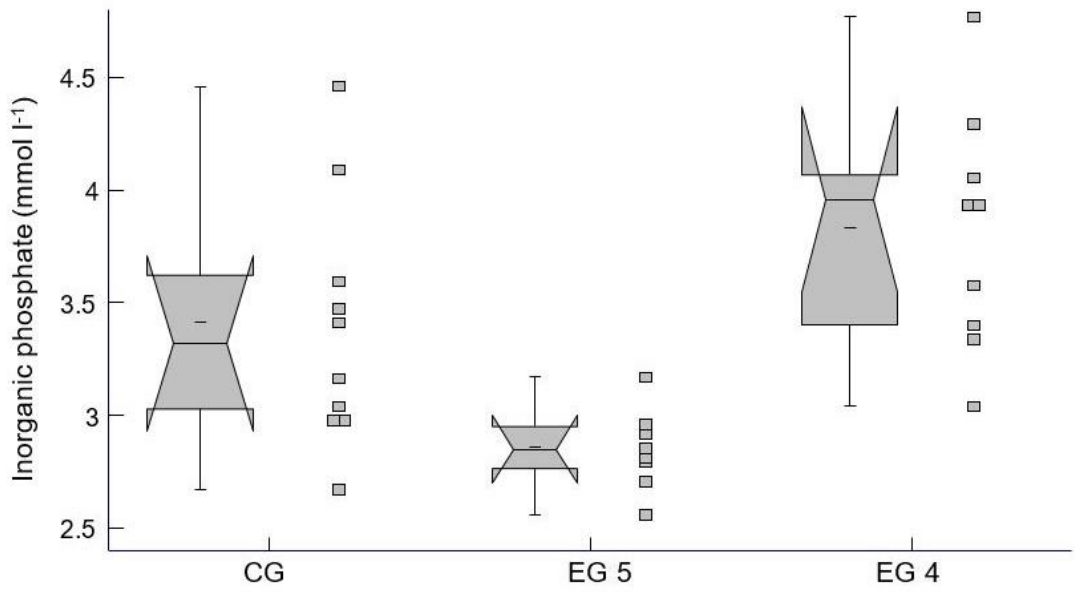

Inorganic phosphate (mmol l-1)
4.5
4
3.5
3
2.5
CG
EG 5
EG 4

Fig 10

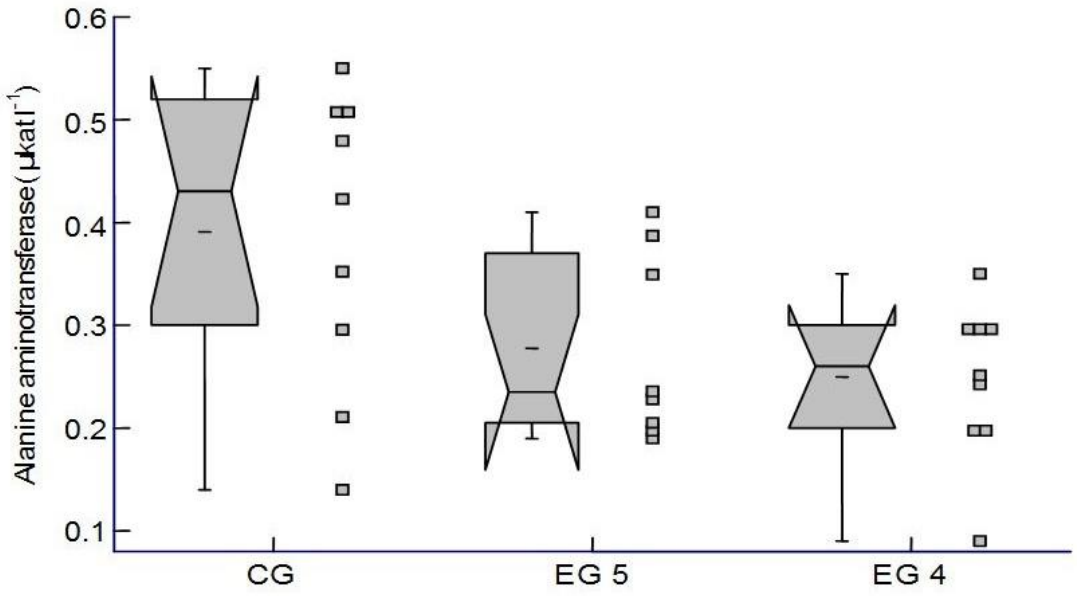

Alanine aminotransferase (µkat l-1)
0.6
0.5
0.4
0.3
0.2
0.1
CG
EG 5
EG 4

Fig 11

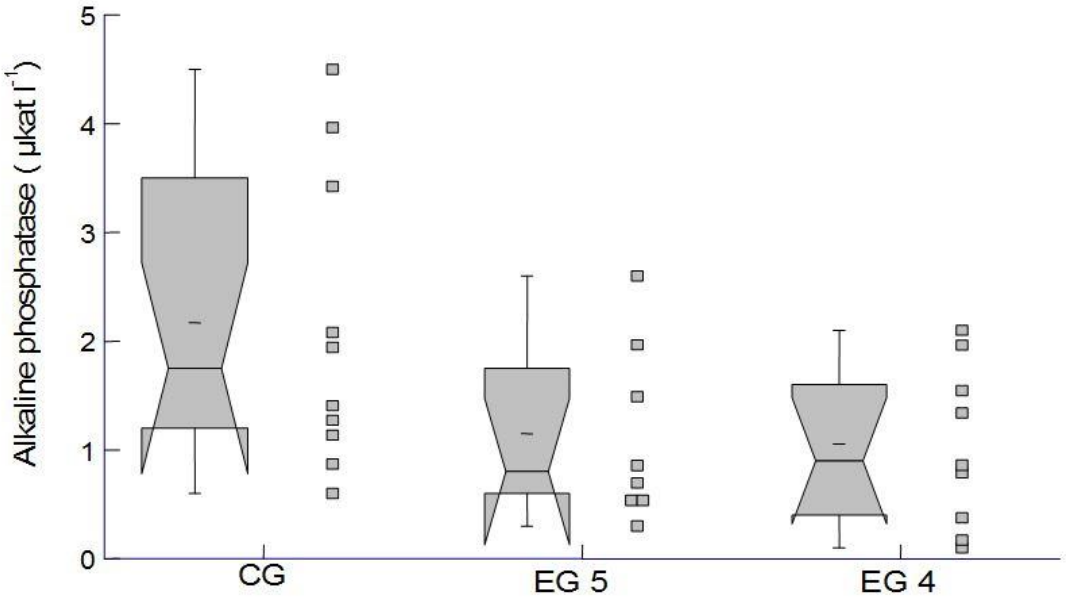


Fig 12

Figs 1-12: *Oncorhynchus mykiss*. Biochemical values in experimental and control rainbow trout offered 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; **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.

The catalytic concentration of AST was lower in EG1 and a reduced catalytic concentration of LD was in EG2 (Fig. 5). Comparisons between the two experimental groups showed a significant ($p \leq 0.05$) increase in the catalytic concentration of AST in EG2 (4.76 – 5.93 [EG2] vs 1.2 – 6.77 [EG1]) (Table 2).

Tab 2: *Oncorhynchus mykiss*. Comparison of biochemical parameters of control rainbow trout (CG) injected with DMSO and fish injected with 50 mg kg $^{-1}$ di(2-ethylhexyl)phthalate (DEHP) (EG 1), 200 mg kg $^{-1}$ DEHP (EG 2), 2 µg kg $^{-1}$ 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) (EG 3), 50 mg kg $^{-1}$ hexachlorocyclohexane (γ-HCH) (EG 4) and 5 mg kg $^{-1}$ γ-HCH (EG 5). Mean ± standard deviation; *probability.

Test	CG	**EG 1**	*t* test	**EG 2**	*t* test	**EG 3**	*t* test	**EG 4**	*t* test	**EG 5**	*t* test
TP g l^{-1}	32.5 ± 3.66	31.8 ± 6.42	0.805	31.3 ± 5.4	0.621	38.9 ± 5.89	0.022*	37.8 ± 11.49	0.247	29.7 ± 4.21	0.202
BUN mmol l^{-1}	0.4 ± 0.17	0.5 ± 0.19	0.363	0.5 ± 0.12	0.558	0.3 ± 0.1	0.132	0.5 ± 0.21	0.692	0.3 ± 0.12	0.115
UA µmol l^{-1}	60.5 ± 17.63	65.1 ± 13.72	0.523	58.7 ± 13.14	0.791	60.5 ± 16.2	1	74.7 ± 17.3	0.094	19.1 ± 12.6	0.000*
CREA µmol l^{-1}	13.8 ± 8.4	12.4 ± 3.73	0.616			15.2 ± 5.8	0.134	16.1 ± 6.69	0.531	17.5 ± 5.57	0.311
GL mmol l^{-1}	4.79 ± 0.86	4.41 ± 0.87	0.340	4.45 ± 0.72	0.346	5.28 ± 0.36	0.127	3.97 ±0.49	0.184	5.45 ± 1.18	0.184
P mmol l^{-1}	3.41 ± 0.54	4.52 ± 0.73	0.001*	4.29 ± 0.64	0.004*	4.04 ± 0.48	0.014*	3.83 ± 0.53	0.108	2.86 ± 0.18	0.011*
Ca_t mmol l^{-1}	3.12 ± 0.34	2.8 ± 0.2	0.028*	2.77 ± 0.21	0.015*	3 ± 0.17	0.36	2.83 ± 0.4	0.116	2.83 ± 0.22	0.063
ALT µkat l^{-1}	0.39 ± 0.14	0.18 ± 0.05	0.002*	0.18 ± 0.05	0.002*	0.17 ± 0.06	0.002*	0.25 ± 0.07	0.024*	0.28 ± 0.09	0.077
AST µkat l^{-1}	6.78 ± 2.88	3.69 ±1.82	0.015*	5.45 ± 0.43	0.207	7.32 ± 0.71	0.601	6.5 ± 3.76	0.865	4.29 ± 2.16	0.065
ALP µkat l^{-1}	2.17 ± 1.35	1.01 ± 0.48	0.027*	0.83 ± 0.25	0.012*	1.77 ± 0.86	0.442	1.06 ± 0.75	0.044*	1.15 ± 0.8	0.080
LD µkat l^{-1}	19.34 ± 6.83	15.93 ± 4.68	0.217	12.9 ± 0.96	0.022*	19.89 ± 7.18	0.868	16.81 ± 6.58	0.450	13.63 ± 4.15	0.072

TP: total protein; BUN: blood urea nitrogen; UA: uric acid; CREA: creatinine; GL: glucose; P: inorganic phosphate; Ca_t: total calcium; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; LD: lactate dehydrogenase

The post-mortem examination of the fish showed a slight accumulation of a serous fluid in the peritoneal cavity in the majority of fish in EG1 and ascites in one fish in EG2.

Histological examination revealed eosinophilic droplets in the epithelium of renal tubules and small variability of fat vacuoles of hepatocytes, especially in EG2 fish.

TCDD

The biochemical profile of this highly persistent organic pollutant, has markedly increased TP and P levels (Fig. 6 and 7) and doubly decreased ALT activity (Fig. 8).

γ-HCH

Fish in EG5 had much lower levels of UA and P (Fig. 9 and 10) and fish in EG4 had a decreased catalytic concentration of ALT and ALP (Fig. 11 and 12). Significant differences ($p \leq 0.05$) between the experimental groups (Table 2) were observed in the rate of increase in glycaemia (3.36 - 4.69 [EG5] vs 3.67 – 7.36 [EG4]) and phosphoraemia (2.56 – 3.17 [EG5] vs 3.04 – 4.77 [EG4]), and there was a tendency towards hyperuricaemia (4.09 – 42.2 [EG5] vs 49.2 – 98.3 [EG4] in fish injected with a higher concentration of γ-HCH.

Marked post mortem changes occurred mainly in fish from EG4, which showed different levels of ascites accompanied in 50% of the fish by hyperaemia on the pyloric caeca and in visceral fat. Enlarged kidney and liver were observed in two cases in fish from EG5.

In EG4 one fish died after 18 days and in EG5 two fish died after 19 days with signs of exophthalmos and a with a large quantity of clear or turbid fluid in the abdominal cavity. There was an intensive hyperaemic demarcation around the point of injection.

Histological examination of the fish injected with γ-HCH at a higher concentration revealed an increased activation of sinusoidal cells in the liver, a fibrous thickening of bile duct walls and a periductal inflammatory cellulisation (Fig. 13a and b). The inflammation infiltrate was well bounded and patches of oedematous seepage were observed around the bile ducts with the presence of inflammatory cells in the thin connective tissue. There were no regressive changes in hepatocytes.

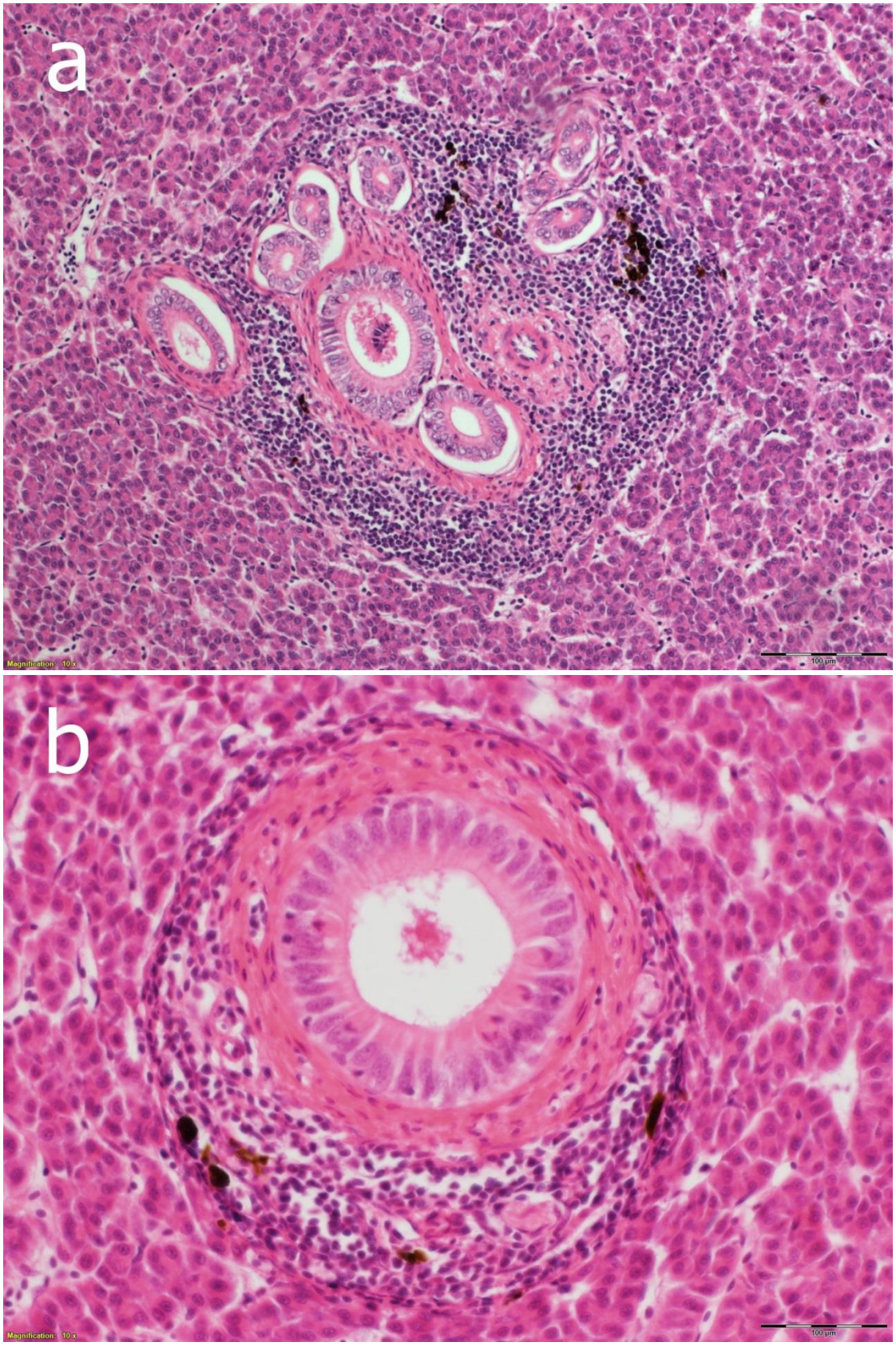
The parasitological and bacteriological examination of the fish at the end of the experiment was negative.

Haematological and genotoxic tests

Erythrocyte polychromasia was observed in 20% to 50% of the fish in both experimental groups injected with γ-HCH. The significant ($p \leq 0.01$) increase in polychromatophilic erythrocytes was up to 0.35, compared to 0.04 in the CG fish.

A significant ($p \leq 0.01$) neutrophilic leucocytosis was a typical feature of the DEHP- and TCDD-injected fish, with a highly increased occurrence of metamyelocytes, compared to the CG fish (0.13 [EG2], 0.18 [EG1] vs 0.01 [CG]) and myelocytes (0.09 [EG3] vs 0.01[CG]). A significant increase in the segmented forms was recorded in the fish from EG3 (0.03) and EG5 (0.03) vs CG 0.01). Compared to the CG fish (0.95), the proportion of lymphocytes was 0.84 in the fish from EG2, 0.76 in the fish from EG1 and 0.88 in the fish from EG3.

The occurrence of micronuclei in all experimental groups as well as the control group ranged between 0.8 and 2.1 ‰ (Table 3).



Figs 13a,b: *Oncorhynchus mykiss*. Cholangitis. (a) Bile duct hyperplasia with associated influx of inflammatory cells. (b) Bile duct showing mild influx of inflammatory cells.

Tab 3: *Oncorhynchus mykiss*. The number of micronuclei per 1000 erythrocytes in individual rainbow trout

	Experimental group					
	CG	EG1	EG2	EG3	EG4	EG5
			n			
	10	10	10	10	9	8
Individual fish	2	5	1	0	2	3
	4	1	1	1	1	1
	2	0	1	2	1	2
	1	3	0	1	0	0
	3	4	0	1	1	4
	1	1	1	1	1	1
	1	2	1	1	2	0
	1	3	2	1	1	2
	1	1	0	0	3	
	2	1	1	0		
Mean (‰)	1.8	2.1	0.8	0.8	1.3	1.6

Discussion and conclusions

As to the results of the analytes used, their major parameters are discussed below. These include, in the case of DEHP testing, the impact on mineral metabolism, manifesting itself as an increase in the level of P and decrease in the concentration of Ca_t; and as for enzyme activities, we focus on the reduction of the catalytic concentrations of both aminotransferases, ALP and LD. To explain the mechanism of the occurrence of γ-HCH-induced hypouricaemia, we would like to draw attention to the impact of this gamma isomer of HCH on reduced UA production or on the reduction of UA reabsorption in the distal tubule, or on the reduction of the tubular secretion of UA, as known in homoeothermic vertebrates given uricosurics (Racek 1999). γ-HCH, like DEHP, reduced the catalytic concentration of ALT and ALP, while having a reverse impact on the level of P.

Hyperproteinaemia in TCDD could be caused by glucocorticoids, particularly cortisol, affecting the metabolism of proteins (Smith 1991).

It is remarkable that all the tested pollutants left the level of GL unchanged, although many authors assert that an increased GL level in blood plasma is a response of the fish to acute toxicity of pollutants (Svobodová 1971; Srivastava 1981; Singh & Srivastava 1982; Mishra & Srivastava 1983; Natarajan 1989), including organophosphates (Gill *et al.* 1990; Balint *et al.* 1995; Sancho *et al.* 1997; Ceron *et al.*1997; Lusková *et al.* 2002). It is of course possible that GL might vary significantly in other periods of the experiment.

Significant differences in GL levels were only observed between the two tested concentrations of γ-HCH (5 or 50 mg kg^{-1}). Mourad *et al.* (1999) demonstrated this in their trials where *Tilapia zillii* were subjected to sub-lethal lindane concentrations of 10 and 20 mg l^{-1}. GL level decreased after 3, 6 and 24 hr, whereas hyperglycaemia was noticed after 48, 96 and 168 hr of exposure. Effects of an acute exposure to lindane on the liver carbohydrate metabolism of rainbow trout exposed to lindane at 0 or 0.05 mg l^{-1} were examined by Soengas *et al.* (1997). A significant increase in plasma glucose was observed in the treated fish, compared to the control after 6 – 12 hr.

All the tested pollutants affected the metabolism of P and, in particular, all suppressed the catalytic activities of the enzymes under study. The increase in the level of plasma phosphate may be due to its release from the destroyed skeleton. The lower activity of transaminase may also be due to a disorder in amino acid metabolism. An increased phosphataemia (7.46 ± 0.243 vs 6.5 ± 0.094) was also recorded when testing a technical mixture of Delor 103 (Řehulka 2002). Trichlorobiphenyl administered i.p. to rainbow trout at a concentration of

0.24 mg kg^{-1} had, after five days of exposure, an effect similar to that observed in the blood serum in chicks after oral administration of trichlorobiphenyl Delor 103 at concentrations of 5, 50 and 100 mg kg^{-1} (Lopuchovský 1986).

Similar results in the differential leucocyte count were obtained in rainbow trout after two months of exposure to phenol at a concentration of 2 mg l^{-1} (Własow 1985) and in carp during pesticide toxicity testing (Svobodová & Pečená 1988).

The γ-HCH concentrations tested by us did not cause histological alterations in the fish liver, as are described for pesticides, especially not those described by Gonzáles de Canales *et al.* (2009), who tested the toxicity of lindane to *Sparus aurata* exposed to 16 μg l^{-1} for 15 days (fatty degeneration and vacuolisation), or by Ortiz *et al.* (2003) who tested toxicity to *Mugil* sp., *Cyprinus carpio* and *Barbus* sp. after an accidental discharge of lindane into the Barbate River (Cádiz, SW Spain) (the hepatic cells appeared compactly arranged with a strong cytoplasmic vacuolisation [steatosis] and an increased basophilia within the cytoplasm of some hepatocytes, hepatocellular necrosis with parenchymal vacuolisation, hypertrophy of hepatocytes, haemorrhages and widening of blood sinusoids), and also Braunbeck *et al.* (1990), who observed hepatic steatosis in zebra fish *Brachydanio rerio* induced by long-term exposure to γ-HCH. However, we should also pay attention to the histopathological changes in the gallblader of *Catla catla* treated with 1.2% lindane for 30 days, as described by Tripathi *et al.* (2012) (crystallization of bile and deformities in the normal structures of epithelial lining and muscular layer).

Eosinophilic droplets have also been noted in salmonids by some authors in association with toxicants (Fergusson 2006).

Our results suggest that, in addition to detecting the environmental exposure to pollutants by measuring the activities of liver detoxification enzymes, including cytochrome P4501A (CYP1A), glutathion-dependent enzymes and UDP-glucuronosyltranspherase, it is helpful also to study metabolic changes. Learning metabolic disorders by using multiple tests also indicates that it would be useful to study certain metabolites' regulatory mechanisms, as demonstrated in the case of phosphoraemia, calcaemia and glycaemia. The findings obtained when examining carbohydrate metabolism provide evidence that there are good reasons for measuring GL dynamics during trials. For a basic indication aimed at confirming the disorders of osseous metabolism, it will be necessary to test repeatedly the variation in the levels of P and Ca_t. The results of testing the activities of both transaminases together with the pathological finding in the kidney provide an impulse for analysing the amino acid spectrum. The results of mineral metabolism, especially P, suggest that the role of catalytic concentration of ALP as an indicator of skeleton disorders should be reviewed. Both concentrations of DEHP suggest that MNT should be used to review mutagenic risks.

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