ORIGINAL ARTICLE

Effects of low-concentrations of simazine on early life stages of common carp (*Cyprinus carpio* L.)

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Abstract

OBJECTIVES: The aim of this study is to assess the toxicity of simazine in different developmental stages of common carp (*Cyprinus carpio*) on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, and Fulton's condition factor during and at the conclusion of the test.
DESIGN: The toxicity tests were performed on carp according to OECD 210 methodologies. The developmental stages of carp were exposed to simazine at four concentrations, 0.06, (reported concentration in Czech rivers), 60, 600, and 3 000 $\mu g.l^{-1}$ for 36 days and compared to carp in a non-treated control group.
RESULTS: Simazine in concentration 0.06 $\mu g.l^{-1}$ had no effect on early life stages of carp. Simazine in concentration 600 and 3 000 $\mu g.l^{-1}$ caused decrease of mass and total length of carp. Fish exposed to three highest levels of simazine showed alteration of tubular system of caudal kidney. On the basis of histopatological changes the values of LOEC = 60 $\mu g.l^{-1}$, NOEC = 0.06 $\mu g.l^{-1}$ for simazine were estimated.
CONCLUSIONS: Chronic simazine exposure of early-life stages of common carp affected their growth rate, and histology. Some of the changes were observed only at higher exposures (600, 3 000 $\mu g.l^{-1}$), but change founded in caudal kidney was affected in fish exposed to the second lowest concentration tested (i.e., 60 $\mu g.l^{-1}$), which is about 10 $\mu g.l^{-1}$ higher than reported in Colorado rivers in recent years. Concentrations of simazine in World rivers have been reported to generally vary in the range 0.0003–49.20 $\mu g.l^{-1}$.

Abbreviations:

NOEC	- no observed effect concentration
LOEC	- lowest observed effect concentration
48hLC50	- lethal concentration
ANOVA	- analysis of variance
ANC4.5	- acid neutralization capacity
CODMn	- chemical oxygen demand
FWC	- Fulton's weight condition factor
OECD	- Organization for Economic Cooperation and Development

INTRODUCTION

During the several centuries since the industrial revolution, humankind's activities have caused strong alterations in the structure and function of their environment. The increasing production of pesticides that enter the environment has become a real threat not only for plants or animals, but also for humans themselves. Negative consequences of anthropogenic activities include the input of toxic, persistent and bioaccumulative compounds to the environment (Haluzova *et al.* 2010; Mikulikova *et al.* 2011; Plhalova *et al.* 2011).

Simazine (6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine) was introduced by a Swiss company J. R. Geigy in 1956 and is a member of the triazine family compounds (a six-membered ring containing three carbon and three nitrogen atoms). It is a selective systemic herbicide used to control germinating annual grasses and broad-leaved weeds in a variety of crops. In addition, simazine is used on shelterbelts, nursery stock, woody ornamentals, woodlots, woodland and Christmas tree plantations, and site preparation for conifer planting (Arufe *et al.* 2004; Velisek *et al.* 2009).

Simazine is the second most commonly detected pesticide in surface and ground waters in the U.S., Europe, and Australia, presumably due to relatively high persistence in soil (up to 3 years) and water (half-life 50–176 days) and to a combination of solubility (3.5 mg.l^{-1} at 20 °C), low absorption (soil organic carbon-water partitioning coefficient – 135 ml.g^{-1}) (Inoue *et al.* 2006). Its degradation products are detected less frequently than atrazine and other triazine pesticide in the aquatic environment. The highest concentration reported in surface water in the Czech Republic is 0.06 µg.l^{-1} (Velisek *et al.* 2009). In Europe simazine levels can reach values up to 5.0 µg.l^{-1} (Beitz *et al.* 1994). In USA simazine levels can reach values up to 49.20 µg.l^{-1} (U.S. EPA 1994).

Currently simazine and another seven s-triazines have been identified as relevant in a study of the prioritization of substances dangerous to the aquatic environment in the member states of the European Community. Simazine is included in the EU Priority Pollutants List and the US Environmental Protection Agency's List. According to Commission regulation (EU) No 196/2010 of 9 March 2010, amending Annex I to Regulation (EC) No 689/2008 of the European Parliament and of the Council concerning the export and import of dangerous chemicals simazine use is banned in the countries of the European Union. Although the effects of acute and sub-chronic exposure of fish to atrazine, another s-triazine herbicide, have been well-documented, there is a dearth of data on the sub-chronic toxicity of simazine at environmentally realistic concentrations in early life stages of common carp. The aim of the present study was to describe lethal and sublethal effects of simazine on embryos and larvae of common carp using a 36 day embryo larval toxicity test. Toxicity tests with early life stages of aquatic organisms have been proposed as a faster and more cost-efficient way of testing chemicals and environmental samples. Moreover, experience shows that these developmental stages of fishes are often the most sensitive to toxic effects, although the various embryonic and larval stages differ in their susceptibility as a result of physiological and biochemical differences (McKim 1995; Velisek *et al.* 2012a). Common carp was selected because it is the most often bred fish in the Czech Republic. That is why it is often used for the toxicity tests of chemicals and for the contamination monitoring of surface waters (Dobsikova *et al.* 2006). The toxicity of simazine was assessed on the basis of mortality, early ontogeny, occurrence of morphological anomalies, growth rate, and Fulton's condition factor during and at the conclusion of the test.

MATERIALS AND METHODS

Experimental animals

Fertilized eggs of common carp (*Cyprinus carpio* L.) were obtained from the breeding station of the Research Institute of Fish Culture and Hydrobiology in Vodnany, University of South Bohemia (Czech Republic). Eggs were produced according to standard methods of artificial reproduction by mating 15 females with 25 males (full-factorial scheme of crossing) as described by Kocour *et al.* (2005).

Experimental protocol

The trial was carried out using the modified test design of Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals 210 (Fish, Early-life stage toxicity test). At 24 h post-fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into each crystallization basins containing tested solution of simazine (Sigma Aldrich, Czech Republic, chemical purity 99.5%) and also into the control dish. Four ascending concentrations of tested solutions and control were used, each with 100 fertilized eggs in triplicate groups. The concentrations were marked as follows: 0.06 µg.l^{-1} (reported environmental concentration in Czech rivers – group 1 – E1), 60 µg.l^{-1} (group 2 – E2), 600 µg.l^{-1} (group 3 – E3), and 3000 µg.l^{-1} (group 4 – E4). The simazine concentrations of 60 µg.l^{-1}, 600 µg.l^{-1}, and 3000 µg.l^{-1} corresponded to the 0.15% of 48-hour half lethal concentration (48h LC50), 1.5% 48h LC50 and 7.5% 48h LC50 for juvenile common carp (*Cyprinus carpio*).

Water parameters

Aerated tap water was used in the present study, with the following parameters: dissolved oxygen >93%, temperature 19.3–21.1 °C, pH 7.2–8.1, $ANC_{4.5}$ 1.00 mmol.l^{-1}, COD_{Mn} 1.0 mg.l^{-1}, total ammonia 0.01 mg.l^{-1}, NO_3^- 6.30 mg.l^{-1}, NO_2^- 0.02 mg.l^{-1}, sum of Ca^{2+}+Mg^{2+} 10.1 mg.l^{-1}. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature

were measured daily. Simazine concentrations were checked daily by high performance liquid chromatography. Water samples were assayed using the method of Katsumata *et al.* (2005). The values measured did not differ from the value stated for test purposes by more than 7%.

Experimental protocol

The basins were placed in a laboratory (open-air conditions) with the natural light exposure (16:8 h light: dark), and the arrangement of basins was random. The exposure water for each treatment was renewed twice daily by gently draining each chamber then adding new solution slowly to prevent disturbing the embryos and larvae. Observations of hatching, survival, and behavior were made twice daily and dead embryos and larvae were collected. When able to feed, larvae were given freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii ad libitum twice daily prior to water exchange. The nauplii were rinsed with tap water to avoid contaminating the exposure water with chloride. During and at the conclusion of the trial, samples of embryos and larvae were collected to monitor development, occurrence of morphological anomalies, rate of length and weight, Fulton's weight condition factor (FWC = body weight (g)/standard lenght (cm)^3 × 100), and the length–weight relationship. Samples were collected at days 8, 19, 25, 32, and 36. Samples were fixed in 4% formalin, with 10 specimens per replicate (i.e., 30 per group).

Determination of developmental periods and stages followed Penaz *et al.* (1983), who described nine embryonic (E1–E9), six larval (L1–L6), and two juvenile stages (J1–J2). Final measurements included accumulated mortality, basic length parameters for fish with no cranial–skeletal deformities (TL, total length; SL, standard length), and mass. The length parameters were measured under a stereomicroscope (Olympus SZ61/SZ51) using a micrometer (accuracy of 0.01 mm). Weight, to 0.1 mg, was measured by using a Mettler-Toledo balance. The trial schedule was as follows: day 1, trial beginning (1 day before: fertilization of eggs); day 5–6, hatching completed; day 9, beginning of exogenous nutrition (*A. salina*); day 36, end of the trial (at that time, the majority of fish in the control group had reached the first juvenile stage).

The mean specific growth rate (SGR) for fish in each of the experimental groups was calculated for the period beginning at day 8 (the first sampling time) and ending at day 36 (end of the trial). The following equation was used (OECD 2000).

$$SGR = \frac{\overline{\ln w_2} - \overline{\ln w_1}}{t_2 - t_1} \cdot 100$$

where SGR = mean specific growth rate in the group, w_1 = mass of one fish at time t_1 individually (μg), w_2 = mass of one fish at time t_2 individually (μg), $\ln w_1$ = mean value of the $\ln w_1$ values, $\overline{\ln w_2}$ = mean value of the $\ln w_2$ values, t_1 = time (days) – first sampling time, t_2 = time (days) – end of exposure.

The inhibition of specific growth rate in each experimental group was calculated using the following formula according to OECD number 215 (OECD 2000):

$$I_x[\%] = \frac{SGR_x\ (control) - SGR_x\ (group)}{SGR_x\ (control)} \cdot 100$$

where I_x = inhibition of specific growth in selected experimental group after x days of exposure, SGR_x (*control*) = mean specific growth rate in the control group, SGR_x (*group*) = mean specific growth rate in selected experimental group.

Statistical analysis

The statistical software program STATISTICA (version 8.1 for Windows, StatSoft) was used to compare differences among test groups. Prior to analysis, all measured variables were evaluated for normality (Kolmogorov-Smirnov test) and homoscedasticity of variance (Bartlett's test). If these conditions were satisfied, a one-way ANOVA was employed to determine whether there were significant differences in measured variables among experimental groups. When a difference was detected ($p<0.05$), Dunnett's multiple range test was applied. If conditions for ANOVA were not satisfied, a non-parametric test (Kruskal-Wallis) was used.

Evaluation of LOEC and NOEC

For the evaluation of LOEC, and NOEC values, the probit analysis was used on the basis of histopatological changes of cranial kidney in the test groups and the EKOTOX 5.1 software (Ingeo Liberec) was used.

Histopathology examination

Histopathology was evaluated in all experimental groups at the samples days. Five whole fish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, and stained with hematoxylin and eosin. Histological changes in samples of skin, gills, caudal and cranial kidney and liver were examined by light microscopy.

RESULTS

Hatching began 5 day after the onset of exposure, on days 5 to 6. The majority of eggs in all treatment groups hatched by day 6. No significantly negative effects of simazine on hatching and embryos viability were observed compared to control.

Accumulated mortality

Cumulative mortality of common carp samples exposed to simazine and the control sample was between 7–11% (Figure 1). Simazine in the tested concentrations had no negative influence on the early-life stages of common carp mortality.

Tab. 1. Growth rate and fish mortality results of the 36 day embryo-larval toxicity test on common carp after simazine exposure.

Fish Group	Control	E1	E2	E3	E4
Simazine (μg.l^{-1})	–	0.06	60	600	3000
m_8 (Mean ± SD, mg)	1.84±0.21	1.88±0.33	1.77±0.23	1.64±0.16	1.71±0.21
m_{36} (Mean ± SD, mg)	94.95±22.88	90.61±26.46	104.24±29.71	67.74±14.76*	65.59±20.78*
SGR	14.00	13.69	14.40	13.21	12.78
I (%)	–	2.21	–2.28	5.60	8.71
Total mortality (%)	11	8	7	8	7

m_8, m_{36} = Mean fish mass in selected group after 8 and 36 days exposure; SGR = mean specific growth rate in selected group; I = inhibition of specific growth in selected group; SD = standard deviation. *Experimental groups significantly (p<0.05) different from the control group.

Length and weight growth parameters

Mass and total length of fish related to simazine concentration in water are depicted in Figures 2 and 3. From 32 days of exposure, fish exposed to the two highest tested groups E3 – 600 μg.l^{-1}, and E4 – 3000 μg.l^{-1} simazine showed significantly (p<0.05) lower mass compared with controls. By day 36, fish exposed to the highest tested concentration of simazine showed significantly lower (p<0.05) total length compared with controls. Simazine in the tested concentrations had no negative influence on FWC. Specific growth rates and inhibition of growth were calculated for 36 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to the highest simazine concentration (3000 μg.l^{-1}) was 8.71 % compared to control.

Early ontogeny

No significant differences (p<0.05) in early ontogeny of carp embryos and larvae were noticed among test groups. Early ontogeny indicators of all tested groups were comparable to the control. Furthermore, no significant differences in the type and occurrence of morphological abnormalities were observed in tested fish embryos and larvae during the test.

Histopatology

The most histological changes were observed in caudal kidney in groups E2, E3 and E4 at all sampling time compared to control fish and group E1. Fish exposed to three higher levels of simazine showed alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules (Figure 4). No histopathological changes were demonstrated in skin, gills, liver and cranial kidney following exposure to simazine. On the basis of histopatological changes of cranial kidney in the experimental groups, values of LOEC = 60 μg.l^{-1} simazine, NOEC = 0.06 μg.l^{-1} simazine were estimated.

DISCUSSION

The most sensitive method of assessing the influence of chemicals on early life stages of fish, since fish are exposed through the all critical periods of early devel-

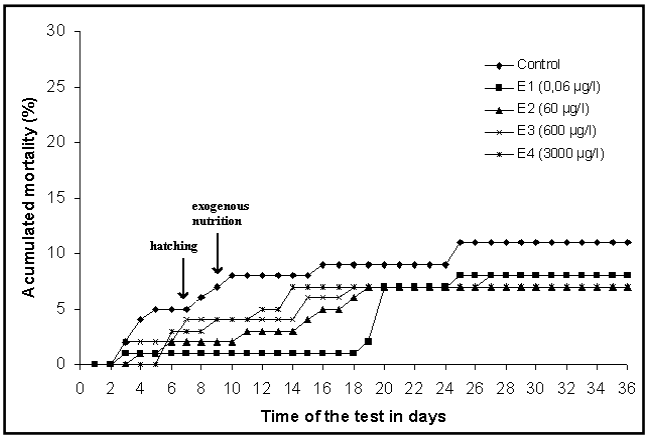

Fig. 1. Accumulated mortality (percentage) of common carp embryos, larvae, and juveniles after simazine exposure.

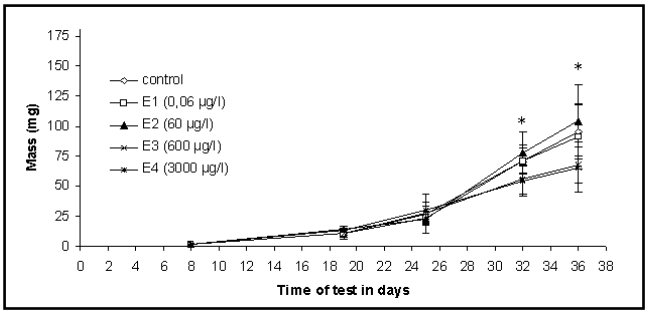

Fig. 2. Mean mass of common carp larvae (juveniles) after simazine exposure. *Experimental groups significantly (p<0.05) different from the control group.

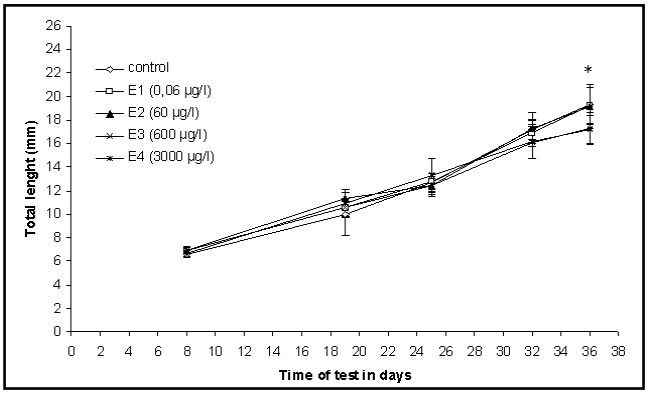

Fig. 3. Total length of common carp larvae (juveniles) after simazine exposure. *Experimental groups significantly (p<0.05) different from the control group.

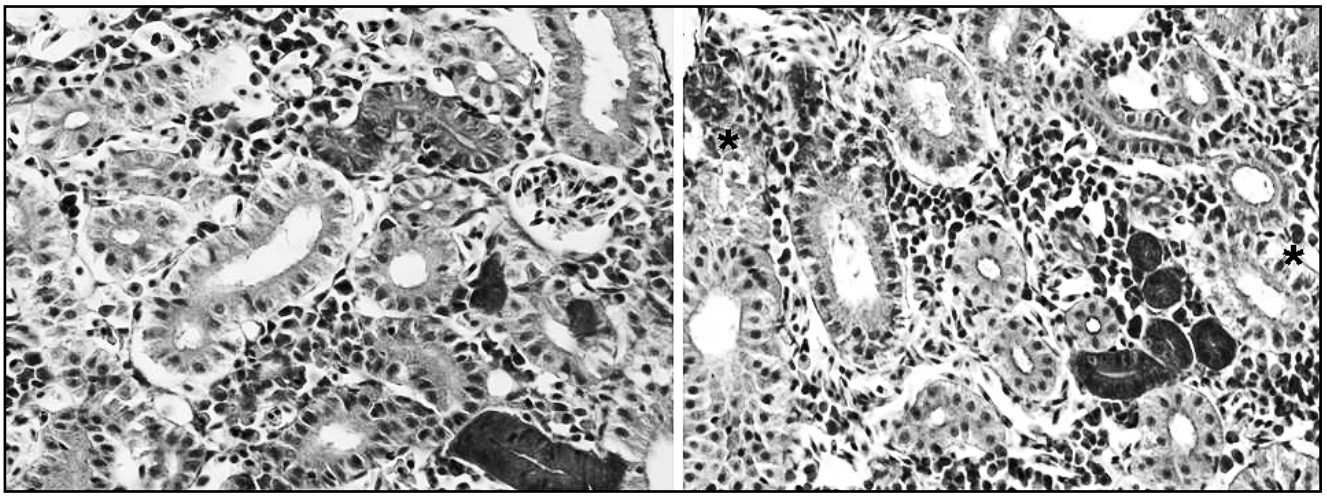

Fig. 4. Caudal kidney of carp (*Cyprinus carpio* L.) after simazine exposure. Haematoxylin and eosin, × 400. A – control group 1, B – group E4 (concentration 3000 μg.l^{-1}of simazine). The stars indicate destructed tubular epithelium.

opment: hatching, swim-up, first-feeding, early development of gills, and metamorphosis (McKim 1995). Studies of the embryonic development of fishes are important, not only to increase knowledge of the developmental processes, but also to understand species-specific adaptations and their ecological value in the course of speciation. Also, the economic relevance for aquaculture and fishery biology is generally accepted (Meijide & Guerrero 2000). Aquatic toxicologists have convincingly defended the usefulness of particularly the advantages of fish early life stage tests as a fairly rapid, lowcost and comparable tool to screen test chemicals and effluents (Woltering 1984). Although we have information on the toxicity of the simazine in adult stages of fish, we do not have too much information on the toxicity of this compound in early life stages of fish. The results of this study provide further data on chronic exposure to simazine for consideration in risk assessment. The findings contribute to improved knowledge of the toxic profile of simazine at actual concentrations in Czech river on early life stage of carp.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). The present study revealed no significant negative effects of simazine on hatching, embryo viability, morphology, mortality of embryos and larvae and early ontogeny at the concentrations tested (0.06–3000 μg.l^{-1}). In the present study, beginning on day 32 of exposure, fish exposed to the two highest tested groups simazine showed significantly lower mass compared with controls. Simazine in highest concentration (3000 μg.l^{-1}) caused decrease of total length of carp after 36 day exposure. Growth represents an integration of a variety of physiological and environmental factors. It provides a sensitive gauge of environmental conditions and is important for reviewing the success with which organisms adapt to their environment (Bengtsson 1974). Growth reductions might delay maturation and reproduction as well as increase the susceptibility of young fish to predation and disease. Their ability to obtain food and to compete for suitable habitats might also be reduced (Rosenthal & Alderdica 1976; Woltering 1984). Plhalova *et al.* (2009) and Velisek *et al.* (2012a) reported a significant decrease growth after terbutryn exposure in zebrafish (*Danio rerio*), respectively in carp.

Little is known about the histopathological changes in development stages of carp after triazine exposure. Generally, triazine pesticides have a direct effect on kidney structure and function in freshwater fish (Velisek *et al.* 2009; 2010; 2011; 2012a,b). The caudal kidney of carp exposed to simazine in the three highes concentrations showed alteration of tubular system of caudal kidney. The kidney is important for the maintenance of a stable internal environment with respect to water and sodium chloride, excretion, and, partially, for the metabolism of pesticides (Ortiz *et al.* 2003). It is evident that renal alteration was related to simazine exposition, while the degree of kidney alteration was not affected by liver. On the basis of our findings it is possible to describe simazine as a primary nephrotoxic substance. Histopathological tissue changes in cranial kidney were similar to the changes found in rainbow trout, sea bream and common carp by other authors (Fischer-Scherl *et al.* 1991; Arufe *et al.* 2004; Oropesa *et al.* 2009).

Histopathological changes were used for estimation of NOEC and LOEC. The values for NOEC and LOEC were estimated at 0.06 and 60 μg.l^{-1} simazine, respectively. It appears that simazine may not be a serious problem for early-life stages of carp in the wild in the Czech Republic. Because we found, that simazine in real concentration in Czech rivers has not influence on early-life stages of carp. But changes founded in caudal kidney were found in fish exposed to the second lowest concentration tested (i.e., 60 μg.l^{-1}), which is

about 10 μg.l^{-1} higher than reported in Colorado rivers in recent years. Concentrations of simazine in World rivers have been reported to generally vary in the range 0.0003–49.20 μg.l^{-1} (Beitz *et al.* 1994; U.S. EPA 1994).

Chronic simazine exposure of early-life stages of common carp affected their growth rate, and histology. Some of the changes were observed only at higher exposures (600, 3000 μg.l^{-1}), but change founded in caudal kidney was affected in fish exposed to the second lowest concentration tested (i.e., 60 μg.l^{-1}). Aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. For detailed elucidation of simazine effects further research is necessary. This research should be focused not only on the studies of effects of simazine alone, but in view of possible synergic or potentiation effect.

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Potential Conflicts of Interest: *None disclosed.*

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