Effects of subchronic exposure to simazine on zebrafish (*Danio rerio*)

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Abstract

OBJECTIVES: Simazine is a triazine herbicide which has been used for a long period in agriculture and in the aquatic environment for control of weeds and algae. The aim of this study was to investigate the effects of subchronic exposure to simazine on growth and the development of histopathological changes in selected organs (gills, kidney, liver) in *Danio rerio*.

METHODS: Juvenile growth tests were performed on *D. rerio* according to the OECD method No. 215. Fish at the age of 20 days were exposed to the environmental simazine concentration commonly detected in Czech rivers (0.06 $\mu g.L^{-1}$) and a range of sublethal concentrations of simazine (0.6, 6.0 and 60.0 $\mu g.L^{-1}$) for 28 days.

RESULTS: There were no significant differences ($p<0.05$) between the specific growth rates (r) of the test groups and those of both control groups. Histopathological examination revealed pathological changes in fish exposed to a simazine concentration of 60.0 $\mu g.L^{-1}$. The values of NOEC and LOEC of simazine were 6.0 $\mu g.L^{-1}$ and 60.0 $\mu g.L^{-1}$.

CONCLUSIONS: The environmental concentration of simazine in Czech rivers did not have any effects on the growth and development of histopathological changes in *D. rerio*.

Abbreviations:

DMSO	- dimethyl sulfoxide
GC/IT-MS	- gas chromatography with ion trap mass spectrometry
NOEC	- no observed effect concentration
LC50	- 50% lethal concentration
LOEC	- lowest observed effect concentration

ORIGINAL ARTICLE

INTRODUCTION

Due to the extensive use of herbicides in agriculture and their persistence, such herbicides are present in surface and ground waters. As one of these herbicides, simazine and its metabolites should therefore be considered a potential risk to many non-target organisms as well as a threat to drinking water quality (Strandberg & Scott-Fordsmand 2002; Katsumata *et al.* 2005).

Simazine (2,4-bis(ethylamino)-6-chloro-*s*-triazine, molecular weight – 201.7) belongs to the triazine group of herbicides and is widely used for pre- and post-emergence control of annual grasses and broad-leaved weeds in many crops including pome fruit, stone fruit, citrus, vines, maize, beans, and non-cropped land. It is also used as an aquatic herbicide and algicide for the control of algae and submerged weeds. Simazine is a selective systemic herbicide which acts as a photosynthesis inhibitor. It is absorbed by leaves and roots, translocated acropetally in the xylem and accumulates in the apical meristem (Tucker *et al.* 1983; Roberts *et al.* 1998; Strandberg & Scott-Fordsmand 2002; Noppe *et al.* 2007).

According to the European Commission (1999) simazine and some other s-triazines have been identified as relevant with respect to the prioritisation of substances dangerous to the aquatic environment in the member states of the European Community (Arufe *et al.* 2004). Preparations containing simazine have not been registered in the Czech Republic since 2004, but simazine is still detected in the aquatic environment in the Czech Republic. The highest concentration of simazine in surface water reported in 2008 (data of the Czech Hydrometeorogical Institute) was 0.06 $\mu g.L^{-1}$. The presence of simazine in water was also frequently detected in other European countries (Noppe *et al.* 2007); even higher levels of simazine residues (about 2.0 $\mu g.L^{-1}$) were found in Spain (Rodríguez-Mozaz *et al.* 2004; Belmonte *et al.* 2005).

The aim of this study was to investigate the long-term effects of subchronic exposure to environmental and other sublethal concentrations of simazine on growth and on the formation of histopathological changes in selected organs (gills, kidney, liver) in *Danio rerio*, and to make an estimation of the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) of simazine.

MATERIAL AND METHODS

Experimental fish

Tests of simazine toxicity were performed on *Danio rerio*, which is one of the model organisms most commonly used in toxicity tests (Lele & Krone 1996; Lawrence 2007; Dolezelova *et al.* 2009; Plhalova *et al.* 2009). 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).

The subchronic toxicity test

The tests were performed on *D. rerio* at the age of 20 days according to OECD No. 215 Fish, Juvenile Growth Test with simazine of 99.5% chemical purity (Sigma Aldrich, Czech Republic). The test compound was dissolved in water using dimethyl sulfoxide. The concentration of DMSO in all tanks reached 0.01%. The fish were randomly distributed into 30 L glass aquaria, 40 specimens per each. The experiment was conducted in a flow-through system and the volume of test solutions was replaced twice a day. The fish were exposed to a range of sublethal concentrations of simazine (0.06 $\mu g.L^{-1}$ – environmental concentration in Czech rivers, 0.6, 6.0 and 60.0 $\mu g.L^{-1}$) for 28 days. Each test on a simazine treated group was performed in duplicate. One control group was kept in dilution water only and another control group was maintained in dilution water with the DMSO solvent at a concentration of 0.01%. The average initial weight of fish used in the experiment was 0.011 ± 0.002 g. The fish were fed with dried *Artemia salina* without nutshells to the amount of 8% of their body weight per day; the food ration was based on initial fish weights and was recalculated after 14 days. At the end of the tests, fish were weighed and their tank-average specific growth rates were determined. Food was withheld from the fish 24 h prior to weighing. During the tests, living conditions were checked at 24-hour intervals and the number of dead fish was recorded in each concentration. The mean values for water quality were: temperature 23 ± 2 °C, oxygen saturation above 60%, pH 7.98–8.33. The basic chemical parameters of dilution water used were: COD_{Mn} (chemical oxygen demand) 1.4–1.9 $mg.L^{-1}$; total ammonia below the limit of determination (<0.04 $mg.L^{-1}$); NO_3^- 24.5–31.4 $mg.L^{-1}$; NO_2^- below the limit of determination (<0.02 $mg.L^{-1}$); Cl^- 18.9–19.1 $mg.L^{-1}$; Σ Ca ± Mg 14 $mg.L^{-1}$.

Tank-average specific growth rates were calculated using the following formula according to the OECD No. 215:

$$r = \frac{\overline{\log_e W_2} - \overline{\log_e W_1}}{t_2 - t_1} * 100$$

r - tank-average specific growth rate

W_1, W_2 - weights of a particular fish at times t_1 and t_2 respectively

$\overline{\log_e W_1}$ - average of the logarithms of the values W_1 for the fish in the tank at the start of the study period

$\overline{\log_e W_2}$ - average of the logarithms of the values W_2 for the fish in the tank at the end of the study period

t_1, t_2 - time (days) at the start and end of study period

Statistical analysis

Data were subjected to Kruskal-Walllis one-way ANOVA and subsequently to Dunnett`s test in order to assess the statistical significance of differences in tank-average fish specific growth rates between test groups with different concentrations and those of the control groups. The estimation of the LOEC and NOEC was based on ANOVA followed by Dunnett`s test for identification of the lowest concentration at which these differences of specific growth rate were (were not) significant at a 0.05 probability level, and, further, on the results of histopathological examination and the assessment of fish behaviour.

Determination of simazine

Sample preparation was based on simple liquid-liquid extraction into cyclohexane. The separation, identification and quantification of simazine were based on the GC/IT-MS method (gas chromatography with ion trap mass spectrometry). A Varian 450-GC (Varian Inc., USA) gas chromatograph and VF-5ms (30 m × 0.25 mm) column were used for the separation of simazine. A Varian 220-MS (Varian Inc., USA) ion trap mass spectrometer was used for identification and quantification. Chromatographic and MS conditions were based on the method described by Perreau and Einhorn (2006). All solvents were GC/MS-grade purity (Chromservis, s.r.o., CZ). A certified standard of simazine was purchased from Dr. Ehrenstorfer GmbH (Germany). The detection limit (3σ) of simazine was 0.02 $\mu g.L^{-1}$. Expanded uncertainty was 7.0% on condition that the coefficient of expansion was k = 2.

Histopathological examination

The fish were prepared for histopathological examination (on selected organs – gills, kidney, liver), fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 μm, and stained with haematoxylin and eosin (HE).

RESULTS

Fish behaviour and mortality.

The fish were exposed to 4 sublethal concentrations of simazine. The effects of the environmental concentration in Czech rivers (0.06 $\mu g.L^{-1}$) and concentrations 10-fold (0.6 $\mu g.L^{-1}$), 100-fold (6.0 $\mu g.L^{-1}$) and 1 000-fold (60.0 $\mu g.L^{-1}$) higher were assessed. We did not notice any changes in fish behaviour in simazin-treated groups in comparison with both control groups. Mortality in all simazine-exposed groups and in the control groups did not exceeded 5% during the 28-day experimental period.

Growth rate.

No significant differences ($p>0.05$) were found when the specific growth rates of fish from particular simazine concentrations were compared to both control groups.

Histopathology

Histopathological examination revealed pathological changes in fish exposed to simazine at a concentration of 60.0 $\mu g.L^{-1}$. Marked lesions were observed in the gills, represented by hypertrophy and hyperplasia of epithelial cells with lamellar fusion, hyperaemia, desquamation of epithelial cells, and the presence of multiple foci of coagulation necrosis (Figure 1). Moderate dystrophic lesions were evident in the liver. There were morphological signs of an initial cell injury represented by swelling and hydropic vacuolar degeneration of

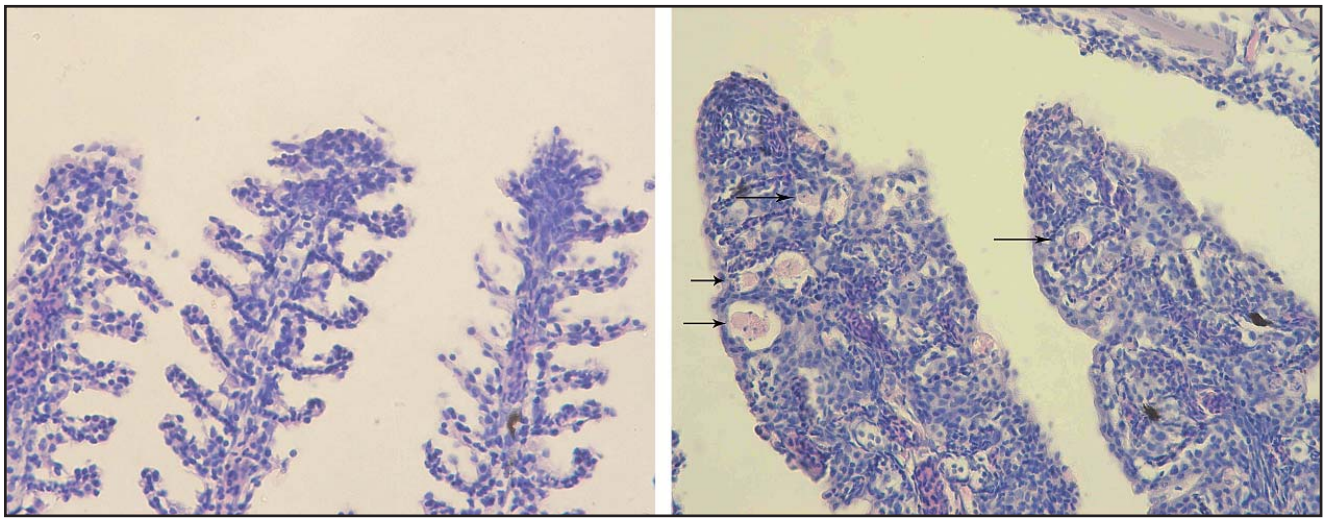

Fig. 1. Hypertrophy, hyperplasia and desquamation of epithelial cells and multiple foci of coagulation necrosis in the gills of *D. rerio* exposed to a simazine concentration of 60.0 $\mu g.L^{-1}$ for 28 days (HE, 400×).

hepatocytes (Figure 2). Mild dystrophic changes were observed in the caudal kidney. There was coagulation of the apical part of the cytoplasm of epithelial cells of the renal tubules (Figure 3). All affected organs were histopathologically compared with tissue sections from both control groups. Tissues and organs of the fish in experimental groups exposed to simazine at the concentrations of 0.06 μg.L^{-1}, 0.6 μg.L^{-1} and 6.0 μg.L^{-1} exhibited no pathomorphological changes.

Validity of the tests

Our test met all conditions required by the OECD – mortality in both control groups was below 10%, the final weight of control fish was higher than 150% of the initial weight, the dissolved oxygen concentrations were at least 60%, the water temperature did not differ by more than ±1 °C among test aquariums, and test substance concentrations were above 80% (ranging from 81% to 93%) of the measured initial concentration.

DISCUSSION

Due to the low solubility of simazine (Piletska *et al.* 2005) we used dimethyl sulfoxide (DMSO) as a dissolution reagent. Dimethyl sulfoxide is the most commonly used delivery system for water-insoluble chemicals in aquatic bioassays (Hallare *et al.* 2006; Machova *et al.* 2009). The concentration of DMSO reached 0.01% in all simazine test concentrations and DMSO control. We did not find any significant differences between the control group with dilution water only in comparison with the control group with DMSO. Hallare *et al.*

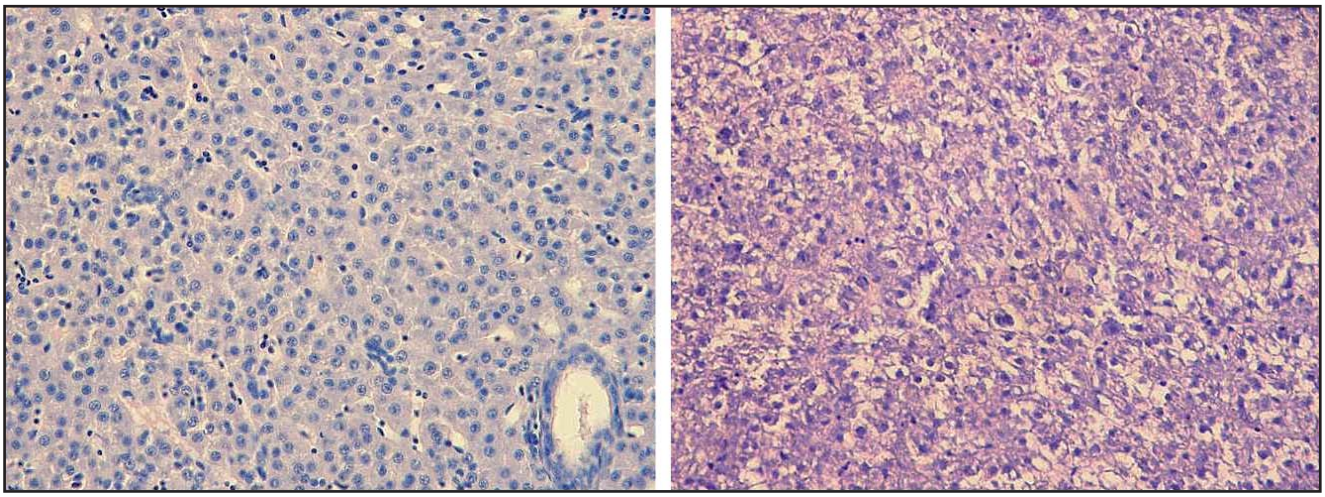

Fig. 2. Dystrophic lesions and swelling and hydropic vacuolar degeneration of hepatocytes in the liver of *D. rerio* exposed to a simazine concentration of 60.0 μg.L^{-1} for 28 days (HE, 400×).

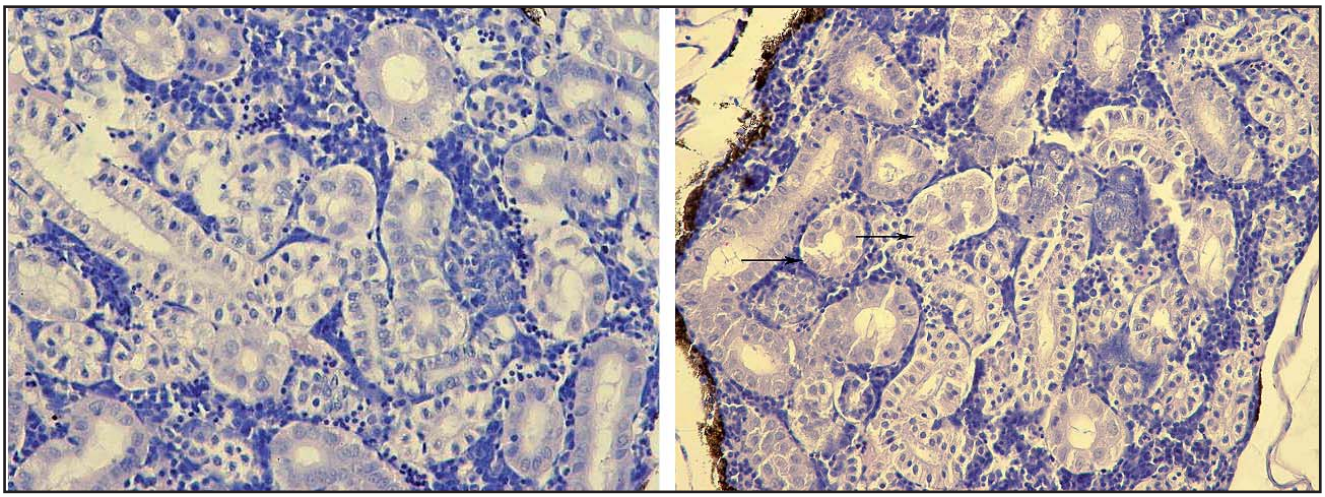

Fig. 3. The coagulation of apical part of cytoplasm of epithelial cells of the renal tubules in the kidney of *D. rerio* exposed to a simazine concentration of 60.0 μg.L^{-1} 28 days (HE, 400×).

(2006) reported that DMSO may be used as a carrier solvent in zebrafish embryo assays at levels below 1.5% v/v (for stress protein analysis of the exposed embryos the solvent concentration should be below 0.01% v/v). Machova *et al.* (2009) revealed a significantly lower mean body weight of larvae of common carp (*Cyprinus carpio*) in 5% DMSO when compared to the control after 29 days of exposure.

During our test we did not observe any abnormalities in fish behaviour and feed consumption in any test concentration (i. e. 0.06 $\mu g.L^{-1}$, 0.6 $\mu g.L^{-1}$, 6.0 $\mu g.L^{-1}$ and 60.0 $\mu g.L^{-1}$ of simazine) or in either of the controls. Likewise, Velisek *et al.* (2009) reported normal feeding habits and no abnormal behaviour exhibited in common carp exposed to simazine at concentrations of 0.06 $\mu g.L^{-1}$, 4.0 $\mu g.L^{-1}$, 20.0 $\mu g.L^{-1}$ and 50.0 $\mu g.L^{-1}$. The exposure period was the same as in our study, i. e. 28 days. Also Oropesa *et al.* (2009) found no signs of respiratory distress in common carp exposed to simazine of 45.0 $\mu g.L^{-1}$ for a period of 90 days. Tucker *et al.* (1983) described a reduction in fish production and poorer feed conversion in ponds with simazine of 1.3 $mg.L^{-1}$ in comparison with control ponds. The fish ceased to feed after the application of simazine and feeding did not resume until harvest. Our results did not demonstrate any differences in specific growth rates between control and test groups.

Nevertheless, the histopathological examination revealed pathological changes in several tissues. The changes were discovered in the gills, liver and in the caudal kidney, but only in the highest test concentration of simazine (60.0 $\mu g.L^{-1}$). Other authors (Oropesa-Jimenez *et al.* 2005; Oropesa *et al.* 2009; Velisek *et al.* 2009) also described pathological changes in the liver, kidney and gills in common carp exposed to low concentrations of simazine similar to those in our study. Velisek *et al.* (2009) reported significant histopathological changes (steatosis, hyperaemia and necrosis) in fish liver caused by simazine concentrations of 4.0 $\mu g.L^{-1}$ and 20 $\mu g.L^{-1}$. The above described lesions and even more pronounced lesions (massive coagulation necrosis and a degradation of physiological cell structure) were identified at a simazine concentration of 50 $\mu g.L^{-1}$. Arufe *et al.* (2004), who studied the acute effects of a commercial formulation containing simazine, detected pathological changes in the liver of yolk sac larvae of gilthead seabream (*Sparus aurata*) after 72 hours of exposure, but the fish were exposed to a simazine concentration several-fold higher than those in other long-term studies (4.5 $mg.L^{-1}$).

In the light of the above findings, simazine NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) were determined in the present study. For the exposure period of 28 days the values of NOEC and LOEC were 6.0 $\mu g.L^{-1}$ and 60.0 $\mu g.L^{-1}$, respectively. Arufe *et al.* (2004) reported simazine NOEC of 2.25 $mg.L^{-1}$ and LOEC of 4.50 $mg.L^{-1}$ for *Sparus aurata* on the basis of larval survival and growth. However, these values were obtained for an exposure of 72 hours.

On the basis of our results, it was concluded that the environmental concentration of simazine detected in Czech rivers (0.06 $\mu g.L^{-1}$) had no effect on growth or on the development of histopathological changes in *D. rerio*. Our finding is in accordance with Velisek et. al (2009), who did not observe any effects of this simazine concentration on the biochemical, hematological or histopathological profile of common carp (*C. carpio*).

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