ORIGINAL ARTICLE

Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in early life stages of common carp (*Cyprinus carpio* L.)

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Abstract

OBJECTIVES: The aim of the study was to investigate effects of the triazine herbicide prometryne on early life stages of common carp Cyprinus carpio as indicated by oxidative stress and antioxidant indices.

DESIGN: Toxicity tests were performed according to OECD 210 methodologies. Common carp larvae and embryos exposed for 35 days to prometryne at three concentrations, 0.51 (reported concentration in Czech rivers), 80 (1% 96 h LC50), and 1200 (15% 96 h LC50) $\mu g\,l^{-1}$, were compared to carp in a non-treated control group. Activity of superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR), as well as levels of thiobarbituric acid reactive substances (TBARS) were assessed.

RESULTS: Chronic exposure of early life stages of carp to prometryne showed no effect on growth and mortality rates. Levels of oxidative damage in fish test groups showed no significant differences from the controls. Glutathione reductase activity at exposure 0.51 $\mu g\,l^{-1}$ was significantly increase ($p<0.01$) compared with controls and other exposures.

CONCLUSION: The chronic exposure to prometryne showed no influence on oxidative stress. Differences from control fish was observed in GR activity in exposure prometryne 0.51 $\mu g\,l^{-1}$.

Abbreviations

CAT	- catalase
GR	- glutathione reductase
ROS	- reactive oxygen species
SOD	- superoxide dismutase
TBARS	- levels of thiobarbituric acid reactive substances

INTRODUCTION

Environmental pollution, an increasing worldwide concern (Abrantes *et al.* 2010), can be attributed to a variety of sources resulting from industrial and agricultural technologies (Figueiredo-Fernandes *et al.* 2006). Agricultural development has led to growth in the use of chemical agents for pest control. These can disperse in the environmental media, and directly or indirectly threatening human health and the environment (Bermudez-Saldana *et al.* 2005; Abrantes *et al.* 2010). Specifically rivers, lakes, ponds, and reservoirs are predisposed to receiving and accumulating contaminants discharged in industrial sewage, as well as agriculture runoff (Ceyhun *et al.* 2010).

Triazine herbicides are among pollutants frequently monitored in the aquatic environment. They are relatively persistent, with detectable levels in drinking water, foods, and fish (Plhalova *et al.* 2011). Some evidence has linked the mode of triazine compound toxicity in mammals to a disruption of the vitamin metabolism (Kamrin 1997). Triazine herbicides have the ability to disrupt energy metabolism leading to symptoms such as difficulty walking, tremors, convulsions, paralysis, slowed breathing, and diarrhea (Hayes & Laws 1991).

Prometryne, 2,4-bis (isopropylamino)-6-methylthio-s-triazine, a selective herbicide of the s-triazine family, has been utilized as a pre- or post-emergence controller of annual grasses and broadleaf weeds in a variety of crops, including cotton, celery, pigeon peas, and dill (U.S. EPA 1996a; Kamrin 1997). Prometryne was first registered in 1964 by Ciba Crop Protection (U.S. EPA 1996a). Prometryne has a soil half-life of 60 days and persists for up to three months. Following multiple annual applications of the herbicide, prometryne activity can persist for 12–18 months after the final application (U.S. EPA 1996b). The maximum concentration of prometryne in Czech rivers is 0.51 $\mu g\,l^{-1}$ (Czech Hydrometeorological Institute). In Europe, concentrations of 0.190 to 4.40 $\mu g\,l^{-1}$ have been reported in surface waters (Vryzas *et al.* 2011) and exceeding 1 $\mu g\,l^{-1}$ in ground water (Papadopoulou-Mourkidou *et al.* 2004). Studies indicate that prometryne poses an acute risk to non-target terrestrial and aquatic plants. Five-day EC50 of prometryne for algae (*Selenastrum capricornutum*) is 0.023 $mg\,l^{-1}$ (U.S. EPA 1996a). Prometryne is slightly to moderately toxic to fish. Acute toxicity (96 h LC50) in rainbow trout (*Oncorhynchus mykiss*) is 2.9 $mg\,l^{-1}$ (U.S. EPA 1996b); for sheepshead minnow (*Cyprinodon variegatus*), 5.1 $mg\,l^{-1}$ (Kegley *et al.* 2010); for bluegill sunfish (*Lepomis macrochirus*), 7.9 $mg\,l^{-1}$; and for common carp (*Cyprinus carpio*), 8 $mg\,l^{-1}$ (Popova 1976). Chronic prometryne toxicity in freshwater fish has not been investigated to a significant extent. Adverse effects on growth have been reported in fathead minnows (*Pimephales promelas*) exposed to 1 to 2 $mg\,l^{-1}$ prometryne for 32 days (Erickson & Turner 2002). Triazine herbicides are among the most commonly used pesticides in the world. Prometryne is widely used in China, Australia, Canada, New Zealand, South Africa, and the United States, but is banned in Europe (Kegley *et al.* 2010).

In recent years, concerns about the persistence, mobility, and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in water and in various environmental compartments (Chapadense *et al.* 2009). It is necessary to study the long-term effects of these substances on non-target organisms, especially fish, which are an important component of the aquatic environment and the human diet. Oxidative stress, along with antioxidant systems, may be a good indicator of prometryne accumulation in fish tissue. Although the effects of acute and sub-chronic exposure of fish to triazine herbicides have been well documented, there is a scarcity of data on the chronic exposure to these compounds at environmentally realistic concentrations with respect to oxidative stress and antioxidant responses in the fish species typical of Central Europe. The aim of the present study was to investigate effects of long-term exposure of common carp to low prometryne concentrations with respect to oxidative stress and the antioxidant defense system.

MATERIALS AND METHODS

Experimental animals

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

Experimental design

The trial was carried out using the modified test design of the 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 randomly selected fertile eggs were transferred to crystallization basins for each containing one of three concentrations of prometryne (Sigma Aldrich, Czech Republic, chemical purity 99.3%) and to a control dish. The trial was done in triplicate groups. The concentrations were: Group 1, 0.51 $\mu g\,l^{-1}$ (reported environmental concentration in Czech rivers); Group 2, 80 $\mu g\,l^{-1}$ (1% 96 h LC50); and Group 3, 1200 $\mu g\,l^{-1}$ (15% 96 h LC50). The test baths consisted of continuously gently aerated tap water with the following parameters: dissolved oxygen >93%, temperature 19.3–21.1 °C, pH 7.56, $ANC_{4.5}$ 3.3 $mmol\,l^{-1}$, COD_{Mn} 1.1 $mg\,l^{-1}$, total ammonia < 0.02 $mg\,l^{-1}$, NO_3^- 5.55 $mg\,l^{-1}$, NO_2^- < 0.01 $mg\,l^{-1}$, PO_4 0.228 $mg\,l^{-1}$, Ca_2^+ 49.10 $mg\,l^{-1}$, and Mg_2^+ 13.60 $mg\,l^{-1}$. Oxygen saturation, pH, and temperature were mea-

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

The basins were placed in a laboratory (open-air conditions) with ambient light exposure (16:8 h light:dark). The arrangement of basins was random. The exposure water for each treatment was renewed twice daily by gently draining each chamber and adding new solution slowly to prevent disturbing the embryos and larvae. Observations of hatching, survival, and behavior were made twice daily. Dead embryos and larvae were collected, and the numbers recorded for each concentration. When able to feed, larvae were given freshly hatched brine shrimp (*Artemia salina*) nauplii *ad libitum* twice daily prior to water exchange. The nauplia were rinsed with tap water to avoid contaminating the exposure water with chlorine.

Samples of carp early life stage and preparation of post-mitochondrial supernatant

The trial ended at 35 days when fish were weighed and measured for total length. Samples were immediately frozen and stored at –80 °C for analysis. Frozen tissue samples were weighed and homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and a second centrifuged at 4 °C to obtain the post-mitochondrial supernatant for other antioxidant analyses.

Indices of oxidative stress

The TBARS method described by Lushchak *et al.* (2005) was used to evaluate lipid peroxidation.

Antioxidant parameters

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined spectrophotometrically by the method of Marklund & Marklund (1974). The catalase (CAT; EC 1.11.1.6) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers & Sizer (1952). Glutathione reductase (GR) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg & Mannervik 1975). One unit of CAT or GR activity is defined as the amount of the enzyme that consumes 1 mol l^{-1} of substrate or generates 1 mol l^{-1} of product per min. Activity was expressed in international units (or milliunits) per mg of protein.

Protein estimation

Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

The software program STATISTICA (version 8.0 for Windows, StatSoft) was used to compare differences among the test groups. Prior to analysis, all measured variables were checked for normality (Kolmogorov-Smirnov test) and homoskedasticity of variance (Bartlett's test). If these conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. When a significant difference was detected ($p<0.05$), Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a non-parametric test (Kruskal-Wallis) was used.

RESULTS

Length, weight, and mortality

Length and weight growth parameters and total mortality of carp early life stages after 35 days exposure to prometryne are shoved in Table 1. Fish groups exposed to the selected prometryne concentrations did not differ in weight, total length, or mortality rate compared with controls.

Oxidative stress

Effects of chronic exposure to prometryne on TBARS in the homogenate carp early life stages are given in Table 2. The test groups were not significantly different from the control group in TBARS in homogenates.

Antioxidant response

Effect of chronic exposure to prometryne on antioxidants responses (SOD, CAT, GR) in homogenate of carp early life stages are given in Table 3. Exposure Group 1, showed significantly higher GR activity than the controls and other exposures tested to prometryne ($p<0.01$). No differences from controls were observed in SOD and CAT activity of exposed groups.

Tab. 1. Total length, weight, and mortality of early life stages carp after 35 day exposure to prometryne.

Fish Group Prometryne (µg l^{-1})	Control –	1 0.51	2 80	3 1 200
Total length (mm)	19.93±1.92	19.31±2.09	18.35±1.61	18.77±1.45
Weight (mg)	106.73±32.52	90.98±29.88	80.01±19.83	84.77±20.79
Total mortality (%)	14	17	13	29

Tab. 2. Effects of chronic exposure to prometryne on level of thiobarbituric acid reactive substances (TBARS, nmol/mg protein) activity in homogenates of carp early life stages.

Fish Group Prometryne (µg l^{-1})	Control –	1 0.51	2 80	3 1 200
TBARS	0.40±0.06	0.43±0.04	0.39±0.03	0.38±0.01

Tab. 3. Effect of chronic exposure to prometryne on superoxide dismutase (SOD, nmol NBT/min/mg protein), catalase (CAT, μmol H_2O_2/min/mg protein) and glutathione reductase (GR, nmol NADPH/min/mg protein) activity in homogenate of carp early life stages.

Fish Group Prometryne (μg l^{-1})	Control –	1 0.51	2 80	3 1 200
SOD	0.28±0.03	0.24±0.03	0.27±0.03	0.26±0.06
CAT	0.61±0.13	0.51±0.16	0.50±0.16	0.49±0.12
GR	0.34±0.10	0.90±0.19**	0.43±0.38	0.26±0.15

** Experimental groups significantly (p<0.01) different from the control group.

DISCUSSION

Studying the toxicity of pesticides to early life stages of fish is useful as embryos and larvae are often the most sensitive to toxic effects, although they may differ in susceptibility due to physiological and biochemical differences (McKim 1995). We observed chronic exposure to prometryne at concentrations commonly occurring in the aquatic environment on the early life stages of carp. No significant effect was observed on total length, weight, and mortality the fish. Effects of the triazine herbicide terbutryne at environmental concentrations on early life stages of carp were studied by Velisek *et al.* (2012). The fish exposed to terbutryne at 0.02 μg l^{-1} showed significantly lower weight and overall length, and at 2 mg l^{-1}, high mortality compared to controls was found. Terbutryne at 0.02 μg l^{-1} had no affect growth on *Danio rerio* (Plhalova *et al.* 2009). Mikulikova *et al.* (2011) found the environmental concentration of terbuthylazin (380 ng l^{-1}) affected biometric indices of carp experimentally exposed for 91 days.

The main objective of this study was to determine the influence of prometryne on carp oxidative stress and antioxidant parameters. This knowledge has a great importance for environmental and aquatic toxicology. Oxidative stress can be evoked by numerous chemicals including some pesticides. Pro-oxidant action on fish can be used to assess water pollution (Slaninova *et al.* 2009). Contaminant-stimulated reactive oxygen species (ROS) production, and resulting oxidative damage, may be a significant mechanism of toxicity in aquatic organisms exposed to pollution (Livingstone 2003). Oxidative stress occurs when there is an imbalance between pro-oxidant and antioxidant production, leading to the generation of ROS (Nwani *et al.* 2010). Environmental xenobiotics are known to alter antioxidant defense systems and to cause oxidative damage in aquatic organisms by ROS production (Monteiro *et al.* 2006). Among the ROS produced, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (OH·) at high concentrations can react with biological macromolecules potentially leading to lipid peroxidation, enzyme inactivation, and possibly cell death (Kevin *et al.* 2005). At low concentrations effects are less pronounced. Lipid peroxidation determined by the formation of TBARS is one of the commonly used markers of oxidative stress (Nwani *et al.* 2010). In the present study, no elevated levels of TBARS in response to the exposure to prometryne were observed. Toni *et al.* (2010) described increased TBARS levels in brain of carp exposed to the herbicide bispyribac-sodium at 20.87 μg l^{-1} after 7, 21, and 72 days. Changes were observed in muscle after 21 days. Levels were increased in liver after 72 days. Moreas *et al.* (2007) observed effects of the herbicides clamazone (0.5 mg l^{-1}) and propanil (3.6 mg l^{-1}) on *Leporinus obtusidens*. The TBARS levels were lower in brain and muscle, and higher in liver, after 30 days exposure. Responses to oxidative stress may differ with respect to species, age, habitat, feeding, behavior, duration of exposure, particular tissues, and concentration of the herbicide tested.

Virtually in all tissues of vertebrates contain ROS continuously generated during metabolism. Living cells have developed a variety of defense mechanisms to neutralize the damaging effects of free radicals of ROS (Zhang *et al.* 2004). The antioxidant defense system includes enzymes such as superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, glutathione-S-transferase, and glucose-6-phosphate dehydrogenase (Menezes *et al.* 2011a). These antioxidants scavenge free radicals to prevent oxidative damage. SOD and CAT systems provide a first line of defense against ROS (Nwani *et al.* 2010). Superoxide dismutase catalyses the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by CAT. This enzyme removes the hydrogen peroxide, which is metabolized to oxygen and water (Menezes *et al.* 2011b). Glutathione reductase also provides a first line of defense against oxidative damage by maintaining cytosolic concentrations of gluthatione (Stephensen *et al.* 2002). In this study, change was observed only in GR activity. At tested concentration 0.51 μg l^{-1} levels of GR was high compared to the control group and other tested concentrations. Regulatory mechanisms of GR activity in fish have not been fully elucidated. It has been reported that oxidized glutathione content is a critical factor in GR induction (Stephensen *et al.* 2002). Generally, elevated GR activity reflects the oxidation of reduced glutathione, which is converted to glutathione, the substrate of GR activity (Elia *et al.* 2006). Santos & Martinez (2012) evaluated the effects of atrazine on *Prochilodus lineatus* as indicated by antioxidant biomarkers in liver. Antioxidant enzymes SOD and CAT were significantly reduced in fish exposed to atrazine concentrations of 2 and 10 μg l^{-1} for 24 and 48 h. On the other hand, activity of GR significantly increased in fish exposed to these concentrations, in both experimental periods. Nwani *et al.* (2010) exposed *Channa punctatus* to atrazine at concentrations of 4.238, 5.3, and 10.6 mg l^{-1} for 15 days. Activity of SOD, CAT, and

GR were increased in liver in a concentration dependent pattern, suggesting the use of these antioxidants as potential biomarkers of contamination exposure in freshwater fish. Numerous studies have demonstrated that exposure to triazine herbicides affects the antioxidant defense system in fish, causing an imbalance between reactive oxidative system production and elimination and resulting in oxidative stress and organism damage (Stara *et al.* 2012). These studies provide evidence that the biochemical responses are dependent on stressor type, species, and duration of exposure (Nwani *et al.* 2010).

This study focused on the effect of chronic exposure to prometryne on early life stages of carp, using biomarkers of oxidative stress and antioxidant enzymes. Chronic exposure to prometryne significantly increased GR activity in fish. The use of these biomarkers to determine damage in fish and pollution of the aquatic environment were shown to be an important tool for detecting adverse effects of pesticides such as triazine herbicides, even at low concentrations.

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Potential Conflicts of Interest: *None disclosed.*

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