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ORIGINAL ARTICLE

Effects of terbuthylazine on early life stages of common carp

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Abstract

OBJECTIVES: The aim of this study was to assess the toxicity of terbuthylazine 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 methodology. The developmental stages of carp were exposed to terbuthylazine at four concentrations, 2.9 (reported environmental concentration in Czech rivers); 70; 1,400; and 3,500 $\mu g.L^{-1}$ for 35 days and compared to carps in a non-treated control group.

RESULTS: Terbuthylazine in concentration 1,400 and 3,000 $\mu g.L^{-1}$ caused significant ($p<0.01$) decrease of mass, total length and delayed in development of carp. Fish exposed to terbuthylazine showed alteration of tubular system of caudal kidney. On the basis of histopathological changes the values of LOEC = 2.9 $\mu g.L^{-1}$ terbuthylazine were estimated.

CONCLUSIONS: Chronic terbuthylazine exposure of early-life stages of common carp affected their growth rate, early ontogeny and histology. Some of the changes were observed only at higher exposures, but change founded in caudal kidney was affected in fish exposed to the real environmental concentration tested (i.e., 2.9 $\mu g.L^{-1}$).

Abbreviations

96hLC50	- lethal concentration
$ANC_{4.5}$	- acid neutralization capacity
COD_{Mn}	- chemical oxygen demand
FWC	- Fulton's weight condition factor
LOEC	- lowest observed effect concentration
OECD	- Organization for Economic Cooperation and Development
SD	- standard deviation
SGR	- specific growth rate
TL	- total length

INTRODUCTION

Sources of pollution constitute a problem of increasing concern all over the world (Abrantes *et al.* 2010). Increased environmental pollution can be attributed to a variety of factors resulting from different industrial and agricultural technologies (Figueiredo-Fernandes *et al.* 2006). Effects of the residues of various substances persisting in the aquatic environment, the most important of those being pesticides, also are monitored. From among pesticides, the most frequently found are residue of triazine herbicides. Triazine herbicides are among the most commonly used pesticides in the world. Moreover, some of triazine pesticides are prohibited in European countries. Triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community (European Commission 1999) and they are included in the EU Priority Pollutants List (EP 2013).

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine), a triazine herbicide, is a selective systemic herbicide which acts as a photosynthesis inhibitor. It is used as a broad spectrum herbicide in maize, sorghum, vines, citrus, coffee, potatoes, legumes, and forestry. Terbuthylazine was registered in the United States in 1975. Terbuthylazine has very similar chemical structure to atrazine. The difference is only iso-butyl and tert-butyl substituent on the amino group (Roberts *et al.* 1998). The minimum difference in structure affects the decomposition reactions of these substances in the environment that led to a ban on atrazine in the European Union. Terbuthylazine is used as a substitute for atrazine since the end of 2006.

Terbuthylazine is stable to hydrolysis, and to aqueous photolysis. It degrades very slowly under aerobic aquatic conditions, and will persist under most aquatic conditions The half-life of terbuthylazine was reported to 8 days at pH 1, 86 days at pH 5, >200 days at pH 9 in water at 20 °C (Roberts *et al.* 1998). The fate of residues in aerobic and anaerobic aquatic conditions is similar. The major metabolites of terbuthylazine are the de-chlorinated and N-dealkylated products, which are more mobile than the parent, and exhibit some herbicidal activity when they retain the chlorine atom on the triazine ring plus one alkyl group (WHO 2003). Terbuthylazine levels can reach values up to 2.9 $\mu g.L^{-1}$ in Europe rivers (Buser 1990; Brambilla *et al.* 2003; CHMI 2014).

Studies of the toxicity of various triazine herbicides to aquatic organisms indicate that it can cause growth retardation and morphological, biochemical, haematological, histopathological, and antioxidant enzymes alteration (Stara *et al.* 2013, 2014; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a, b, 2015), but less is known about the specific effects of terbuthylazine in real concentration on fish. The toxicity of terbuthylazine 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 carp 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 described by Kocour *et al.* (2005).

Water parameters

Aerated tap water was used in the present study, with the following parameters: dissolved oxygen >95%, temperature 19.1–20.5°C, pH 7.3–8.0, $ANC_{4.5}$ 1.11 $mmol.L^{-1}$, COD_{Mn} 1.2 $mg.L^{-1}$, total ammonia 0.02 $mg.L^{-1}$, NO_3^- 6.00 $mg.L^{-1}$, NO_2^- 0.01 $mg.L^{-1}$, sum of Ca^{2+} + Mg^{2+} 9.81 $mg.L^{-1}$. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature were measured daily. Terbuthylazine concentrations were checked daily by high performance liquid chromatography. Water samples were assayed using the method of Papadopoulos *et al.* (2012). The values measured did not differ from the value stated for test purposes by more than 6%.

Experimental protocol

The trial was carried out using the modified test design of Organization for Economic Cooperation and Development Guidelines for Testing of Chemicals No. 210 (OECD 2013). At 24 h post-fertilization, unfertilized eggs were discarded, and 100 eggs were randomly transferred into each crystallization basins containing tested solution of terbuthylazine (Sigma Aldrich, Czech Republic, chemical purity 99.4%) 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: 2.9 $\mu g.L^{-1}$ (real environmental concentration – group 1 – E1); 70 $\mu g.L^{-1}$ (group 2 – E2); 1,400 $\mu g.L^{-1}$ (group 3 – E3); and 3,500 $\mu g.L^{-1}$ (group 4 – E4). Selected terbuthylazine concentrations of 70; 1,400; and 3,500 $\mu g.L^{-1}$ corresponded to the 1% of 96 hour half lethal concentration (96hLC50), 20% 96hLC50 and 50% 96hLC50 for carp.

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 water for each treatment was renewed daily by gently draining each chamber and adding new solution slowly to avoid disturbing embryos and larvae. Control of hatching, and mortality, were made twice daily, and dead fish were removed. From 6 day larvae were fed freshly hatched brine shrimp *Artemia salina* nauplii *ad libitum* one daily.

On days 7, 14, 21, 28, and 35 samples of fish (30 per concentration groups and control) were collected

to monitor development, occurrence of morphological anomalies, growth rate, Fulton's weight condition factor (FWC), and the length/mass relationship. Determination of development periods and stages followed Penaz *et al.* (1983). Final evaluations included accumulated mortality, mass and total length (TL) of fish. The total length was measured by stereomicroscopy using a micrometer. Mass to 0.1 mg was measured with a Mettler-Toledo balance.

The experiment schedule was: day 1, trial initiation (1 day post-fertilization); day 7, hatching complete; day 9, initiation of exogenous feeding; day 35, end of the experiment. To 35 day, the majority of control fish had become first stage juveniles.

The mean specific growth rate (SGR) for fish in each experimental group was calculated for the period from day 7 to day 35 and compared with controls using the method described by OECD (2000).

Evaluation of 35 day LC50 and LOEC

For the evaluation of 35 day LC50 values, a probit analysis EKOTOX 5.1 software (Ingeo Liberec) was used based on mortality at different terbuthylazine concentrations. For the evaluation of LOEC value, the probit analysis was based on histopathological changes at different terbuthylazine concentrations.

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.

Statistical analysis

One-way analysis of variance was conducted to compare differences among the test groups using the software program Statistica 12 for Windows (StatSoft). The differences in cumulative mortality among groups were assessed using contingency tables (χ^2).

RESULTS

Hatching began 5 day after the onset of exposure, on days 5 to 7. The majority of eggs in all treatment groups hatched by day 7. No significantly negative effects of terbuthylazine on hatching and embryos viability were observed compared to controls.

Accumulated mortality

Cumulative mortality of common carp samples exposed to terbuthylazine and the control sample was between 10–58% (Figure 1). Significant ($p<0.01$) differences in total accumulated mortality were found in fish exposed to the two highest terbuthylazine concentration (1,400 and 3,500 µg.L^{-1}), compared with controls. Based on mortality in the experimental groups, terbuthylazine concentrations were estimated at day 35 to be LC50 = 2,992 µg.L^{-1}.

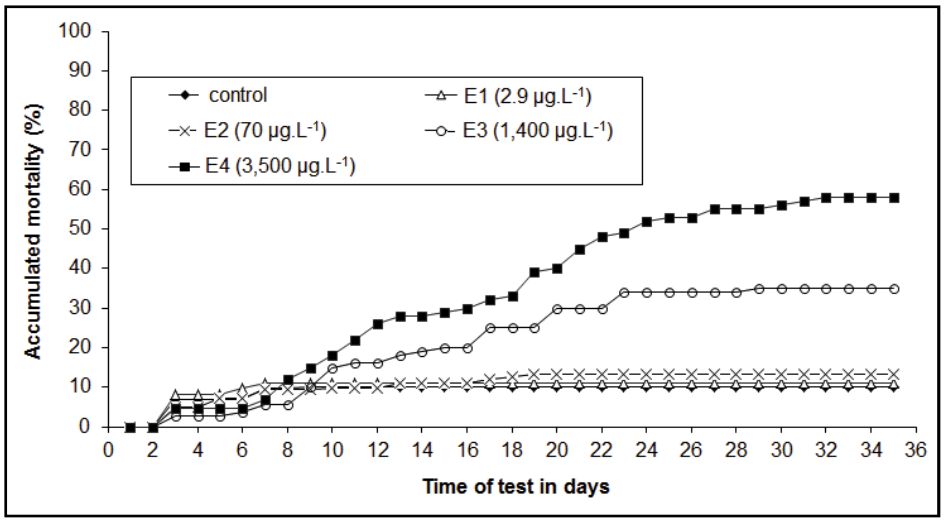

Fig. 1. Accumulated mortality (percentage) of common carp embryos, larvae, and juveniles after terbuthylazine exposure.

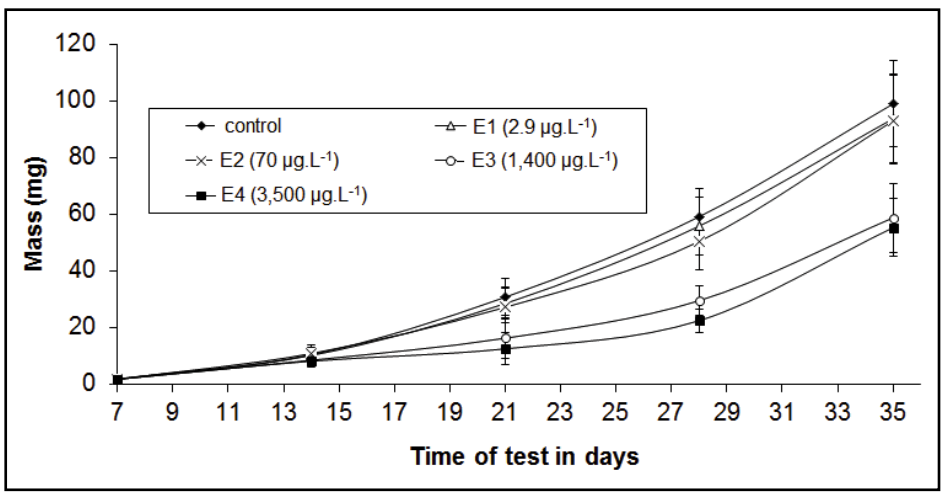

Fig. 2. Mean mass of larvae and juveniles common carp after terbuthylazine exposure. Data are means ± standard deviation (SD).

Tab. 1. Growth rate and fish mortality results of the 35 day embryo-larval toxicity test on common carp after terbuthylazine exposure.

Fish Group	Control	E1	E2	E3	E4
Terbuthylazine (μg.L^{-1})	–	2.9	70	1,400	3,500
m_7 (mean ± SD, mg)	1.72±0.18	1.67±0.24	1.60±0.21	1.58±0.19	1.59±0.25
m_{35} (mean ± SD, mg)	98.85±20.18	93.59±24.36	92.84±20.51	58.64±18.14*	55.16±21.12*
SGR	14.47	14.38	14.50	12.92	12.67
I (%)	–	0.62	–0.21	10.71	12.44
Total mortality (%)	10	11	13	35	58

m_7, m_{35} = mean fish mass in selected group after 7 and 35 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<0.01) different from the control group.

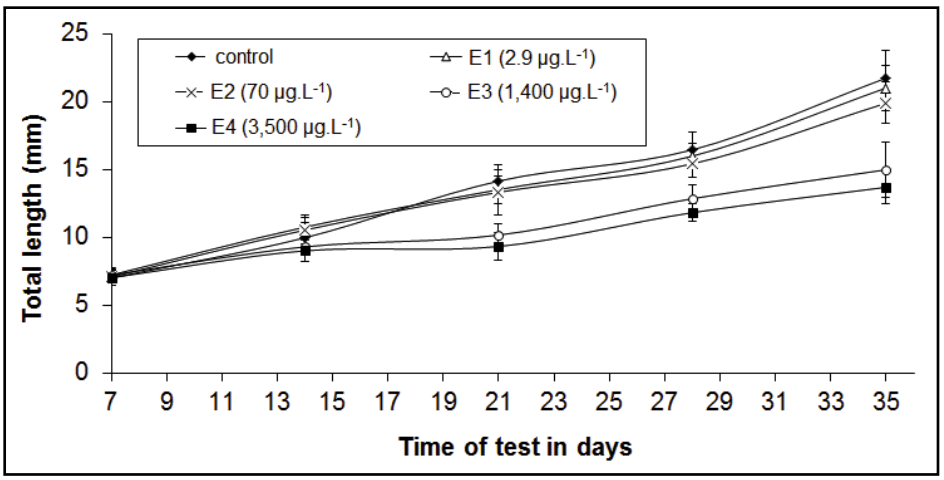

Fig. 3. Total length of larvae and juveniles common carp after terbuthylazine exposure. Data are means ± standard deviation (SD).

Tab. 2. Developmental periods during the 35 day embryo–larva toxicity test on common carp.

Fish Group	Control	E1	E2	E3	E4
Terbuthylazine (μg.L^{-1})	-	2.9	70	1,400	3,500
Times (day)					
7	ec9–L1	ec9–L1	ec9–L1	ec9–L1	ec9–L1
14	L2–L3b	L2–L3b	L2–L3b	L1–L3a	L1–L2
21	L4a–L5	L4a–L5	L4a–L5	L3a–L4a	L3a–L3b
28	L5–L6	L5–L6	L5–L6	L4a–L4b	L3b–L4b
35	J1	J1	J1	L4b–L6	L4b–L5

Length and weight growth parameters

Mass and total length of fish related to terbuthylazine concentrations in water are depicted in Figures 2 and 3. From 21 days of exposure, fish exposed to the two highest tested groups E3 – 1,400 μg.L^{-1}, and E4 – 3,500 μg.L^{-1} terbuthylazine showed significantly (p<0.01) lower mass and total length compared with controls. Terbuthylazine in the tested concentrations had no negative influence on FWC. Specific growth rates and inhibition of growth were calculated for 35 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to the two highest tested concentrations (1,400 and 3,500 μg.L^{-1}) was 10.71% and 12.44%, respectively, compared to control.

Early ontogeny

Fish from two highest tested concentrations (1,400 and 3,500 μg.L^{-1}) of terbuthylazine were significantly (p<0.01) delayed in early development compared with the control group (Table 2). No significant differences in the type and occurrence of morphological abnormalities were observed in tested embryos and larvae of carp during the test.

Histopatology

The histological changes were observed only in caudal kidney in all experimental groups compared to control fish. Fish exposed to terbuthylazine showed alteration of tubular system included destruction of tubular epithelium with or without casts, vacuolization of tubular epithelia and disintegration of glomerules. On the basis of histopathological changes of caudal kidney in the experimental groups, values of LOEC = 2.9 μg.L^{-1} terbuthylazine were estimated.

DISCUSSION

Triazines are serious pollutants of the aquatic environment that can have harmful effects on aquatic organisms alteration (Stara *et al.* 2013; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a). Aquatic organisms are subjected to prolonged exposure of small doses of contaminants in water that do not cause death but which could induce retardation of growth, early developmental and histopatological changes (Stepanova *et al.* 2012;

Stara *et al.* 2013; Koutnik *et al.* 2014; Velisek *et al.* 2013, 2014a,b, 2015). The results of this study provide further data on long term exposure to terbuthylazine for consideration in risk assessment. The findings contribute to improved knowledge of the toxic profile of terbuthylazine at actual concentrations in the Czech rivers on early life stage of carp.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). Based on cumulative mortality, the 35 day LC50 was estimated at 2,992 $\mu g.L^{-1}$ terbuthylazine. The values obtained were lower than that reported in short-term tests on adult specimens, 7 $mg.L^{-1}$ terbuthylazine (Tomlin 2002). Terbuthylazine acute toxicity (96hLC50) values for the goldfish (*Carassius auratus*), 9.4 $mg.L^{-1}$ (Hartley & Kidd 1987); for rainbow trout (*Oncorhynchus mykiss*), is 3.8–4.6 $mg.L^{-1}$; for bluegill sunfish (*Lepomis macrochirus*), 7.5 $mg.L^{-1}$; and for common carp (*Cyprinus carpio*), 7 $mg.L^{-1}$ (Tomlin 2002).

The present study revealed no significant negative effects of terbuthylazine on hatching and embryos viability, morphology at the concentrations tested (2.9–3,500 $\mu g.L^{-1}$). In the present study, beginning on day 21 of exposure, fish exposed to the two highest tested groups terbuthylazine showed significantly lower mass and total length compared with controls. Growth reductions after terbuthylazine exposure might delay maturation and reproduction as well as increase the susceptibility of young fish to predation and disease. This is in agreement with studies Davies *et al.* (1994) who found growth reduction of common galaxias (*Galaxias maculates*) after exposure to low concentrations of atrazine. Plhalova *et al.* (2009) reported growth reduction of zebrafish (*Danio rerio*) after 28 days of exposure to terbutryne. Growth reduction were reported in carp after simazine (Velisek *et al.* 2012a), terbutryne (Velisek *et al.* 2012b) and terbuthylazine-2-hydroxy (Velisek *et al.* 2014b).

The advantage of toxicity tests on early-life stages of fish is the opportunity to observe developmental and morphological changes during exposure of xenobiotics. These tests are a sensitive method, since it covers two developmental stages (embryo and larvae) which differ in susceptibility as a result of physiological and biochemical differences (Penaz *et al.* 1983; McKim 1985; Stepanova *et al.* 2012). In our study terbuthylazine in concentrations 1,400 and 3,500 $\mu g.L^{-1}$ cause delayed in ontogenetic development. These findings are in accord with other studies which describe delay of ontogenetic development in common carp after exposure to terbuthylazine (Stepanova *et al.* 2012), prometryne (Velisek *et al.* 2015), terbutryne (Velisek *et al.* 2012b) and terbuthylazine-2-hydroxy (Velisek *et al.* 2014b).

Triazine pesticides have a direct effect on kidney structure and function in freshwater fish (Velisek *et al.* 2012a, 2014b, 2015). The caudal kidney of carp exposed to terbuthylazine all tested concentrations showed alteration of tubular system of caudal kidney. On the basis of our findings it is possible to describe terbuthylazine 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; Velisek *et al.* 2012a, 2014b, 2015). Histopathological changes were used for estimation of LOEC. The value for LOEC was estimated at 2.90 $\mu g.L^{-1}$ terbuthylazine. It appears that terbuthylazine may be a serious problem for early-life stages of carp in the polluted rivers of terbuthylazine. Because we found, that terbuthylazine in real concentration in Czech rivers has influence on caudal kidney of early-life stages of carp. According to our results of embryolarval test on carp, histopathological changes in caudal kidney seem to be the most sensitive parameter.

Chronic terbuthylazine exposure of early-life stages of common carp affected their growth rate, early ontogeny and histology. Some of the changes were observed only at higher exposures (1,400 and 3,500 $\mu g.L^{-1}$), but change founded in caudal kidney was affected in fish exposed to the real environmental concentration tested (i.e., 2.9 $\mu g.L^{-1}$). According to results of this present study, the histopathological changes in caudal kidney could provide useful information for evaluating the physiological effects on early life stages carp, but the application of these findings will need more detailed laboratory study before they can be established as special indicators for monitoring aquatic environment contaminated to triazine pesticides.

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