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

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Key words: herbicide; fish; biometric indices; biochemical indices; xenobiotic metabolizing enzymes; haematological indices; antioxidant indices

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...
60 μg/l and 550 μg/l, while albumin ($p<0.01$) rises 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. Cerurolusamin 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 terbuthylazine 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 terbutylazine-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 formulaion. 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 200 l). 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 length 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]²) x 100. The hepatosomatic index (HSI) was calculated according to the formula HSI = liver weight/b. w. x 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 x 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).

**GSII, 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 x 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
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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 Spearman’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. 1. Spearman’s correlation coefficient between selected parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Spearman’s corr. coefficient</th>
<th>Parameters</th>
<th>Spearman’s corr. coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>alb × TP</td>
<td>0.259*</td>
<td>CF × TP</td>
<td>0.196*</td>
</tr>
<tr>
<td>TP × TAG</td>
<td>0.330**</td>
<td>glc × K⁺</td>
<td>0.248*</td>
</tr>
<tr>
<td>TP × Ca</td>
<td>0.488**</td>
<td>alb × Ca</td>
<td>0.508**</td>
</tr>
<tr>
<td>HSI × TAG</td>
<td>0.424**</td>
<td>Na × Cl⁻</td>
<td>0.634**</td>
</tr>
<tr>
<td>HSI × TP</td>
<td>0.035</td>
<td>TAG × Ca</td>
<td>0.059</td>
</tr>
<tr>
<td>CF × TAG</td>
<td>0.168</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

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).

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

Parameters were described by mean of 22–28 determinations and its confidence interval. 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 ($p<0.05$).
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 inhibition 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-
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 terbutylazine 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. Triglycerides 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 terbutylazine 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 terbutylazine 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 terbutylazine 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 \textit{Oncorhynchus mykiss} by Dabrowski \textit{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 \textit{et al.} (1994) reported an increase in ceruloplasmin activity after a single i. p. injection of \textit{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 \textit{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 \textit{et al.} (2008) did not reveal effects of metribuzine-based pesticide formulation on EROD and CYP in \textit{C. carpio} after 28 days of exposure. Effects of atrazine on CYP in \textit{D. rerio} were reported by Dong \textit{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 \textit{C. carpio} by terbuthylazine is possible, as well as activation of adaptation mechanisms resulting in a decline to background values before day 28. Tarja \textit{et al.} (2003) studied effects of temperature on detoxification of terbutryn and terbuthylazine in rainbow trout \textit{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. Salaberría \textit{et al.} (2009) examined effects of a single i. p. injection of atrazine on hepatic metabolism in \textit{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. Wiegang \textit{et al.} (2000; 2001) studied effects of atrazine exposure on detoxification enzymes in embryos of \textit{D. rerio}. Egaas \textit{et al.} (1993) reported no differences in activities of GST and EROD in \textit{O. mykiss} exposed to atrazine of 10 ppb for 14 days.

**Histological examination**

Velisek \textit{et al.} (2009; 2010) examined samples of liver, spleen, cranial and caudal kidney of \textit{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 \textit{et al.} (2005) reported histopathological changes in the gills of \textit{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 \textit{C. carpio}. The environmental concentration of terbuthylazine enhanced biometric indices of the fish, while the other pesticide-treated groups were not affected. Further 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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