The effects of Click 500 SC (terbuthylazine) on common carp *Cyprinus carpio* under (sub)chronic conditions

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Abstract

OBJECTIVES: Effects of the herbicide formulation Click 500 SC (terbuthylazine 500 g/l) on common carp *Cyprinus carpio* were assessed through biometric, biochemical, haematological and antioxidant indices, induction of xenobiotic metabolizing enzymes and histological examination of selected tissues.
DESIGN: The fish were exposed to the formulation with terbuthylazine concentrations of 380 ng/l (environmental concentration); 60 µg/l and 550 µg/l for up to 91 days. Haematological indices were assessed using unified methods of haematological examination in fish. Biochemical indices in plasma were measured by biochemical analyzer, ferric reducing ability of plasma (FRAP) and ceruloplasmin activity were determined spectrophotometrically. Concentration of total cytochrome P450, glutathione-S-transferase activity and glutathione content were assessed spectrophotometrically in liver. Activity of liver ethoxyresorufin-O-deethylase (EROD) activity was measured spectrofluorimetrically. Histopathological examination of liver, skin, gills, spleen, cranial and caudal kidney was performed by light microscopy.
RESULTS: An increase ($p<0.05$) was observed in hepatosomatic index and condition factor in fish from the environmental concentration. A decrease ($p<0.05$) in haemoglobin and mean corpuscular haemoglobin concentration (MCHC) was found in fish treated with terbuthylazine of 550 µg/l. There was a decline in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) ($p<0.05$) in terbuthylazine of 60 µg/l and 550 µg/l. Triglycerides (TAG) ($p<0.01$) were elevated in all pesticide-treated groups. Alanine aminotransferase (ALT) ($p<0.01$) and phosphorus ($p<0.05$) decreased in fish exposed to terbuthylazine of

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60 µg/l and 550 µg/l, while albumin ($p<0.01$) rised in the same groups. An elevation in natrium ($p<0.05$) in terbuthylazine of 550 µg/l and a rise in protein ($p<0.01$) in the concentrations of 380 ng/l and 550 µg/l were observed. Correlations between several indices were significant. Ceruloplasmin activity and FRAP were augmented ($p<0.01$) in the highest concentration tested. Examined xenobiotic detoxification systems were not significantly affected by the exposure. Non-specific histopathological changes were found in the gills and skin of the test fish.

CONCLUSION: The fish treated with terbuthylazine developed a disorder in several haematological and plasma biochemical indices. The levels of markers of oxidative stress increased in response to the exposure. Examined systems involved in detoxification of xenobiotics did not reflect long-term contact with the herbicide. Detected histological lesions were non-specific. The environmental concentration of terbuthylazin affected biometric indices of the test fish.

Abbreviations:

alb	- albumin
ALT	- alanine aminotransferase
AST	- aspartate aminotransferase
b. w.	- body weight
CF	- condition factor
CYP	- cytochrome P450
DMSO	- dimethylsulfoxide
EROD	- ethoxyresorufin-O-deethylase
FRAP	- ferric reducing ability of plasma
glc	- glucose
GC/IT-MS	- gas chromatography with ion trap mass spectrometry
GSH	- glutathione
GST	- glutathione-S-transferase
Hb	- haemoglobin
HSI	- hepatosomatic index
i. p.	- intraperitoneal
LDH	- lactate dehydrogenase
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
MCV	- mean corpuscular volume
PPD	- p-phenylenediamine
PCV	- packed cell volume, haematocrit
RBC	- red blood cell count
SL	- standard length
TAG	- triglycerides
TP	- total protein
WBC	- white blood cell count
XME	- xenobiotic metabolizing enzymes

INTRODUCTION

Terbuthylazine (6-chloro-N-(1,1-dimethylethyl)-N-ethyl-1,3,5-triazine-2,4-diamine) is a triazine compound used as a broad spectrum pre- or postemergence herbicide in maize, sorghum, vines, citrus, potatoes, legumes and in forestry. It acts as a photosynthesis inhibitor (Roberts *et al.* 1998). The usage of terbuthylazine-based plant protection products in Europe has increased manifold since withdrawal of authorisations for those containing atrazine in 2004, resp. 2007 (2004/248/EC). Terbuthylazine is becoming a frequent contaminant of the aquatic environment (Carafa *et al.* 2007; Fava *et al.* 2010), its half-life in riverwater ranges from 196 to 331 days (Navarro *et al.* 2004). In the Czech Republic the maximum concentration of terbuthylazine detected in 2009 was 2.6 µg/l and 0.21 µg/l in the surface water and in a water-supply reservoir, resp. (Kodes *et al.* 2010). However, there is only scarce information on its influence on fish. The long-term exposure to other triazines, such as terbutryn, simazine or metribuzine has been shown to e.g. affect haematological and biochemical profile of *Cyprinus carpio* (Modra *et al.* 2008; Velisek *et al.* 2009), growth of *Danio rerio* (Plhalova *et al.* 2009) and induce histopathological changes in fish tissues (Oropesa-Jimenez *et al.* 2005; Velisek *et al.* 2009). Adverse effects of triazine compound atrazine on fish have been studied extensively (e.g. Neskovic *et al.* 1993; Saglio & Tijasse 1998; Spano *et al.* 2004).

The aim of our study was to assess the long-term influence of a commercial terbuthylazine-based formulation on common carp *C. carpio* through biometric, biochemical, haematological and antioxidant indices, induction of xenobiotic metabolizing enzymes and histological examination of selected tissues. The concentrations of terbuthylazine tested included the environmental concentration detected in the Czech Republic.

MATERIAL AND METHODS

Test solutions

The experiment was conducted in a flow-through system. The test concentrations were prepared by dissolving an appropriate amount of the formulation in 10 ml of 10% dimethylsulfoxide (DMSO) and application into the stock tanks with dechlorinated tap water, from which it flowed into the test tanks with fish (pH 7.1–7.9; temperature 18.5–21.5 °C; oxygen saturation ≥ 60%). The solution volume (200 l) was exchanged twice a day. The volume of DMSO was identical for all concentrations tested. The control fish were subjected to dechlorinated tap water with the same concentration of DMSO (0.0005%). The active substance of Click 500 SC is terbutylazine (500 g/l). The producer does not disclose other components of the formulation. Test solutions were sampled and the concentrations of terbuthylazine were measured 13 times within the experiment. Actual concentrations were as follows; 380 ng/l – environmental concentration (group A); 60 µg/l (group B) and 550 µg/l (group C). These are further used to refer to the test groups. Terbuthylazine concentrations exceeded 80% of nominal concentrations over the test period.

Test animals

Common carp *C. carpio* (135.8 ± 32.4 g, SL: 17.1 ± 1.4 cm) were obtained from a commercial fish farm. After 14-day acclimation to laboratory conditions (a photoperiod of 12/12), the fish were randomly dis-

tributed into ten tanks (volume of 200l). The fish were fed commercial pellets (Coppens International bv) at a total rate of 1.5% body weight. The feed amount was recalculated once a month. The fish from each concentration were sampled (8 to 14 specimens) on day 28; 63 and 91. Individual blood samples were taken by cardiac puncture and stabilized with aqueous solution of heparin (50 IU per ml of blood). The fish were euthanized, their body weight and standard lenght were recorded. Samples of tissues and the whole liver (hepatopancreas) were removed. The sections of liver which were not used for histological examination were stored at –85 °C until further analyses were performed.

Biometric parameters

The condition factor (CF) of each fish was calculated with the following formula: CF = (b.w. [g]/SL [cm]^3) × 100. The hepatosomatic index (HSI) was calculated according to the formula HSI = liver weight/b. w. × 100.

Haematological parameters

RBC, WBC, PCV, Hb, MCV, MCH and MCHC (for abbreviations see the list of abbreviations) were determined according to Svobodova *et al.* (1991).

Biochemical profile

Analysis of biochemical indices in plasma (glucose, albumin, total protein, triglycerides, total calcium, inorganic phosphorus, chloride, potassium, sodium, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase) was performed using the biochemical analyzer Konelab 20i and commercial test kits (BioVendor).

Ceruloplasmin

Ceruloplasmin activity was determined according to Ceron & Martinez-Subiela (2004) with slight modifications. The analysis was conducted using Varioskan Flash spectral scanning multimode reader (Thermo Scientific). Plasma samples (15 µl) were mixed with PPD solution (100 µl; 41 mg PPD/25 ml of sodium acetate buffer – pH 5.2) in a 96-well plate. The absorbance was recorded every 60 s for 30 min (550 nm, 37 °C). At each point, the absorbance of blank (PPD solution in distilled water) was deducted from the obtained values. The results were expressed as the amount of the absorbance increase per minute × 10,000.

FRAP

The ferric reducing ability of plasma samples was measured on biochemical analyzer Konelab 20i according to Benzie & Strain (1996) including slight modifications (Haluzova *et al.* 2010).

Xenobiotic metabolizing enzymes and biomarkers

Total CYP, glutathione content and activities of EROD and GST were determined in the liver samples.

CYP and EROD activity

Liver samples were homogenised in buffer (pH 7.4), centrifuged (10,000 g, 20 min, 4 °C) and the supernatant was re-centrifuged again (100,000 g, 1 h at 4 °C). The final supernatant was drained and the pellet was washed and resuspended in the buffer. Each suspension was stored at –85 °C until enzymatic assays. Concentrations of microsomal protein were determined according to Lowry *et al.* (1951). The activity of EROD was examined spectrofluorimetrically (Chang & Waxman 1998; Nilsen *et al.* 1998; Rutten *et al.* 1992). Quantities of total CYP were determined spectrophotometrically. For details see Siroka *et al.* (2005).

GSH, GST

After homogenisation in buffer (pH 7.2), liver samples were centrifuged (10,500 g, 20 min, at 4 °C) and the supernatants were used for determination. Protein concentration was quantified with the Bicinchoninic Acid Protein Assay Kit (Sigma–Aldrich) using bovine serum albumin as a standard. The activity of GST was determined spectrophotometrically (Habig *et al.* 1974), glutathione was measured spectrophotometrically using Ellman's method (1959).

Histological examination

Samples of skin, gills, liver, spleen, cranial and caudal kidney of three fish from each concentration tested and from the control group were fixed in buffered 10% neutral formalin and treated for histological examination (hematoxylin and eosin).

Determination of terbuthylazine in water

Gas chromatography with ion trap mass spectrometry (GC/IT-MS) was used for determination of terbuthylazine. Sample preparation was based on simple liquid-liquid extraction into hexan. Separation, identification and quantification of terbuthylazine were based on the GC/IT-MS method. Gas chromatograph Varian 450-GC (Varian Inc., USA) and VF-5ms (30 m × 0.25 mm) column were used for separation of terbuthylazine. Varian 220-MS (Varian Inc., USA) ion trap mass spectrometer was used for identification and quantification. Chromatographic and MS conditions were based on method described by Perreau & Einhorn (2006). All solvents were GC/MS-grade purity (Chromservis, s.r.o., CZ). Certified standard of terbuthylazine was purchased from Dr. Ehrenstorfer GmbH (Germany). Detection limit (3σ) of terbuthylazine was 0.01 µg/l. Expanded uncertainty was 6.0% on condition that coefficient of expansion was k = 2.

Statistical analysis

Standard descriptive statistics was used for the analysis; parameters were described by mean of 15–28 determinations and its confidence interval. Several parameters with non-normal distribution (glucose, Cl^-, K^+, LDH and EROD) were described by geometric mean and

Tab. 1. Spearman's correlation coefficient between selected parameters.

Parameters	Spearman's corr. coefficient	Parameters	Spearman's corr. coefficient
alb × TP	0.259*	CF × TP	0.196*
TP × TAG	0.330**	glc × K^+	0.248*
TP × Ca	0.488**	alb × Ca	0.508**
HSI × TAG	0.424**	Na × Cl^-	0.634**
HSI × TP	0.035	TAG × Ca	0.059
CF × TAG	0.168		

* statistically significant at $p<0.05$
** statistically significant at $p<0.001$

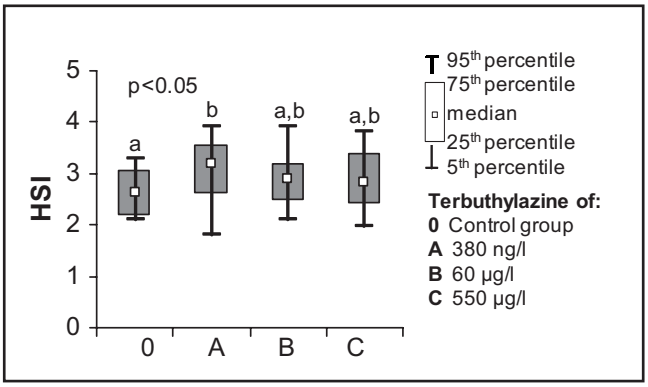

Fig. 1. Hepatosomatic index (HSI) of the fish exposed to Click 500 SC (terbuthylazine), 22–28 determinations.

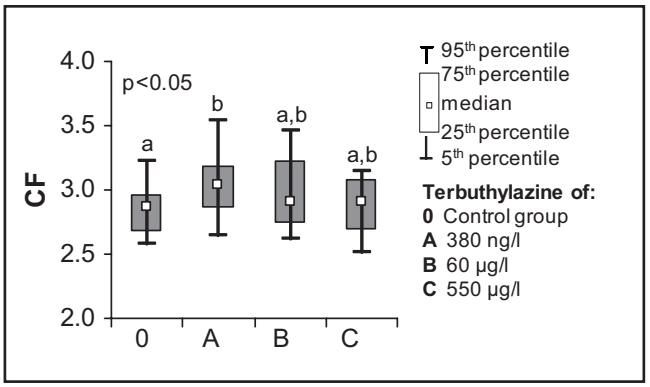

Fig. 2. Condition factor (CF) of the fish exposed to Click 500 SC (terbuthylazine), 22–28 determinations.

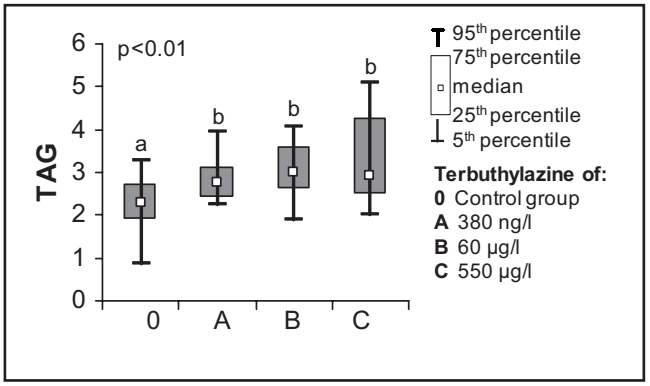

Fig. 3. Triglycerides (TAG) [mmol/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–27 determinations.

its confidence interval. These parameters were transformed before analysis. Differences (for every parameter) between groups, time points and next between groups within each time point and between time points within each group were assessed by ANOVA. Statistical differences between time points (day 28, 63, 91) were not found. For this reason, data from particular concentrations and control were consolidated and further assessed as related to the concentration explicitly. Differences (for every parameter) between groups were assessed by ANOVA. Homogeneous groups were specified by Tukey's HSD test. Subsequently correlation between selected parameters was assessed by Spearmen's rank correlation coefficient. For data analysis IBM Statistics 19 for Windows (Release 19.0.1, IBM Corporation 2010) was used.

RESULTS

Biometric parameters

When compared to the control, there was no effect on HSI and CF in fish exposed to the terbuthylazine concentrations of 60 µg/l and 550 µg/l. In the environmental concentration (380 ng/l) a significant increase ($p<0.05$) in HSI and CF was observed (Figures 1, 2). Correlations between HSI and plasma TAG; CF and plasma total protein were found (Table 1).

Haematological parameters

The exposure did not affect RBC, WBC and PCV, while there was a significant ($p<0.05$) decrease in haemoglobin and MCHC in fish exposed to the highest concentration of terbuthylazine. Mean corpuscular volume and MCH declined significantly ($p<0.05$) in terbuthylazine of 60 µg/l and 550 µg/l (Table 2).

Biochemical profile

TAG ($p<0.01$) were found to increase in all pesticide-treated groups. Alanine aminotransferase ($p<0.05$) and phosphorus ($p<0.01$) were revealed to decline in fish exposed to terbuthylazine of 60 µg/l and 550 µg/l, while albumin ($p<0.01$) increased in the same groups (Figures 3–6). There was an elevation in natrium ($p<0.05$) in terbuthylazine of 550 µg/l treated fish and a rise in TP ($p<0.01$) in the terbuthylazine groups of 380 ng/l and 550 µg/l (Figure 7, 8). Other indices were not affected when compared to the control group (Table 3). Correlations between selected indices are shown in Table 1.

Ceruloplasmin

Ceruloplasmin activity was observed to increase significantly ($p<0.01$) in fish treated with the highest concentration of terbuthylazine (Figure 9).

FRAP

A significant rise ($p<0.05$) in FRAP was found in fish exposed to the highest concentration of terbuthylazine (Figure 10).

Tab. 2. Haematological indices in common carp exposed to to Click 500 SC (terbuthylazine).

Indice	Unit	Control	Group A	Group B	Group C
RBC	[10^{12}/l]	1.59[a] (1.50;1.68)	1.51[a] (1.44;1.58)	1.64[a] (1.57;1.71)	1.60[a] (1.51;1.69)
WBC	[10^{9}/l]	41.0[a] (35.3;46.7)	33.0[a] (28.9;37.1)	34.9[a] (30.3;39.5)	40.0[a] (34.6;45.3)
Hb	[g/l]	82.0[b] (73.7;90.2)	73.9[a,b] (69.7;78.0)	74.4[a,b] (70.4;78.4)	69.1[a] (65.5;72.7)
PCV	[l/l]	0.28[a] (0.26;0.29)	0.28[a] (0.27;0.29)	0.27[a] (0.26;0.28)	0.27[a] (0.26;0.28)
MCV	[10^{-15} l]	175.6[a,b] (166.0;185.1)	188.4[b] (178.7;198.1)	168.6[a] (158.1;179.0)	169.6[a] (158.1;181.0)
MCH	[10^{-12} g]	51.0[a] (45.7;56.2)	50.2[a] (46.7;53.7)	44.1[b] (41.4;46.9)	44.3[b] (40.9;47.8)
MCHC	[g/l]	0.30[b] (0.28;0.32)	0.27[a,b] (0.26;0.29)	0.28[a,b] (0.26;0.29)	0.26[a] (0.25;0.28)

Parameters were described by mean of 15–28 determinations and its confidence interval. Means in the same row lacking a common letter of superscript ([a,b]) differ significantly ($p<0.05$).

Tab. 3. Biochemical indices in plasma of the common carp exposed to Click 500 SC (terbuthylazine).

Indice	Unit	Control	Group A	Group B	Group C
[1]AST	[µkat/l]	2.45[a] (1.97; 2.94)	2.63[a] (2.15; 3.11)	2.41[a] (1.97; 2.86)	2.58[a] (2.24; 2.92)
[2]glc	[mmol/l]	1.50[a,b] (1.39; 1.61)	1.70[b] (1.57; 1.83)	1.51[a,b] (1.38; 1.65)	1.46[a] (1.34; 1.58)
[2]Cl^-	[mmol/l]	103.9[a] (103.4; 104.3)	103.9[a] (103.4;104.4)	103.6[a] (102.9; 104.3)	104.7[a] (103.8; 105.6)
[2]K^+	[mmol/l]	3.50[a,b] (2.96; 4.11)	3.98[b] (3.72; 4.25)	3.52[a,b] (3.38;3.67)	3.22[a] (3.02; 3.42)
[2]LDH	[µkat/l]	3.98[a] (2.59; 5.91)	4.81[a] (3.25; 6.93)	2.54[a] (1.73; 3.60)	3.40[a] (2.36; 4.75)
[1]Ca	[mmol/l]	2.71[a] (2.59; 2.83)	2.77[a] (2.72; 2.83)	2.67[a] (2.62; 2.72)	2.74[a] (2.53; 2.95)

[1]Parameters were described by mean of 22–28 determinations and its confidence interval. [2]Parameters were described by geometric mean of 19–27 determinations and its confidence interval.
Means in the same row lacking a common letter of superscript ([a,b]) differ significantly ($p<0.05$).

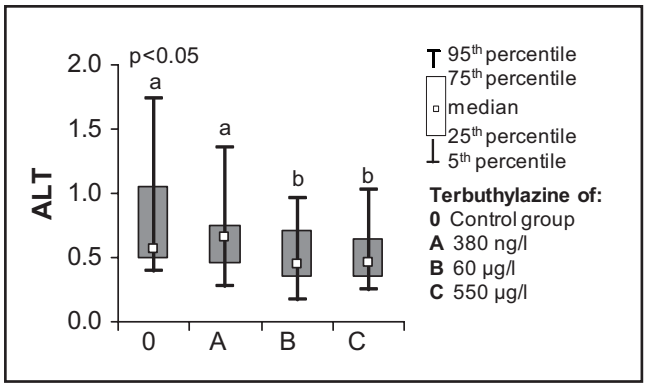

Fig. 4. Alanine aminotransferase (ALT) [µkat/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–27 determinations.

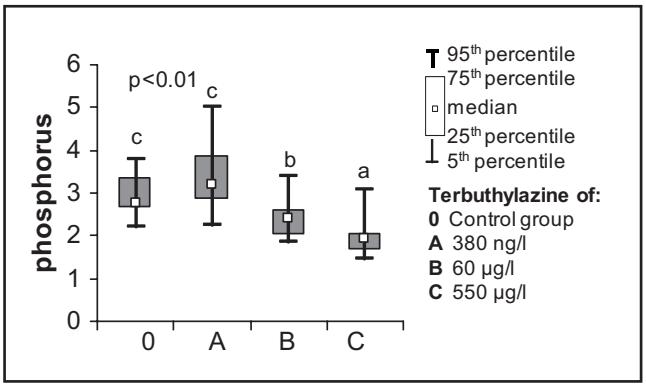

Fig. 5. Phosphorus [mmol/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–27 determinations.

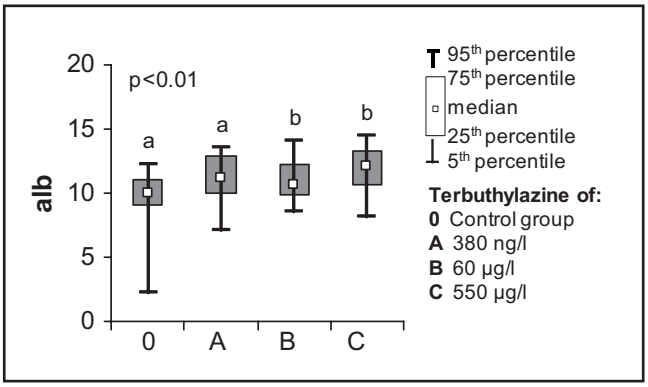

Fig. 6. Albumin (alb) [g/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–28 determinations.

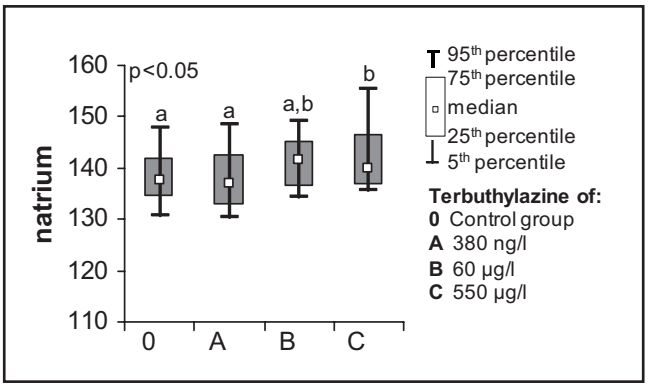

Fig. 7. Natrium [mmol/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 19–27 determinations.

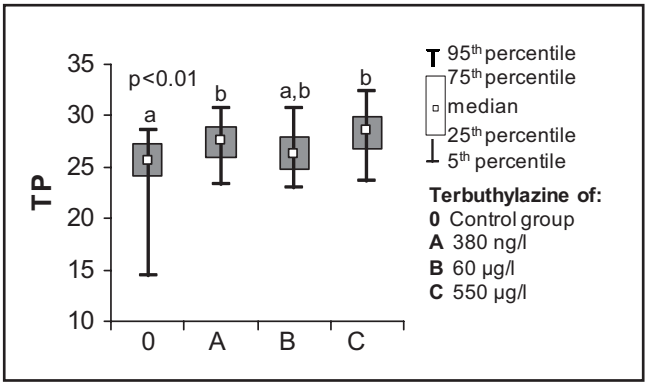

Fig. 8. Total protein (TP) [g/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–27 determinations.

XME. Neither the first nor the second phase systems of detoxification which were analysed were found to be significantly affected by the concentrations tested when compared to the control. (Data not shown.)

Histological examination

A long-term exposure to terbuthylazine of 380 ng/l (environmental concentration), 60 µg/l and 550 µg/l did not result in any specific histopathological changes in the pesticide-treated fish. Remarkable non-specific lesions were present in the gills of fish in the highest concentration tested after 91-day exposure. These can be described as mainly regressive changes of the respiratory epithelium (vacuolisation and desquamation) and venostasis. In skin samples of fish from terbuthylazine of 60 µg/l and 550 µg/l, there was a slight increase in the number of mucous cells. Other tissues (liver, cranial and caudal kidney, spleen) did not develop any significant histopathological changes.

DISCUSSION

Velisek *et al.* (2009; 2010) reported distinct influence of triazine compounds simazine and terbutryn on common carp *C. carpio* after 28 days. Prasad *et al.* (1991) studied effects of atrazine concentration 1.1 mg/l dissolved with acetone on haematological profile in *Tilapia mossambica* within 90 days (exposed fish were sampled on day 7, 15, 30, 60 and 90). The differences from control were concluded to increase with exposure period up to 60 days and recovery was seen on day 90. The data were evaluated using Student's T test, however, the information on sampling of control fish is missing, control data are not connected to any sampling day. Thus, the influence of time and test conditions seems not to have been included into the assessment. In the present study, the effects of terbuthylazine on *C. carpio* were time-independent from day 28 till day 90. Earlier impacts were not monitored.

Biometric parameters

The impact of environmental concentration on HSI and CF was more profound than that of other concentrations tested when compared to the control. The fish did not show any signs of either health impairment or differences in feed consumption in any test concentration of terbuthylazine. Velisek *et al.* (2009; 2010) observed no effects of triazine compounds simazine and terbutryn on biometric parameters in *C. carpio* after 28-day exposure. Meador *et al.* (2011) reported an elevation in growth in juvenile chinook salmon *Oncorhynchus tshawytscha* fed a diet containing low doses of tributyltin for 55 days, while in fish fed the highest tributyltin content diet, there was an inhibion of growth. A biphasic dose-response relationship characterized by a low-dose stimulation and high-dose inhibition is called hormesis (Calabrese 2008). A reduction in HSI and CF caused by exposure to higher concentrations of the formulation was not found in our study, as well as the stimulation observed did not reach 30%. However, the experiment was not designed in a way enabling assessment of hormetic effects (Calabrese 2008).

Haematological profile

Haemoglobin is a sensible indice in fish from polluted areas. Triazines have been reported to have no effect (Modra *et al.* 2008; Velisek *et al.* 2009; 2010) on haemoglobin content after 28-day exposure to sublethal concentrations. Terbuthylazine of 550 µg/l affected pro-

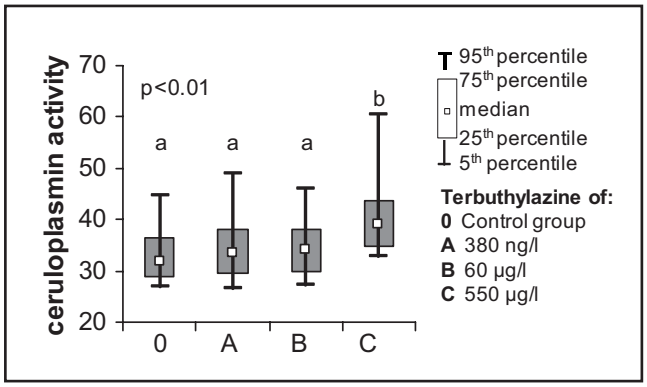

Fig. 9. Ceruloplasmin activity [increase in absorbance per min ×10,000] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 22–28 determinations.

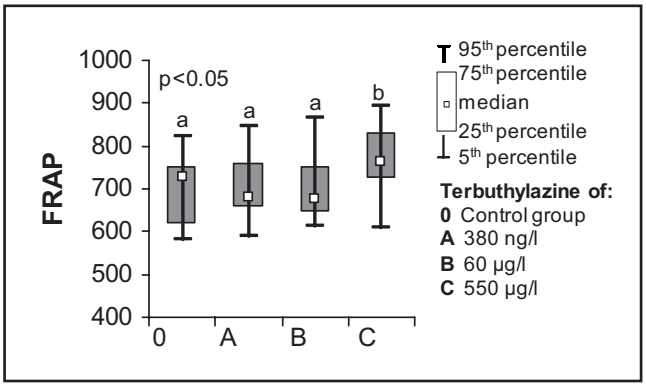

Fig. 10. Ferric reducing ability of plasma (FRAP) value [Fe^{2+} equivalent µmol/l] in plasma of the fish exposed to Click 500 SC (terbuthylazine), 21–26 determinations.

duction of haemoglobin during either erythropoiesis or during maturation of erythrocytes in the blood stream. Mean corpuscular haemoglobin, MCHC and MCV are secondary indices, thus they do not indicate the quality of living conditions so closely. The decrease in MCH (in terbuthylazine of 60 µg/l and 550 µg/l) and MCHC (550 µg/l) reflects changes in haemoglobin.

Biochemical profile

The rise in triglycerides indicates an interference with pathways involved in lipid metabolism. Wagner & Congleton (2004) do not consider plasma TAG a reliable indicator of nutritional status. Nevertheless, Svobodova *et al.* (2009) suggested that *C. carpio* with higher plasma triglycerides has better ability to manage energy resources during winter, which gives a better prognosis for successful over-wintering. Triglycerids are the major energy-storing lipids, they represent an important energy source for the fish. The level of plasma TAG was found to be significantly correlated with HSI. The primary site for long-term storage of TAG is the mesenteric adipose tissue, secondary storage sites are e.g. liver and white muscle (Tocher 2003). Lipid storage in fish liver can be both decreased and increased under toxicant exposure and may lead to liver steatosis (Hinton *et al.* 2008). Steatosis was not found in the examined liver samples. Changes in plasma enzyme activity are used as indicators of tissue injury, environmental stress, or a diseased condition (Ovie *et al.* 2010). Generally, a rise in enzyme plasma levels is reported due to exposure to pollutants. The reduction in ALT activity can be attributed to a direct action of terbuthylazine on the enzyme or its repressor effect on the enzyme synthesis. Significant effects of terbutryn and a metribuzine-based pesticide on ALT were not found under subchronic conditions (Modra *et al.* 2008; Velisek *et al.* 2010). No influence on plasma phosphorus was observed in the above mentioned studies. In the present study phosphorus declined in fish exposed to terbuthylazine of 60 µg/l and 550 µg/l. Signs of reduced phosphorus content in *C. carpio* have been reported to be e.g. reduced growth, poor feed efficiency, skeletal deformity and increased visceral fat (Tacon 1992), however, these symptoms were not observed within the experiment. The disturbance in natrium ions may cause a plasma osmolarity impairment, as it is a major extracellular ion. Osmoregulatory organs include especially gills and urinary system. There was a significant correlation between natrium and chloride ions, whereas plasma chloride ions were not affected by the treatment with pesticide, as well as potassium ions, which showed a significant correlation with plasma glucose. The level of total proteins in the blood plasma has a tendency to decrease in fish after a long-term exposure to pollutants (Svobodova *et al.* 1994). Interestingly, a reverse effect was observed with a significant increase in TP in fish exposed to terbuthylazine of 380 ng/l and 550 µg/l. The inconsistent reaction (a non-significant rise in fish treated with 60 µg/l) could be attributed to large experimental variability in the indice. Total protein reflects protein metabolism, it is an important index of health and condition of fish and depends, first of all, on their nutritive condition (Luskova 1997). This was confirmed by a significant correlation between TP and CF. Significant correlations found between total protein and TAG and total protein and Ca are in accordance with findings of Svobodova *et al.* (2009). Furthermore, a statistically significant correlation between albumin and Ca was observed. A close relationship between albumin and total protein levels could be expected, as albumin constitutes a relevant proportion of total protein.

FRAP

FRAP represents antioxidant power of plasma mediated through generally non-protein antioxidants. Haluzova *et al.* (2010) reported a decreased FRAP in *C. carpio* after 28-day exposure to prochloraz-based

formulation. A decline in FRAP was observed in *Oncorhynchus mykiss* by Dabrowski *et al.* (2004) in sequel to a decrease in water oxygen saturation level. The increase in FRAP in terbuthylazine of 550 µg/l could be caused by elevation in plasma uric acid, which contributes to about 60% of total FRAP value (Benzie & Strain 1996). Nevertheless, the content of uric acid was not determined.

Ceruloplasmin activity

The level of circulating ceruloplasmin increases in response to various stresses and disease-related processes (Cousins 1985). Dunier *et al.* (1994) reported an increase in ceruloplasmin activity after a single i. p. injection of *O. mykiss* with lindan on day 7, 14, 21 (10; 50 and 100 mg/kg) and 28 (only the concentration 100 mg/kg). Test concentrations of terbuthylazine were much lower, thus the response of organism may have not been so distinct and the increase was observed in the highest concentration solely. Ceruloplasmin is a component of fish innate immune system. It has various functions including copper transport, modulation of coagulation, inactivation of biogenic amines and defence against oxidative stress. Despite its ferroxidase activity ceruloplasmin is unlikely to be active in FRAP (Benzie & Strain 1996). Shukla *et al.* (2006) have discussed also prooxidative activities of ceruloplasmin.

XME

Terbuthylazine affected no examined system of the first and second phase of detoxification. Modra *et al.* (2008) did not reveal effects of metribuzine-based pesticide formulation on EROD and CYP in *C. carpio* after 28 days of exposure. Effects of atrazine on CYP in *D. rerio* were reported by Dong *et al.* (2009), who observed a significant elevation in liver CYP on days 10, 15, and 20. Thereafter, on day 25, the values in all concentrations (0,01; 0,1 and 1 mg/l) decreased to approximately the control levels, both in males and females. The strongest induction of P450 content was observed on day 15 in males and day 10 in females at treatment concentrations 1 mg/l. The induction of CYP isoenzymes in *C. carpio* by terbuthylazine is possible, as well as activation of adaptation mechanisms resulting in a decline to background values before day 28. Tarja *et al.* (2003) studied effects of temperature on detoxification of terbutryn and terbuthylazine in rainbow trout *O. mykiss*. Both chemicals decreased enzyme activities during 14 days (7 days of exposure plus 7 days of depuration). In all tests the highest liver EROD activities were observed in fish exposed to a water temperature of 4 °C, and the activities decreased with increasing water temperature. The present experiment was conducted at 18.5–21.5 °C. Salaberria *et al.* (2009) examined effects of a single i. p. injection of atrazine on hepatic metabolism in *O. mykiss*. A dose-dependent decrease in hepatic CYP1A gene expression was found. CYP1A subfamily is responsible for biotransformation of a wide range of xenobiotics. In the same study, no significant effect on expression of GST-pi class gene was discovered. The information on induction of the second phase XME by triazines in fish is scarce and focused on atrazine. Wiegand *et al.* (2000; 2001) studied effects of atrazine exposure on detoxification enzymes in embryos of *D. rerio*. Egaas *et al.* (1993) reported no differences in activities of GST and EROD in *O. mykiss* exposed to atrazine of 10 ppb for 14 days.

Histological examination

Velisek *et al.* (2009; 2010) examined samples of liver, spleen, cranial and caudal kidney of *C. carpio* exposed to simazine, resp. terbutryn for 28 days. Tissue impairment was found in liver and caudal kidney of fish treated with terbutryn of 2; 20 and 40 µg/l. In a study on simazine, histological changes in liver and cranial kidney were revealed in concentrations of 4; 20 and 50 µg/l. In the present study, terbuthylazine of 380 ng/l, 60 and 550 µg/l did not cause similar effects. Oropesa-Jimenez *et al.* (2005) reported histopathological changes in the gills of *C. carpio* exposed to simazine of 45 µg/l for 90 days. Furthermore, the fish exhibited an elevated mucus production within the experiment, however, tissues except of the gills were not histologically examined. Non-specific findings observed in the gills and skin of the terbuthylazine-treated fish represent a common response to irritants.

CONCLUSIONS

Adverse effects of terbuthylazine-based formulation were demonstrated in common carp *C. carpio*. The environmental concentration of terbuthylazine enhanced biometric indices of the fish, while the other pesticide-treated groups were not affected. Futher research is needed to elucidate the underlying mechanism of this effect. Haematological and plasma biochemical parameters were found to be significantly influenced by the concentrations tested, including the environmental one. Disturbances in the measured indices indicate interferences of the test compound with several organ systems and metabolic pathways. Analysed xenobiotic metabolizing systems did not respond to the long-term exposure, therefore other biomarkers should be considered in onward studies. The fish showed no terbuthylazine-specific histopathological lesions. Nevertheless, there were histopathological changes found in the gills and skin of the test fish. The environmental concentration of terbuthylazine was not found to cause tissue impairment.

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REFERENCES

1 2004/248/EC: Commision Decision of 10 March 2004 concerning the non-inclusion of atrazine in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing this active substance.
2 Benzie IFF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem. **239**: 70–76.
3 Calabrese EJ (2008) Hormesis: Why is it important to toxicology and toxicologists. Environ Toxicol Chem. **27**: 1451–1474.
4 Ceron JJ, Martinez-Subiela S (2004) An automated spectrophotometric method for measuring canine ceruloplasmin in serum. Vet Res. **35**: 671–679.
5 Chang TKH, Waxman DJ (1998) Enzymatic analysis of cDNA-expressed human CYP1A1, CYP1A2, and CYP1B1 with 7-ethoxyresorufin as a substrate. In: Phillips, IR, Shephard EA (Eds.): Methods in molecular biology, Vol. 107: Cytochrome P450 protocols. Humana press Inc., Totowa, NJ, pp. 103–109.
6 Carafa R, Wollgast J, Canuti E, Ligthart J, Dueri S, Hanke G, et al (2007) Seasonal variations of selected herbicides and related metabolites in water, sediment, seaweed and clams in the Sacca di Goro coastal lagoon (Northern Adriatic). Chemosphere. **69**: 1625–1637.
7 Cousins RJ (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol Rev. **65**: 238–309.
8 Dabrowski K, Lee KJ, Guz L, Verlhac V, Gabadaun J (2004) Effects of dietary ascorbic acid on oxygen stress (hypoxia or hyperoxia), growth and tissue vitamin concentrations in juvenile rainbow trout (*Oncorhynchus mykiss*). Aquaculture. **233**: 382–392.
9 Dong XL, Zhu LS, Wang JH, Wang J, Xie H, Hou XX, et al (2009) Effects of atrazine on cytochrome P450 enzymes of zebrafish (*Danio rerio*). Chemosphere. **77**: 404–412.
10 Dunier M, Siwicki AK, Scholtens J, Molin SD, Vergnet C, Studnicka M (1994) Effects of lindan exposure on rainbow trout (*Oncorhynchus mykiss*) immunity; III. Effect on nonspecific immunity and B lymphocyte functions. Ecotox Environ Safe. **27**: 324–334.
11 Egaas E, Skaare JU, Svendsen NO, Sandvik M, Falls JG, Dauterman WC, et al (1993) A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the *in vitro* phase II. Atrazine metabolism in some fish, insects, mammals and one plant species. Comp Biochem Physiol. **106C**: 141–149.
12 Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys. **82**: 70–77.
13 Fava L, Orru MA, Scardala S, Alonzo E, Fardella M, Strumia C (2010) Pesticides and their metabolites in selected Italian groundwater and surface water used for drinking. Ann Ist Super Sanita. **46**: 309–316.
14 Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transferases. First enzymatic step in mercapturic acid formation. J Biol Chem. **249**: 7130–7139.
15 Haluzova I, Modra H, Blahova J, Marsalek P, Siroka Z, Groch L, et al (2010) Effects of subchronic exposure to Spartakus (prochloraz) on common carp *Cyprinus carpio*. Neuroendocrinol Lett. **31** (Suppl. 2): 105–113.
16 Hinton DE, Segner H, Au DWT, Kullman SW, Hardman RC (2008) Liver toxicity. In: Di Giulio RT, Hinton DE, editors. The Toxicology of Fishes. Boca Raton: CRC Press, Taylor and Francis Group. p. 327–400.
17 Kodes V, Vejvodova J, Kollerova M (2010) Screening pesticidů v hydrosféře ČR [Screening of pesticides in the hydrosphere of the Czech Republic]. In: Hydrologické dny 2010, Voda v měnícím se prostředí. Czech Hydrometeorological Institute, Hradec Kralove, p. 193–198.
18 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem. **193**: 265–275.
19 Luskova V (1997) Annual cycles and normal values of hematological parameters in fishes. Acta Sc Nat Brno. **31**: 1–70.
20 Meador JP, Sommers FC, Cooper KA, Yanagida G (2011) Tributyltin and the obesogen metabolic syndrome in a salmonid. Environ Res. **111**: 50–56.
21 Modra H, Haluzova I, Blahova J, Havelkova M, Kruzikova K, Mikula P *et al.* (2008) Effects of subchronic metribuzin exposure on common carp (*Cyprinus carpio*). Neuroendocrinol Lett. **29**: 669–674.
22 Navarro S, Vela N, Gimenez MJ, Navarro G (2004) Persistence of four s-triazine herbicides in river, sea and groundwater samples exposed to sunlight and darkness under laboratory conditions. Sci Total Environ. **329**: 87–97.
23 Neskovic NK, Elezovic I, Karan V, Poleksic V, Budimir M (1993) Acute and subacute toxicity of atrazine to carp (*Cyprinus carpio* L.). Ecotox Environ Safe. **25**: 173–182.
24 Nilsen BM, Berg K, Goksøyr A (1998) Induction of cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. In: Philips IR, Shephard EA (Eds.): Methods in molecular Biology. Vol. 107: Cytochrome P450 protocols. Humana press Inc., Totowa, NJ, p. 423–438.
25 Oropesa-Jimenez AL, Garcia-Cambero JP, Gomez-Gordo L, Roncero-Cordero V, Soler-Rodriguez F (2005) Gill modifications in the freshwater fish *Cyprinus carpio* after subchronic exposure to simazine. Bull Environ Contam Toxicol. **74**: 785–792.
26 Ovie KS, Bemigho IR, Gbemi OM (2010) Variations in alanine aminotransferase and aspartate aminotransferase activities in African catfish: *Clarias gariepinus* (Burchell, 1822) at different sublethal concentrations of potassium permanganate. Sci Res Essays. **5**: 1501–1505.
27 Perreau F, Einhorn J (2006) Determination of frequently detected herbicides in water by solid-phase microextraction and gas chromatography coupled to ion-trap tandem mass spectrometry. Anal Bioanal Chem. **386**: 1449–1456**.**
28 Plhalova L, Macova S, Haluzova I, Slaninova A, Dolezelova P, Marsalek P *et al.* (2009) Terbutryn toxicity to *Danio rerio*: Effects of subchronic exposure on fish growth. Neuroendocrinol Lett. **30** (Suppl 1): 242–247.
29 Prasad TAV, Srinivas T, Rafi GM, Reddy DC (1991) Effect *in vivo* of atrazine on haematology and O_2 consumption in fish, *Tilapia Mossambica*. Biochem Int. **23**: 157–161.
30 Roberts TR, Hutson DH, Lee PW, Nicholls PH, Plimmer JR (Eds) (1998) Metabolic pathways of agrochemicals. Part 1: Hebicides and plant growth reulators. 1st ed. The Royal Society of Chemistry, Cambridge, 849 p.
31 Rutten AA, Falke HE, Catsburg JF, Wortelboer HM, Blaauboer BJ, Doorn L, et al (1992) Interlaboratory comparison of microsomal ethoxyresorufin and pentoxyresorufin O-dealkylation determinations: standardization of assay conditions. Arch Toxicol. **66**: 237–244.
32 Saglio P, Trijasse S (1998) Behavioral responses to atrazine and diuron in goldfish. Arch Environ Con Tox. **35**: 484–491.
33 Salaberria I, Hansen BH, Asensio V, Olsvik PA, Andersen RA, Jenssen BM (2009) Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*). Toxicol Appl Pharm. **234**: 98–106.
34 Shukla N, Maher J, Masters J, Angelini GD, Jeremy JY (2006) Does oxidative stress change ceruloplasmin from a protective to a vasculopathic factor? Atherosclerosis. **187**: 238–250.
35 Siroka Z, Krijt J, Randak T, Svobodova Z, Peskova G, Fuksa J, et al (2005) Organic pollutant contamination of River Elbe as assessed by biochemical markers. Acta Vet Brno. **74**: 293–303.
36 Spano L, Tyler CR, van Aerle R, Devos P, Mandiki SNM, Silvestre F, et al (2004) Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). Aquat Toxicol. **66**: 369–379.
37 Svobodova Z, Pravda D, Palackova J (1991) Unified methods of haematological examination of fish. Manuals of Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic.
38 Svobodova Z, Vykusova B, Machova J (1994) The effects of pollutants on selected haematological and biochemical parameters in fish. In: Muller R, Lloyds R, editors. Sublethal and chronic effects of pollutants on freshwater fish. 1st ed. Oxford: Fishing News Books. p. 39–52.

39 Svobodova Z, Smutna M, Flajshans M, Gela D, Kocour M, Machova J, et al (2009) Differences in biochemical profiles among spawners of eight common carp breeds. J Appl Ichthyol. **25**: 734–739.
40 Tacon AG (1992) Nutritional fish pathology. Morphological signs of nutrient deficiency and toxicity in farmed fish. FAO Fisheries Technical Paper, vol. 330. FAO, Rome, Italy. 75 pp.
41 Tarja N, Kirsti E, Marja L, Kari E (2003) Thermal and metabolic factors affecting bioaccumulation of triazine herbicides by rainbow trout (*Oncorhynchus mykiss*) (2003) Environ Toxicol. **4**: 219–226.
42 Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci. **11**: 107–184.
43 van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Phar. **13**: 57–149.
44 Velisek J, Stastna K, Sudova E, Turek J, Svobodova Z (2009) Effects of subchronic simazine exposure on some biometric, biochemical, hematological and histopathological parameters of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett. **30** (Suppl. 1): 236–241.
45 Velisek J, Sudova E, Machova J, Svobodova Z (2010) Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). Ecotox Environ Safe. **73**: 384–390.
46 Wagner T, Congleton JL (2004) Blood chemistry correlates of nutritional condition, tissue damage, and stress in migrating juvenile chinook salmon (*Oncorhynchus tshawytscha*). Can J Fish Aquat Sci. **61**: 1066–1074.
47 Wiegand C, Pflugmacher S, Giese M, Frank H, Steinberg C (2000) Uptake, toxicity, and effects on detoxication enzymes of atrazine and trifluoroacetate in embryos of zebrafish. Ecotox Environ Safe. **45**: 121–131.
48 Wiegand C, Krause E, Steinberg C, Pflugmacher S (2001) Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). Ecotox Environ Safe. **49**: 199–205.