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Original article

Recovery ability of common carp (*Cyprinus carpio*) after a short-term exposure to terbuthylazine

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Abstract

Effects of a high terbuthylazine concentration (3.3 mg/l) on *Cyprinus carpio* were studied using a commercial herbicide formulation Click 500 SC (terbuthylazine 500 g/l). The fish were exposed to the pesticide for 24 h and allowed to recover for 6 days. Biometric parameters, plasma biochemical parameters and biomarkers of oxidative stress as well as histopathological changes in selected tissues were assessed on day 1 and 7. After a 24-h exposure, there were significant alterations found in the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as in the plasma concentrations of glucose, natrium, chlorides, calcium and phosphorus. Hepatosomatic index, plasma albumin and lactate reflected the treatment with a delay. Ion levels and ALT were found to be restored after a 6-day recovery period, which was too short for AST activity and glucose to diminish to the control levels. The histopathological examination revealed disorders in the gills of the exposed fish, however, the changes were not detected after a 6-day recovery period. The study shows high regeneration potential of the fish.

Key words: herbicide, fish, biometric indices, biochemical indices, oxidative stress

Introduction

Many contaminants, including pesticides, which reach surface waters may cause harm not only to aquatic organisms but also to a human as a consumer due to their accumulation in fish. The highest concentrations of pesticides in flowing surface waters can be detected for a short period of time following their application in field. This concentration in natural water is quickly diluted and affected wildlife starts to recover from the toxic attack. The length of this period depends, among others, on pesticide concentration, chemical classification, duration of the exposure, species and category affected. Only few studies have been engaged in the ability of fish to deal with a short-term stress caused by these chemicals.

Terbuthylazine (6-chloro-N-(1,1-dimethylethyl)-N'-ethyl-1,3,5-triazine-2,4-diamine), which belongs to triazine herbicides, has been commonly applied in agriculture especially since atrazin ban in the European countries. It is used as a broad spectrum pre- or postemergence herbicide in maize, sorghum, vines,

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citrus, potatoes, legumes and in forestry. The herbicidal activity of terbuthylazine is based on inhibition of photosynthesis (Roberts et al. 1998). According to State Phytosanitary Administration (2011) terbuthylazine has recently been among the most widely used herbicides in the Czech Republic. Like triazines, whose application is not permitted in the countries of the European Union (simazine, terbutryn and atrazin), terbuthylazine and its degradation products are commonly detected in the surface water as well as in the ground water (e. g. Noppe et al. 2007, Quednow and Puttmann 2007, Fava et al. 2010, Loos et al. 2010).

The aim of the present study was to examine the toxic impact of 24-h terbuthylazine exposure on juvenile common carp (*Cyprinus carpio*) including delayed effects and assess regeneration ability of different organ systems of these fish.

Materials and Methods

Test solutions

The fish were exposed to commercial preparation Click 500 SC (terbuthylazine 500 g/l), the concentration of its active substance terbuthylazine reached 3.3 mg/l. The selected test concentration was derived from 96hLC50 (median lethal concentration, 50% mortality after a 96 h) for preparation (12 mg/L) (Safety Data Sheet 2004) and 96hLC50 of terbuthylazine (4.9-9.4) (Gangolli 1999) for some freshwater fish species. The test concentration was prepared by dissolving the formulation in 10 ml of 10% dimethyl sulfoxide (DMSO) and applied into the stock tanks with dechlorinated tap water, from which it flowed into the test tanks with fish (pH 7.5-8.0; temperature 19.5-21.2°C; oxygen saturation ≥60%). The solution volume (200 l) was replaced twice a day. The concentration of DMSO (0.0005%) was identical both for control (dechlorinated tap water) and the pesticide-treated fish. Test solutions were sampled and the concentrations of terbuthylazine were measured twice during the experiment. The exposure to pesticide lasted for 24 hours, after that the fish were subjected to dechlorinated tap water with DMSO (0.0005%) for another 6 days.

Test animals

The experiment was conducted on common carp *C. carpio* (weight 130.7 ± 27.7 g) obtained from a commercial fish farm. The fish were randomly distributed into 4 tanks (volume of 200 l) equipped with a flow-through system, 19-20 specimen per each. Total 10 control fish and 40 pesticide-treated fish were used for the present study. The fish were fed commercial pellets (Coppens International bv) at a total rate of 1.5% body weight. The test began after 14-day acclimation to laboratory conditions. Six fish of 40 fish treated with pesticide died during the 24-h exposure. After the exposure as well as at the end of the experiment (7 days after the beginning) individual blood samples ($n_1 = 5$ for the control fish, $n_2 = 17$ for the exposed fish) were taken by cardiac puncture and stabilized with aqueous solution of heparin (50 IU per ml of blood). The fish were euthanized and their body weight and standard length were recorded. The whole liver (hepatopancreas) and samples of tissues were removed.

Experimental procedures were in compliance with national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended, and Decree No. 207/2004 Coll., on the Protection, Breeding and Use of Experimental Animals, as amended).

Biometric parameters

The condition factor (CF) of each fish was calculated using the following formula: CF = (body weight [g]/standard length [cm]^3) × 100. The hepatosomatic index (HSI) was calculated with the formula HSI = liver weight/body weight × 100.

Biochemical profile

Determination of biochemical indices (glucose, lactate, total protein, albumin, triglycerides, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), chlorides, natrium, total calcium and inorganic phosphorus) in plasma was conducted using a biochemical analyzer Konelab 20i and commercial test kits (BioVendor).

Biomarkers of oxidative stress

The analysis of ceruloplasmin activity was performed using a Varioscan flash spectral scanning multimode reader (Thermo Scientific) according to Ceron and Martinez-Subiela (2004) with slight modifications (Mikulikova et al. 2011). Results were expressed as the amount of the absorbance increase per minute × 10,000.

Ferric reducing ability of plasma (FRAP) was measured on a biochemical analyzer Konelab 20i ac-

cording to Benzie and Strain (1996) including slight modifications (Haluzova et al. 2010).

Histological examination

Samples of gills, skin, liver, spleen, cranial and caudal kidney of three fish treated with the pesticide and three fish from the control group were fixed in buffered 10% neutral formalin and treated for histological examination (stained with hematoxylin and eosin) following both sampling days.

Determination of terbuthylazine in water

Terbuthylazine was determined by gas chromatography with ion trap mass spectrometry (GC/IT-MS). Sample preparation was based on simple liquid-liquid extraction into hexan. The separation, identification and quantification of terbuthylazine were based on the GC/IT-MS method. A gas chromatograph Varian 450-GC (Varian Inc., USA) and VF-5ms (30 m x 0.25 mm) column were used for terbuthylazine separation, a Varian 220-MS (Varian Inc., USA) ion trap mass spectrometer was employed for identification and quantification. Chromatographic and MS conditions followed method described by Perreau and Einhorn (2006). All solvents were GC/MS-grade purity (Chromservis, s.r.o., CZ), a certified standard of terbuthylazine was purchased from Dr. Ehrenstorfer GmbH (Germany). The detection limit (3σ) of terbuthylazine was 0.01 µg/l, expanded uncertainty was 6.0% on condition that the coefficient of expansion was k = 2. The concentration of terbuthylazin exceeded 90% of the nominal concentration (3.3 mg/l).

Statistical analysis

The results were analyzed using the statistical package Unistat 5.1 (Unistat Ltd., GB). For all variables tested, normality and homogeneity of variances was checked by means of a Shapiro-Wilk test and Bartlett – Box test (Zar 1999). When necessary, logarithmic transformations were used for analysis of variance, though actual mean values are presented in the figures. The data was subjected to a two-way ANOVA with Treatment (Control, Terbuthylazine) and Day of sampling (Day1, Day7) as the main effects along with their interactions included in the model:

$$x_{ijk} = \mu + T_i + D_j + (TD)_{ij} + e_{ijk}$$

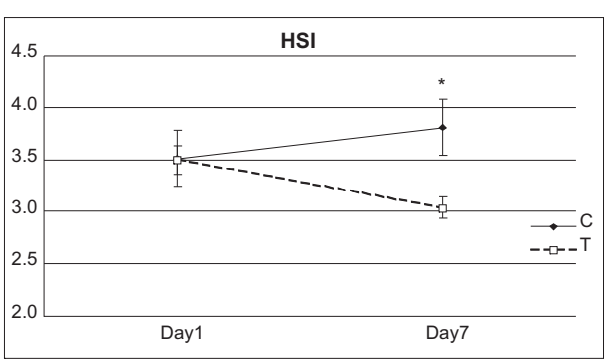
where: x_{ijk} = analyzed measurement, μ = overall mean, T_i = effect of treatment (Control, Terbuthylazine), D_j = effect of Day of sampling (Day1, Day7), $(TD)_{ij}$ = effect of interaction, e_{ijk} = overall error (residual).

Interactions between main effects were analyzed using the general linear model procedure – GLM (HSI, CF, FRAP, ceruloplasmin, total protein, AST, phosphorus, glucose, LDH, lactate, triglycerides, calcium) or Kruskal-Wallis ANOVA (albumin, ALT, chlorides, natrium) and significant differences between means in all possible pairs of groups were evaluated using the Tukey-HSD test or multisample rank sums Tukey-HSD test (Zar 1999).

Results

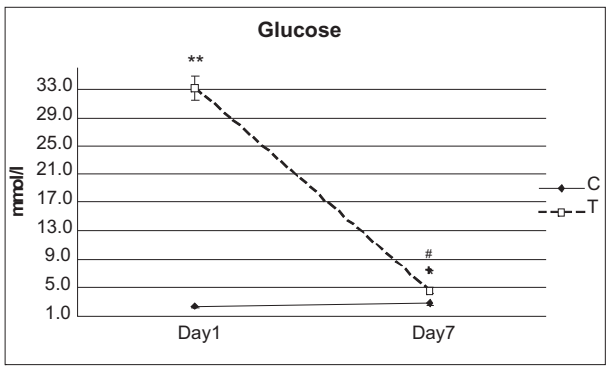
The concentration tested (1/3 96hLC50) was considered sublethal for *C. carpio*, however, the toxicity was probably increased by using DMSO as a solvent. Intensive irritability followed by reduced activity and apathy after several hours were observed in the exposed fish. Six individuals died during 24 hours, necropsy of the dead fish was not conclusive due to quick decomposition of the bodies. During the exposure the fish did not consume the feed. The normal feeding behavior restored 1 day after transfer of the fish into pesticide-free water.

Figures 1-10 show effects of a 24-h exposure to Click 500 SC and subsequent 6-day recovery on HSI, plasma glucose, lactate, ALT, AST, albumin, chlorides, natrium, calcium and phosphorus. The following parameters were not influenced by either exposure to the pesticide or recovery period in water (with DMSO): CF, total protein, triglycerids, LDH, FRAP and ceruloplasmin activity (data not shown).



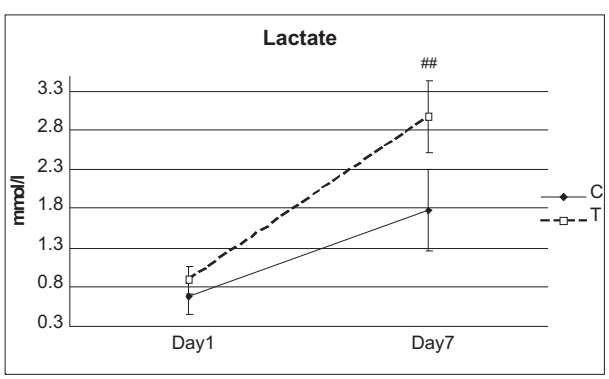
* Means within the same day of sampling differ significantly ($P < 0.05$).

Fig. 1. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on hepatosomatic index.



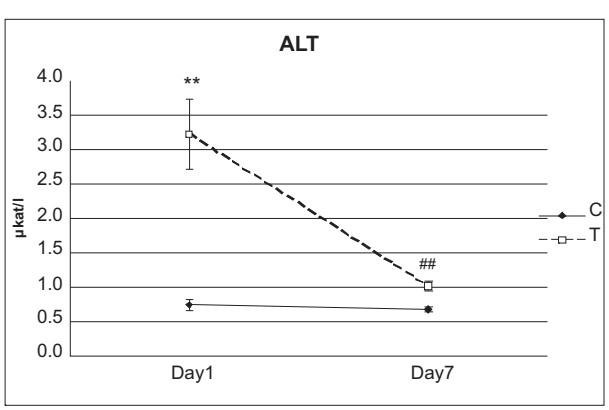
*, ** Means within the same day of sampling differ significantly ($P < 0.05$, $P < 0.01$).
Means within the same treatment differ significantly ($P < 0.05$).

Fig. 2. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma glucose



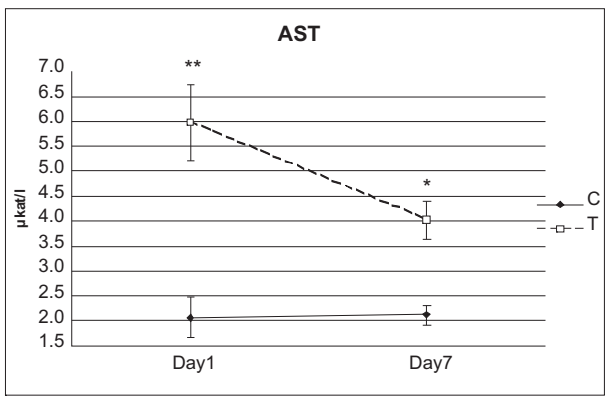
Means within the same treatment differ significantly ($P < 0.01$).

Fig. 3. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma lactate.



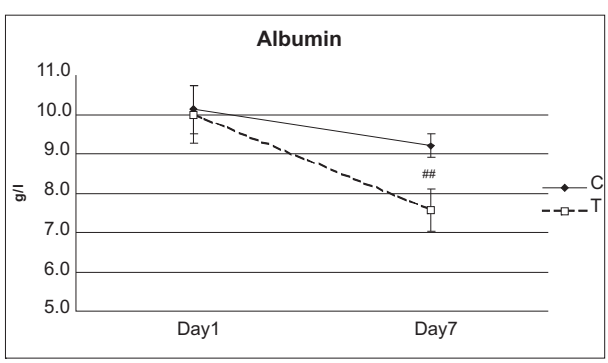
** Means within the same day of sampling differ significantly ($P < 0.01$).
Means within the same treatment differ significantly ($P < 0.01$).

Fig. 4. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma alanine aminotransferase.



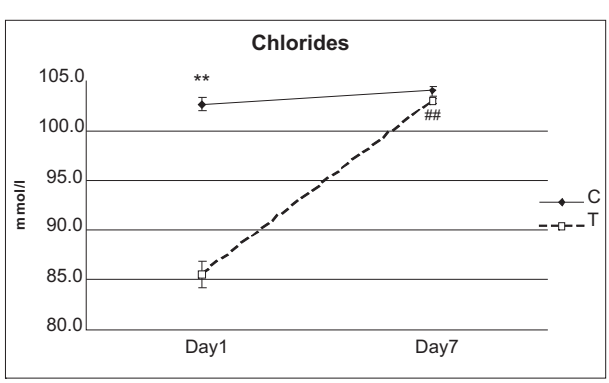
*, ** Means within the same day of sampling differ significantly ($P < 0.05$, $P < 0.01$).

Fig. 5. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma aspartate aminotransferase.



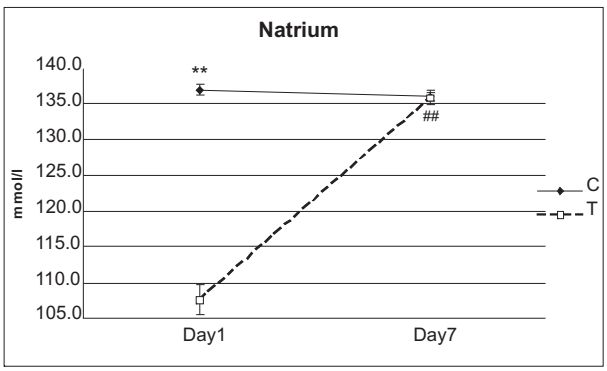
Means within the same treatment differ significantly ($P < 0.01$).

Fig. 6. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma albumin.

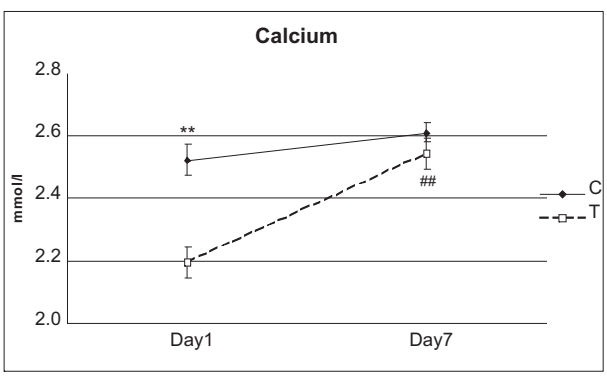


** Means within the same day of sampling differ significantly ($P < 0.01$).
Means within the same treatment differ significantly ($P < 0.01$).

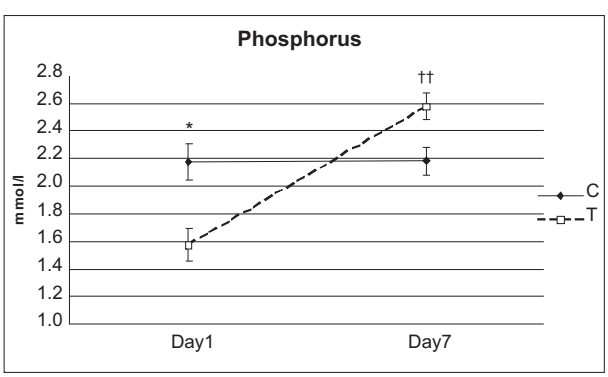
Fig. 7. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma chlorides.



** Means within the same day of sampling differ significantly ($P < 0.01$).
Means within the same treatment differ significantly ($P < 0.01$).
Fig. 8. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma natrium.



** Means within the same day of sampling differ significantly ($P < 0.01$).
Means within the same treatment differ significantly ($P < 0.01$).
Fig. 9. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma calcium.



* Means within the same day of sampling differ significantly ($P < 0.05$).
Means within the same treatment differ significantly ($P < 0.01$).
Fig. 10. The effects of 24-h exposure to Click 500 SC (terbuthylazine) and following 6-day recovery on plasma phosphorus.

Fig. 1-10: C – control fish (dechlorinated water + 0.0005% DMSO); T – fish treated with Click 500 SC (terbuthylazine 3.3 mg/l) + 0.0005% DMSO for 24 h (Day 1) and 6 following days kept in dechlorinated water + 0.0005% DMSO (Day 7).

The histological examination revealed no changes in control tissue samples after 1 day as well as 7 days of the experiment. The fish treated with Click 500 SC for one day showed circulation disorders in gills represented by abundant presence of capillary aneurysms in gill filaments and a local hyperplasia of respiratory epithelium. Above-mentioned findings were not detected after the 6-day recovery period.

Discussion

The present study indicates different reaction time and alteration persistence of various indices measured in fish after a short-term exposure to Click 500 SC. Alanine aminotransferase, AST, glucose, natrium, chlorides, phosphorus and calcium were shown to be affected after 24-h exposure, while HSI, plasma albumin and lactate reflected the exposure with a delay. Ions and ALT activity were observed to be restored after a 6-day recovery period. In contrast, this period was too short for AST activity and glucose to diminish to the control levels.

The tested concentration of terbuthylazine had an impact on feeding behaviour of the fish. However, the lenght of the exposure was insufficient for condition factor to decrease. The fish began to feed again shortly after transfer to the freshwater. On day 7 there was a decline ($P < 0.05$) in HSI of the pesticide-treated fish when compared to the control group. Hepatosomatic index is significantly correlated with storing reserves of glycogen (Svobodova 1977). The consumption of glycogen seems to be the cause of the observed decrease in HSI, as high energy requirements of the tissues for recovery can be expected after the pesticide exposure.

A distinct rise in plasma glucose detected after the exposure reflects intense stress reactions ongoing in the organism (Folmar 1993, Evans and Claiborne 2006). Blood glucose level is a sensitive indicator of discomfort in fish and responds immediatelly to stressor action. Fish have poor ability to control the blood glucose level likely due to insufficient insulin secretion or differences in the activities of glycolytic and gluconeogenic enzymes when compared to mammals (Kumar et al. 2010). The concentration of glucose in our study was still elevated 6 days after exposure to terbuthylazine.

The concentration of plasma lactate was affected with a delay and significantly ($P < 0.01$) increased in the exposed fish during the recovery period in comparison with the first sampling. Under stress conditions, anaerobic pathway is activated and high levels of lactate are produced by reversible conversion of pyruvate in the cell catalysed by lactate dehydrogenase (Kumar et al. 2010). Findings revealed by histological examination imply that the tissue damage was not extensive enough to result in LDH increase.

Alanine aminotransferase and aspartate aminotransferase elevations were attributed to the harm caused by the treatment. Although histological examination did not reveal any impairment of the hepatic tissue, a significant ($P < 0.01$) increase in the activities immediately after pesticide action indicates a relevant damage to the cell membranes. Alanine aminotransferase is predominantly present in hepatocytes, other tissues responsible for AST enhancement are especially muscles and red blood cells (Folmar 1993). The return of ALT to control level in recovery period shows high regeneration potential of liver. The decline of AST activity back to control level was slower and thus could reflect damage of other tissues. An increase of plasma AST and ALT activity during pesticide exposure and a subsequent return to control values (delayed in AST) during 7-days recovery period were recorded by Rao (2006) in *Oreochromis mossambicus*.

Albumin concentration declined in the pesticide-treated fish during the recovery period when compared to the first phase of the test. However, there was no difference in comparison with the control samples. The metabolism of albumin was probably affected, although it was not evident at the total protein levels.

All ions determined followed the same trend; a significant increase ($P < 0.01$ for chlorides, natrium and calcium; $P < 0.05$ for phosphorus) after the exposure with a return to the control levels within 6 days. The changes in chlorides and natrium are in the first place associated with disturbances in the gills functions, which was confirmed also by the results of the histological examination. Both chlorides and natrium are major extracellular ions, whose disorders result in impaired osmolarity. Calcium levels in fish are predominantly regulated by stanniocalcin synthetised by the corpuscules of Stannius. Stanniocalcin is a hypocalcemic factor, it inhibits active Ca^{2+} transport across the branchial epithelium. Other reported actions of stanniocalcin include reduced intestinal Ca^{2+} absorption and stimulation of phosphate reabsorption by renal proximal tubules (Evans and Claiborne 2006). Both stanniocalcin dysregulation and gill impairment could lead to a decreased calcium concentration in blood. An exposure of *Heteropneustes fossilis* to deltamethrin and cypermethrin at a concentration of 80% of 96-h LC50 resulted in a decrease in blood calcium still persisting after 96 hours from the exposure (Srivastav et al. 2009, Mishra et al. 2010).

Effects of a short-term pesticide exposure have been reported by several authors. Velisek et al. (2008) described a significant ($P < 0.01$) decrease in total protein, triacylglycerids, AST, calcium and lactate in blood plasma of *O. mykiss* after a 96-h treatment with a metribuzin-based pesticide Sencor 70 WG at a concentration of 96-h LC50. In that study, glucose, ALT and LDH were not affected when compared to the control group. *C. carpio* exposed to 24-h LC50 of atrazin (18.5 mg/l) for 24 h showed a decline in plasma glucose and total protein (Ramesh et al. 2009).

Effects of a short-term treatment with a high pesticide concentration on FRAP in fish has not been studied yet. In the present study ceruloplasmin activity was not affected by the exposure, though Dunier et al. (1994) observed an increase in this indice 7 days after a single i.p. injection of lindan to *O. mykiss*.

Irritation and injury to the gills are frequent findings in the fish from polluted areas. They negatively affect respiration, ammonia excretion and ion balance of the individual. Histopathological changes in gills of *O. mykiss* exposed to metribuzin (Sencor 70 WG) for 96 h were described as mild proliferation of goblet cells of the respiratory epithelium of the secondary gill lamellae. Other alterations were revealed in the caudal kidney and liver (Velisek et al. 2008).

In conclusion, the results of our study show high regeneration potential of the fish organism. The experiment simulated a pesticide spill in a closed water-system, e.g. a small pond. A further study focused on the course of recovery in more detail is recommended.

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