Effects of metribuzin on rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT: The aim of this study was to assess the effect of metribuzin on rainbow trout (*Oncorhynchus mykiss*). An experimental group of fish was exposed to Sencor 70 WG pesticide product (active substance 70% of metribuzin). The acute semistatical toxicity test lasting 96 h was performed on rainbow trout juveniles. The 96hLC50 value of Sencor 70 WG was 89.3 mg/l. An examination of the haematological and biochemical profile and histopathological tissue examinations were performed on one- to two-year-old rainbow trout after 96 h of exposure to Sencor WG 70 in a concentration of 89.3 mg/l. The experimental group showed significantly lower values ($P < 0.01$) of plasma total proteins, triacylglycerols, aspartate aminotransferase, ammonia, calcium, lactate, alkaline phosphatase, erythrocyte count, haematocrit and significantly higher ($P < 0.01$) values of erythrocyte haemoglobin compared to the control group. A significant decrease ($P < 0.01$) in both the relative and absolute lymphocyte count and a significant increase ($P < 0.01$) in both the relative and absolute count of neutrophile granulocytes were also recorded in the experimental group. The histopathological examination revealed mild proliferation of goblet cells of the respiratory epithelium of secondary gill lamellae and hyaline degeneration of epithelial cells of the renal tubules of the caudal kidney. This alteration of kidney resulted in hypoproteinaemia, followed by the formation of transudate in the body cavity. The metribuzin-based Sencor WG 70 pesticide product was classified among substances harmful to fish.

Keywords: triazine; acute toxicity; haematological profile; biochemical profile of blood; histopathology

Triazines belong to the oldest herbicides, with research on their weed control properties initiated in the early 1950s. As a chemical family, triazines are a group of pesticides with a wide range of use.

Metribuzin is used worldwide as a pre- and post-emergence selective herbicide on grasses and broad-leafed weeds. It is applied to various crops including lucerne, asparagus, maize, potatoes and tomatoes as well as to ornamentals and for landscape maintenance. Metribuzin is applied by various methods including aerial and ground applications and chemigation, (Pauli et al., 1990; Fairchild and Sappington, 2002). Metribuzin was registered as a pesticide for the first time in the U.S. in 1973 (Anderson and Magleby, 1997).

Metribuzin is an asymmetrical triazine herbicide. The systematic name is 4-amino-6-*tert*-butyl-3-(methythio)-1,2,4-triazin-5-one. Common synonyms include Sencor, Bay 94337, and DIC 1468. The empirical formula is $C_8H_{14}N_4OS$ and the mo-

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Projects No. MSM 6007665809 and No. MSM 6215712402) and the Ministry of Environment of the Czech Republic (Project No. SP/2e7/229/07).

lecular weight is 214.3. It is a white, crystalline solid compound with a melting point of 125–126.5°C. It is slightly soluble in water, and soluble to some extent in several organic solvents. Metribuzin is distinct from symmetrical triazines such as atrazine, simazine, ametryn, and prometryn. In the symmetrical triazines, the central ring structure has alternating carbon and nitrogen atoms, whereas metribuzin has two nitrogen atoms and two carbon atoms which are adjacent to each other.

The herbicidal activity of triazines is mediated through the inhibition of photosynthesis (Das et al., 2000) by blocking electron transport during the Hill reaction of photosystem-II (Pauli et al., 1990; DeLorenzo et al., 2001); it binds to a plastoquinine-binding niche on D1 and 32-kD protein encoded by the *psbA* gene of the photosystem-II reaction complex (Das et al., 2000).

Pesticides are recognized as serious pollutants in the aquatic environment with the potential to cause deleterious effects on the biota, especially fish (Verma et al., 1982; Elia et al., 2002).

The extensive use of pesticides contributes to significant improvements in crop yields and farm efficiency. Metribuzin, like other triazine and triazinone herbicides, is prone to run off into surface waters due to its physical and chemical characteristics: water solubility 1.220 mg/l; *K*oc 41; vapour pressure 1.3 mPa; and the soil half-life 30 days (Pauli et al., 1990; Wauchope et al., 1992). Modelling efforts have indicated that metribuzin can reach concentrations as high as 390 g/l in surface water runoff (Pauli et al., 1990). However, the pesticide contamination of fresh water is causing concern with respect to long-term and low-dose effects of pesticides on the public health, as well as to their impact on non-target species. Thus, intensive research on the fate of pesticides in the environment is needed (Sudo et al., 2002; Guasch et al., 2007).

The assessment of the ecotoxicological risks caused by pesticides to ecosystems is based on data on the toxicity and effects of pesticide products on non-target organisms. Fish belong to the group of non-target aquatic organisms. The present paper is a contribution to the assessment of toxicity and effects of a metribuzin-based pesticide on fish.

MATERIAL AND METHODS

The goal was to assess the effect of metribuzin [4-amino-6-*tert*-butyl-3-(methythio)-1,2,4-triazin-5-one] on fish. It was tested in the form of Sencor WG 70 pesticide, the active substance of which was metribuzin in the amount of 70%. The toxic effect was assessed by the results of acute toxicity tests and results of haematological, biochemical and histopathological examination of rainbow trout after exposure to this pesticide.

Acute toxicity

The acute toxicity test on rainbow trout with Sencor WG 70 followed the OECD Direction No. 203 and Methodical Manual ISO 7346/2. Juveniles of rainbow trout (kamloops) of 17.6 ± 3.75 g mean body weight and 128 ± 13 mm mean body length were used for the test. Seven various concentrations and a control were used in the basic test. Ten fish specimens were used for every concentration and also in the control. The test was performed semistatically for 96 h. The bath was changed every 24 h. Basic physical and chemical indices of diluting water used in the acute toxicity test were as follows: acid neutralisation capacity – $ANC_{4.5}$ 1.08 mmol/l; total ammonia 0.03 mg/l; $N0_3^-$ 10.2 mg/l; NO_2^- 0.003 mg/l; PO_4^{3-} 0.02 mg/l; chemical oxygen demand – COD_{Mn} 1.4 mg/l. Water temperatures in the test ranged from 15.9 to 16.2°C, oxygen saturation of water ranged between 86 and 98 %. The LC50, LC0 and LC100 values in the respective time intervals were determined by probit analysis.

Haematological, biochemical and histopathological examination

Haematological, biochemical and histopathological examination of rainbow trout (kamloops) was performed at the end of 96 h acute toxicity test with Sencor WG 70 in a concentration of 89.3 mg/l. At the same time, the control group of trout was examined haematologically, biochemically and histopathologically. Rainbow trout (kamloops) of 290.33 ± 33.62 g average weight and 308 ± 13 mm average body length were used. The test was performed semistatically with the bath exchanged every 24 h. Diluting water had the same physical and chemical parameters as described above. Water temperatures during the test ranged from 14.1 to 14.4°C, oxygen saturation of water was above 60% (ranging from 94 to 99%), pH ranged from 7.96

to 8.24. The test was performed in four aquaria of 200 l volume. Each aquarium was stocked with 15 specimens of one- to two-year-old rainbow trout (one control aquarium, three aquaria with Sencor WG 70 in the concentration of 89.3 mg/l).

Haematological profile after exposure to metribuzin

Heparinised injection needles were used to take samples of blood from the hearts of fish stunned by a blow with a blunt object over the head. To stabilize blood samples, an aqueous solution of heparin sodium salt at 0.01 ml per 1 ml blood was used (Svobodova et al., 1991).

The indices used to evaluate the haematological profile included the erythrocyte count (RBC), haemoglobin concentration (Hb), haematocrit (PCV), mean erythrocyte volume (MCV), mean colour concentration (MCHC), erythrocyte haemoglobin (MCH), leukocyte count (Leuko) and the differential leukocyte count (Leukogram). The procedures were based on Unified Methods for Haematological Examination of Fish (Svobodova et al., 1991).

The results of haematological examinations were tested by the analysis of variance (ANOVA – Tukey's test) using the Statistica 7.0 software.

Biochemical blood plasma profile after exposure to metribuzin

Blood plasma was obtained by the centrifugation of blood samples in a cooled centrifuge (4°C, 837 × g). Biochemical indices determined in the blood plasma included glucose (GLU), total proteins (TP), ammonia (NH_3), triacylglycerols (TAG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), calcium (Ca^{2+}), lactate (LACT), cortisol, cholinesterase (ChE), alkaline phosphatase (ALP) and inorganic phosphate (PHOS). A VETTEST 8008 analyzer (IDEXX Laboratories Inc., U.S.A.) manufactured by Medisoft was used for the biochemical analysis of blood plasma. The analyzer uses dry chemical and colorimetric analysis techniques. Selective test discs (Multi-layer film slides, Kodak) were used for the evaluation by a laser reading bar codes. LACT and ChE were determined with a COBAS MIRA automatic analyser (Hoffman, La Roche Co., Switzerland) using BioVendor tests No. 12061 and 12351. The plasma cortisol level was measured by a commercial radioimmunoassay (RIA) using Cortisol RIA kit from Immunotech Prague (Beckman Coulter Company).

The results of biochemical examination were tested by the analysis of variance (ANOVA – Tukey's test) using the Statistica 7.0 software.

Histopathological examination of tissues

After blood sampling, samples of gills, liver, skin, cranial and caudal kidney and spleen were taken for histopathological examinations. These samples were immediately fixed in 10% formalin, drained and embedded in paraffin. Sections were made of the paraffin blocks and stained with haematoxylin-eosin.

RESULTS

Acute toxicity

On the basis of the tests of acute toxicity to rainbow trout, the 96-hour lethal concentrations of Sencor WG 70 were determined (96hLC50 89.3 mg/l, 96hLC0 48.6 mg/l and 96hLC100 164.1 mg/l). The 96hLC50 is the basic value in the acute toxicity test. For rainbow trout juveniles the 96hLC50 value was 89.3 mg/l of Sencor WG 70 product, which corresponded to 62.51 mg/l of metribuzin. In the course of metribuzin poisoning in rainbow trout, the following clinical symptoms were observed: accelerated respiration, loss of movement coordination, fish lying on their flanks and moving in this position. The subsequent short excitation stage (convulsions, jumps above the water surface, movement in circles) changes into a resting stage and another short-time excitation follows again. In the end, fish fall into damp, moving mainly on their flanks. The respiration is slowed down, and the damp phase and subsequent agony are very long.

The autopsy performed after the acute toxicity test revealed increased amounts of watery mucus on body surfaces, the skin was matt dark in colour and the ventricle expansion was observed. The body cavity contained transudate, and an increased injection of visceral vessels was also obtained (Figure 1).

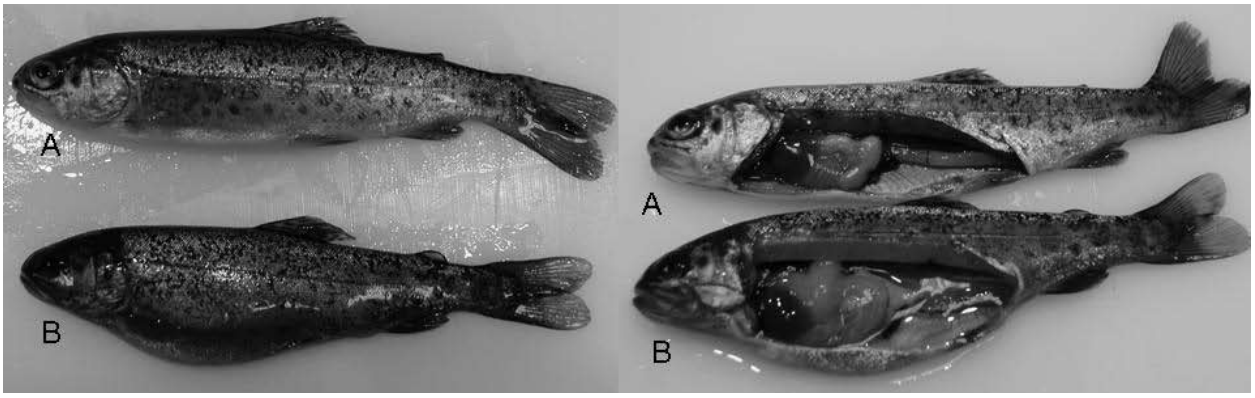

Figure 1. A – control rainbow trout; B – experimental rainbow trout after acute exposure to metribuzin – dark colour body, transudate in the body cavity

Haematological profile after exposure to metribuzin

The results of the erythrocyte profile of control and experimental rainbow trout under study are given in Table 1. Compared to the control specimens, those after the acute exposure to metribuzin had a significantly lower erythrocyte count ($P < 0.01$) and haematocrit, and significantly higher ($P < 0.01$) erythrocyte haemoglobin. The values recorded for Hb, MCV, MCHC, Leuko were comparable in both groups under study.

It was evident that the acute exposure to metribuzin resulted in a significant decrease ($P < 0.01$) in both the relative and absolute lymphocyte count and a significant increase ($P < 0.01$) in both the relative and absolute count of segmented neutrophile granulocytes and band neutrophile granulocytes in the experimental group. The results of examinations of the leukocyte profile of control and experimental rainbow trout are given in Table 2.

Biochemical blood plasma profile after exposure to metribuzin

Table 3 shows the results of the biochemical blood plasma profile of control and experimental rainbow trout under study. The experimental rainbow trout exposed to acute effects of the metribuzin-based pesticide showed a significant ($P < 0.01$) decrease in total proteins, triacylglycerols, aspartate aminotransferase, ammonia, calcium, lactate and alkaline phosphatase in blood plasma. The rest of the indices (GLU, ALT, LDH, CK, cortisol, ChE and PHOS) were comparable in the two groups during the study.

Histopathological examination of tissues

The histopathological examination revealed severe hyaline degeneration of epithelial cells of the renal tubules of the caudal kidney (Figure 2), mild

Table 1. Derived haematological parameters in rainbow trout affected by acute exposure to Sencor WG 70

Indices	Control group (n = 15) $\bar{x}$ ± SD	Experimental group (n = 15) $\bar{x}$ ± SD
Er (T/l)	1.53 ± 0.21[a]	1.19 ± 0.22[b]
Hb (g/l)	50.77 ± 5.73[a]	46.23 ± 6.77[a]
PCV (l/l)	0.40 ± 0.06[a]	0.31 ± 0.06[b]
MCV (fl)	266.62 ± 48.86[a]	295.61 ± 57.61[a]
MCH (pg)	33.70 ± 5.23[a]	39.86 ± 6.93[b]
MCHC (g/l)	127.78 ± 14.26[a]	136.41 ± 19.89[a]

Groups with different alphabetic superscripts differ significantly at $P < 0.01$ (ANOVA)

Table 2. Leukocyte differential counts in rainbow trout affected by acute exposure to Sencor WG 70

Indices		Control group (n = 15) $\bar{x}$ ± SD	Experimental group (n = 15) $\bar{x}$ ± SD
Leukocytes	G/l	26.40 ± 11.66[a]	19.73 ± 8.29[a]
Lymphocytes	%	89.45 ± 8.88[a]	60.05 ± 24.71[b]
	G/l	23.76 ± 11.67[a]	11. 66 ± 7.59[b]
Monocytes	%	4.81 ± 3.77[a]	7.62 ± 6.60[a]
	G/l	1.25 ± 0.94[a]	1.57 ± 1.89[a]
Neutrophile granulocyte segments	%	2.04 ± 2.43[a]	12.71 ± 14.47[b]
	G/l	0.49 ± 0.59[a]	2.61 ± 1.95[b]
Neutrophile granulocyte bands	%	3.71 ± 3.91[a]	18.44 ± 13.31[b]
	G/l	0.90 ± 1.00[a]	3.26 ± 1.74[b]

Groups with different alphabetic superscripts differ significantly at $P < 0.01$ (ANOVA)

proliferation of goblet cells of the respiratory epithelium of the secondary gill lamellae (Figure 3) and hydropic degeneration of hepatocytes around the central veins in the experimental group (at the concentration of 89.3 mg/l Sencor WG 70). No histopathological changes were demonstrated in the tissues (skin, spleen, cranial kidney) of rainbow trout following after the exposure to metribuzin.

DISCUSSION

No mortality of fish was observed in the control aquarium in the course of the 96 h toxicity test of metribuzin-based triazine product Sencor WG 70 on rainbow trout juveniles. The oxygen saturation of water did not drop below 60% in any concentration tested, nor in the control group. The presence

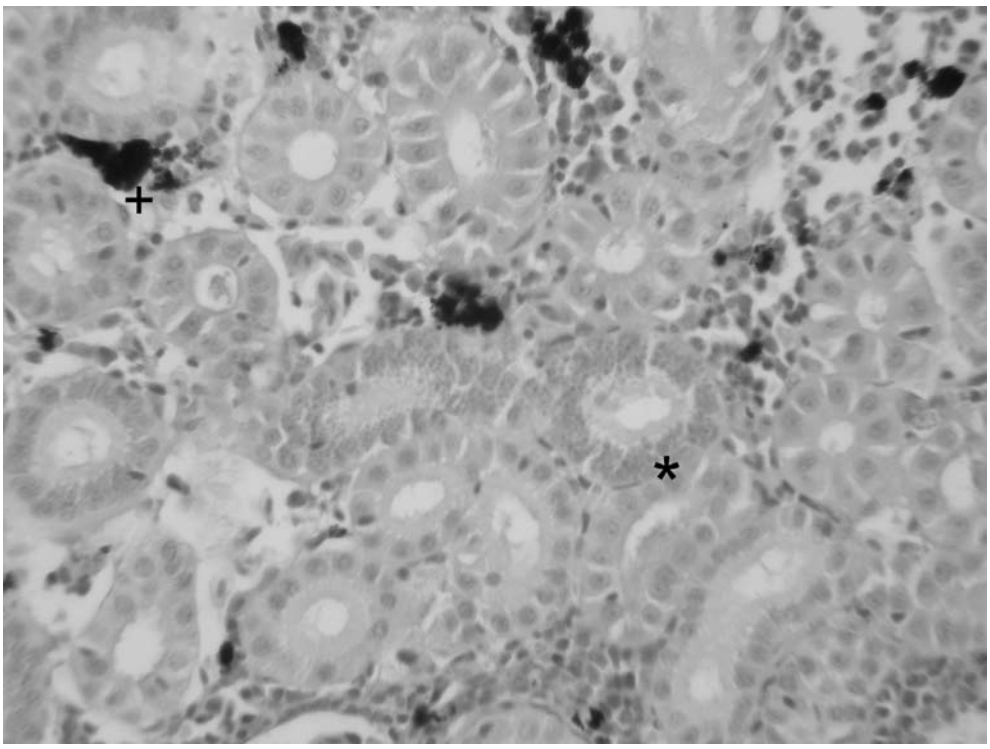

Figure 2. Caudal kidney. Hyaline degeneration of tubular epithelial cells (asterisk). Intertubular haemopoietic tissue and melanomacrophages (cross) are also seen; haematoxylin and eosin, 400×

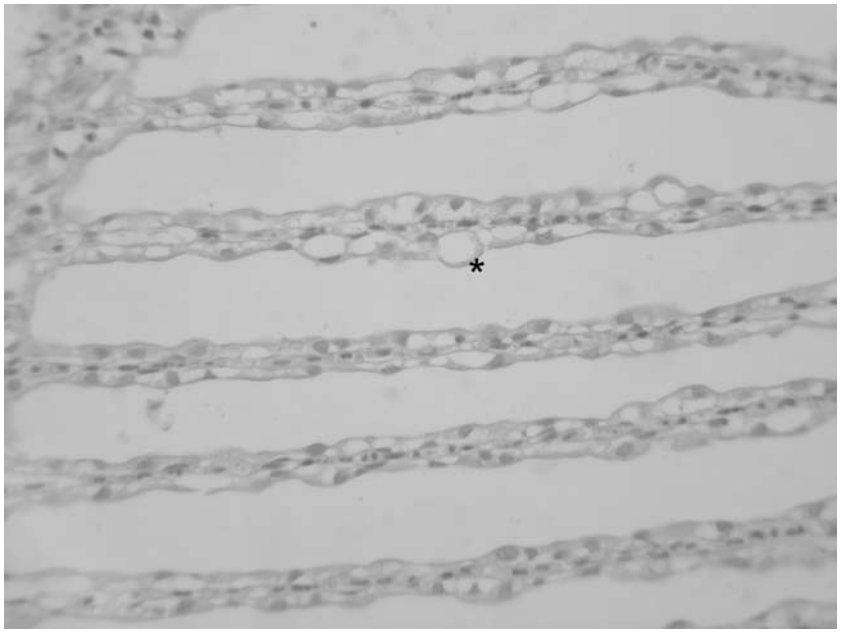

Figure 3. Secondary gill lamellae with mild proliferation of goblet cells. The asterisk shows one of the goblet cells; haematoxylin and eosin, 400×

of the tested substance (above 80% of the nominal concentration) was provided by means of daily exchange of the testing bath. If these conditions are satisfied, the test may be considered valid. On the basis of the observed value of 96hLC50 (89.3 mg/l), the product Sencor WG 70 can be included in a group of substances that are harmful to fish: the risk sentence R52 states that the value of 96hLC50 is 10–100 mg/l (Act No. 356/2003 in the Czech Statute-Books). The value of 96hLC50 for Sencor

Table 3. Derived biochemical indices of blood plasma in rainbow trout affected by acute exposure to Sencor WG 70

Indices	Control group (n = 15) $\bar{x}$ ± SD	Experimental group (n = 15) $\bar{x}$ ± SD
GLU (mmol/l)	3.97 ± 0.67[a]	4.69 ± 2.67[a]
TP (g/l)	48.40 ± 4.90[a]	24.40 ± 9.73[b]
NH_3 (µmol/l)	807.67 ± 100.88[a]	560.53 ± 71.07[b]
TAG (mmol/l)	0.60 ± 0.25[a]	0.15 ± 0.13[b]
AST (µkat/l)	5.33 ± 1.39[a]	3.42 ± 0.94[b]
ALT (µkat/l)	0.28 ± 0.22[a]	1.56 ± 2.58[a]
LDH (µkat/l)	31.09 ± 4.89[a]	32.16 ± 3.31[a]
CK (µkat/l)	23.48 ± 5.16[a]	25.20 ± 4.83[a]
Ca^{2+} (mmol/l)	3.11 ± 0.12[a]	1.83 ± 0.39[b]
LACT (mmol/l)	2.87 ± 1.47[a]	1.68 ± 0.46[b]
Cortisol (mmol/l)	112.87 ± 83.76[a]	117.77 ± 72.69[a]
ChE (µkat/l)	3.00 ± 1.62[a]	2.29 ± 0.96[a]
ALP (µkat/l)	0.94 ± 0.18[a]	0.52 ± 0.17[b]
PHOS (mmol/l)	4.31 ± 0.59[a]	3.97 ± 1.04[a]

Groups with different alphabetic superscripts differ significantly at $P < 0.01$ (ANOVA)

WG 70–89.3 mg/l essentially corresponds to 62.51 mg/l metribuzin. The values observed by us were in agreement with those reported by other authors who determined the toxicity of metribuzin to various species of fish. Mayer and Ellersieck (1986) reported the values of LC50 for rainbow trout between 64 and 76 mg/l metribuzin. Waynon and Finley (1980) determined the value of LC50 42 mg/l for rainbow trout. Hudson et al. (1984) reported the value of LC50 70 mg/l metribuzin for rainbow trout. Fairchild and Sappington (2002) reported the values of 96hLC50 76 mg/l metribuzin for fish.

In the course of metribuzin poisoning in rainbow trout, the following clinical symptoms were observed: accelerated respiration, loss of movement coordination, fish lying on their flanks and moving in this position. Similar changes were also reported by Hussein et al. (1996) in *Oreochromis niloticus, Chrysichthyes auratus* and by Saglio and Trijasse (1998) in *Carassius auratus* following the acute poisoning with atrazine.

Haematological and biochemical profiles of blood can provide important information about the internal environment of the organism (Masopust, 2000). The main haematological response of rainbow trout to the acute effect of metribuzin-based product was a significantly ($P < 0.01$) lower erythrocyte count, haematocrit, lymphocyte count and significantly higher ($P < 0.01$) erythrocyte haemoglobin and neutrophile granulocyte count compared to the control group. The reduction in erythrocyte count, haematocrit value and higher erythrocyte haemoglobin of rainbow trout in the present study can be attributed to the following factors: (1) haemodilution of blood due to the damage of fish organs (Morgan et al., 1980; Sweilum, 2006), and (2) the haematological parameters Ht, RBC and Hb, whose changes can be interpreted as a compensatory response that improves the O_2 carrying capacity to maintain the gas transfer, also indicate a change in the water-blood barrier for gas exchange in gill lamellae (Jee et al., 2005). The haematological results indicated a decrease in nonspecific immunity. Similar changes in the haematocrit value, lymphocyte, erythrocyte and monocyte counts and in segmented neutrophile granulocytes were also reported by Svobodova and Pecena (1988) in carp following the acute poisoning with atrazine.

The main biochemical response of rainbow trout to the acute effect of metribuzin-based product was a significant ($P < 0.01$) decrease in total proteins, triacylglycerols, aspartate aminotransferase, ammonia, calcium, lactate and alkaline phosphatase compared to the control group. The activities of plasma enzymes (ALT, AST, LDH and CK) are also used as a relevant stress indicator (Svoboda, 2001). Mekkawy et al. (1996) observed a significant increase ($P < 0.05$) in GLU and a significant decrease ($P < 0.05$) in TP levels in *Oreochromis niloticus* and *Chrysichthyes auratus* after acute exposure to atrazine in a concentration of 3 mg/l. Davies et al. (1994) reported a decrease in TP in rainbow trout after acute exposure to atrazine in a concentration of 50 µg/l. Prasad and Reddy (1994) observed a decrease in serum calcium in Mozambique tilapia (*Tilapia mossambica*) after exposure to atrazine. On the other hand, Neskovic et al. (1993) found an increase in ALT activity in rainbow trout after exposure to atrazine and Waring and Moore (2004) recorded an increase in cortisol levels in Atlantic salmon (*Salmo salar*) after exposure to atrazine.

We observed teleangiectasiae of the hyaline degeneration of epithelial cells of renal tubules of the caudal kidney and mild proliferation of goblet cells of the respiratory epithelium of the secondary gill lamellae. Histopathological changes are suggestive of disorders in the cellular metabolism of ions. The proliferation of gill goblet cells is a non-specific reaction to toxic irritation. A similar change like hyperplasia of gill epithelial cells was also reported by Neskovic et al. (1993) in common carp (*Cyprinus carpio*) exposed to atrazine in a concentration 1 500 µg/l. A similar change like hyperplasia of gill epithelial cells was also reported by Oropesa-Jimenaz et al. (2005) in carp following the acute poisoning with simazine. Gross morphological anomalies in the gill epithelium of yearling coho salmon (*Oncorhynchus kisutch*) exposed to the herbicide atrazine (15 µg/l for 114 h) included necrosis, desquamation, hypertrophy and hyperplasia, and teleangiectasiae (Meyer and Hendricks, 1985). On the other hand, (Biagianti-Risbourg and Bastide, 1995) reported that atrazine affects different tissues in fish, particularly the liver tissue which shows a substantial increase in the size of lipid inclusions followed by lipoid degeneration, enlargement of secondary lysosomes, mitochondrial malformation and vacuolization, and a reduction in glycogen content.

After the acute toxicity test to metribuzin we performed an autopsy and observed a transudate in the body cavity. We can assume that the increasing escape of proteins occurred due to the damage of epithelial cells of renal tubules. As a result

marked hypoproteinaemia (from 48.40 ± 4.90 g/l to 24.40 ± 9.73 g/l) was found in the blood plasma, which caused the formation of transudate in the body cavity. Svobodova et al. (1987) reported a transudate in the body cavity in rainbow trout after acute exposure to atrazine.

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Received: 2007–08–24
Accepted after corrections: 2008–05–23

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