The effects of atrazine exposure on early life stages of common carp (*Cyprinus carpio*)

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**Abstract**  
**OBJECTIVES:** Atrazine and its chloro-s-triazine metabolites are found in surface water and groundwater of the Czech Republic, although their use has been banned since 2005. The objective of the study was to determine the toxic effects of atrazine at an environmentally relevant concentration of atrazine, 0.3 μg.L⁻¹ and at concentrations of 30, 100, and 300 μg.L⁻¹ on morphometric and condition characteristics, development of early life stages, and antioxidant defense enzymes of common carp.  
**METHODS:** The embryo-larval toxicity test was performed according to the OECD Guidelines 210 (Fish, Early-life Stage Toxicity Test).  
**RESULTS:** Atrazine exposure showed no effect on morphometric and condition characteristics or histology. Exposure at 0.3 μg.L⁻¹ was associated with significantly increased activity of glutathione peroxidase, glutathione S-transferase, superoxide dismutase, and catalase compared to control. Activity of glutathione reductase was slightly higher at a concentration of 0.3 μg.L⁻¹, with significantly lower (p<0.05) activity observed in groups exposed to 30, 100, and 300 μg.L⁻¹ compared to the group exposed to 0.3 μg.L⁻¹. The level of oxidized lipids was slightly higher in groups exposed to atrazine at 100 and 300 μg.L⁻¹ compared to controls.  
**CONCLUSIONS:** Atrazine has a significant influence on the biotransformation enzyme and oxidative defense enzymes of early life stages of common carp. The lowest observed effect concentration (LOEC) was 0.3 μg.L⁻¹.

**Abbreviations:**  
ANC4.5 - Acid Neutralizing Capacity  
CAS - Chemical Abstracts Service Registry Number  
CAT - Catalase  
CODMn - Chemical Oxygen Demand  
FCF - Fulton’s condition factor  
GPx - Glutathione peroxidase  
GR - Glutathione reductase  
GST - Glutathione S-transferase  
I - Inhibition of specific growth  
LC₅₀ - Median lethal concentration  
LOEC - Lowest observed effect concentration  
OECD - Organization for Economic Cooperation and Development  
ROS - Reactive oxygen species  
SD - Standard deviation  
SE - Standard error  
SGR - Specific growth rate  
SOD - Superoxide dismutase  
TBARS - Thiobarbituric acid reactive substances  
TL - Total length  
W - Weight
INTRODUCTION

Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine) is a selective systemic herbicide belonging to the s-triazine family that inhibits photosynthesis. It was first registered in the United States in 1958 and has been used in agriculture to kill weeds in crops including maize, sorghum, sugarcane, winter wheat, pasture, hay, and minor crops (guava, macadamia nuts) and to kill grassy weeds and submerged vegetation in stagnant and slow running waters (Hussein et al. 1996; Kimbrough & Litke 1996; U.S. EPA 2001; WHO 2010). Atrazine and its chloro-s-triazine metabolites (deethylatrazine, deisopropylatrazine, and diaminochlorotriazine) are present in surface water and groundwater as a result of the use of atrazine as a pre-emergent or early post-emergent herbicide (WHO 2010).

This herbicide continues to be one of the most widely used in the United States, China, Brazil, and Argentina (Zhou et al. 2008; Svratz et al. 2012; Xing et al. 2012a). In compliance with the European Commission Decision 2004/248/EC, the Czech Republic has banned use of atrazine since 1 August 2005. According to the Czech Hydrometeorological Institute, this substance is a current contaminant of rivers in the Czech Republic detectable at concentrations ranging from 0.3 to 1 μg.L⁻¹.

Degradation of atrazine can take place through photolysis and by microorganisms in surface water followed by hydrolysis to the chloro-substituent (half-life is >100 days at 20°C), but degradation decreases with increasing water depth. Atrazine has low solubility and can be stable and persist in groundwater. The presence of atrazine and its metabolites in surface water is most likely to be intermittent, particularly in flowing water, but groundwater contamination will usually be persistent (Burnside et al. 1963; Howard 1989; WHO 2010). Owing to its persistence in the environment, minimal, but essentially chronic, effects are associated with atrazine exposure in certain aquatic systems (Hussein et al. 1996; Solomon et al. 1996). The issue of contamination by atrazine has increased in importance in recent years (Du Preez et al. 2005; Murphy et al. 2006).

In aquatic ecosystems, the ecotoxicological relevance of atrazine has been widely studied in various aquatic animals, including fish (Huber 1993; Solomon et al. 1996; Elia et al. 2002; Phyu et al. 2006; Dong et al. 2009; Mhadhbi & Beiras 2012; Mhadhbi et al. 2012; Xing et al. 2012a). Most studies have focused on acute toxicity of atrazine and its adverse effects on fish: reduced sperm production; increased renal excretion of Na⁺, Cl⁻, and protein in rainbow trout and carp; disruptions of normal behavior; damage to kidney and gill epithelium; and decreased ability to withstand warm temperatures (Fischer-Scherl et al. 1991; Nesovic et al. 1993; Steinberg et al. 1995; Hussein et al. 1996). Critical development of tissues and organs in embryos and larvae can be easily disrupted by exposure to toxic compounds (Foekema et al. 2008; Kammann et al. 2009; Praskova et al. 2011). Early life stages of fish are generally regarded as the most sensitive to toxic agents (Hutchington et al. 1998; Wiegand et al. 2001; Hostovsky et al. 2012; Svratz et al. 2012) and are ideal models for determining responses to environmental contaminants. The prevalence of triazine pesticides in the aquatic environment, and their potential for adverse effects, makes them strong candidates for toxicological studies. The objective of the present study was to determine the effects of atrazine on early life stages of common carp with respect to morphometric and condition characteristics, histopathology, and antioxidant defense enzymes.

MATERIAL AND METHODS

Experimental protocol

Embryo-larval toxicity testing was performed according to OECD guidelines 210 (Fish, Early-life Stage Toxicity Test). Fertilized eggs of common carp were obtained from Rybnikarstvi Pohorelice a.s. Eggs were produced according to standard methods of artificial reproduction (Kocur et al. 2005).

A semi-static trial with solution replacement twice daily was used. Replacement was conducted with care to avoid interfering with development of embryos and larvae. Temperature range was 21.3–22.9°C, pH was 8.1–8.9, and dissolved oxygen saturation was >60%; conditions were monitored daily.

Eggs were collected 24 h post-fertilization and groups of 100 were transferred into each of fifteen 900 ml crystallization dishes. Three groups of 100 eggs were exposed to atrazine (Sigma–Aldrich, Czech Republic; chemical purity – 98.9%) at the reported environmental concentration in the Czech Republic of 0.3 μg.L⁻¹ and three to each of the concentrations of 30, 100, 300 μg.L⁻¹ and in atrazine-free tap water as control. Concentrations during the test did not decrease <80% of the nominal concentration.

Day 1 post-fertilization was designated as the beginning of the embryo-larval toxicity test, and the test was terminated at 33 days, when all larvae in the control group had become juveniles. Larvae were fed ad libitum twice daily on fresh Artemia salina nauplii. The hatching rate and mortality were monitored also twice daily. Dead embryos and larvae were removed and recorded.

Sampling was done on days 6, 12, 19, 26, and at the conclusion of the test on day 33. From each group, 30 specimens (day 6 and 12), 15 specimens (day 19 and 26), and 45 specimens (day 33) were collected and stored in 4% formaldehyde. Samples were used for monitoring developmental stages, morphological abnormalities, and morphometric and condition characteristics: total length (TL) (stereomicroscopically to 0.01 mm using a micrometer), weight (W) (to 0.1 mg), Fulton’s condition factor (FCF), specific growth rate (SGR), and inhibition of specific growth (I).
Sample specimens were assigned to developmental stages according to Penaz et al. (1983): nine embryonic (E1–E9), six larval (L1–L6), and two juvenile (J1–J2) stages.

**Water parameters**
The basic physical and chemical parameters of the dilution water used in the embryo-larval toxicity test were, acid neutralizing capacity (ANC4.5), 3.6–3.7 mmol.L–1; chemical oxygen demand (CODMn), 1.4–1.9 mg.L–1; total ammonia, below the limit of determination (<0.04 mg.L–1); nitrates, 12.1–13.6 mg.L–1; nitrites, below the limit of determination, <0.01 mg L–1; Cl–, 17.5 to 18.5 mg L–1; and Σ Ca + Mg, 3.06 mmol L–1.

**Morphometric and condition characteristics**
Fulton’s condition factor was calculated at each sampling time.

\[
FCF = \frac{W \times 10^5}{TL^3}
\]

\(W=\) weight (mg), TL = total length (mm).

The mean specific growth rate (SGR) was calculated for each group beginning on day 6 (the first sampling time) and on day 33 (completion of the test).

\[
SGR = \frac{\ln W_2 - \ln W_1}{T_2 - T_1} \times 100
\]

\(W_1 =\) weight (mg) at time \(T_1\) (6 day), \(W_2 =\) weight (mg) at time \(T_2\) (33 day), \(T_1 =\) first sampling time, \(T_2 =\) end of the test).

The inhibition of specific growth rate (I) was calculated as follows:

\[
I \ [\%] = \frac{SGR (\text{control}) - SGR (\text{group})}{SGR (\text{control})} \times 100
\]

**Histology**
Thirty additional specimens from each group were taken for histological examination at the end of the test. Samples were fixed in buffered 4% formaldehyde, dehydrated, embedded in paraffin wax, sectioned (cross section) at 4 μm, and stained with hematoxylin and eosin. Histology of skin, gill, kidney, and liver was assessed by light microscopy.

**Evaluation of antioxidant defense enzymes and lipid peroxidation**
At the end of the test, a further 30 specimens from each test and control group were killed and weighed, homogenized with phosphate buffer (pH 7.2), and stored at ~85 °C until analysis. The catalytic concentrations of glutathione S-transferase (GST), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were measured spectrophotometrically (Habig et al. 1974; Carlberg & Mannervik 1975; Aebi 1984; Flohe & Gunzler 1984; Ewing & Janero 1995). Protein concentrations were determined by a Bicinchoninic Acid Protein Essay Kit (Sigma–Aldrich, St. Louis, MO, USA) using bovine serum albumin as standard (Smith et al. 1985). To assess lipid peroxidation in the samples, malondialdehyde was measured by lipid peroxidation – thio-barbituric acid reactive substances (TBARS) method by Lushchak et al. (2005).

**Determination of atrazine**
Gas chromatography with ion trap tandem mass spectrometry (GC/IT-MS) was used for the determination of atrazine. A sample preparation was based on simple liquid-liquid extraction into cyclohexane.

Separation, identification and quantification were based on the GC/IT-MS method. A Varian 450-GC gas chromatograph (Varian Inc., USA) and VF-5ms (30 m × 0.25 mm) column were used for separation. 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 & Einhorn (2006). All solvents were of GC/MS-grade purity (Chromservis, s.r.o., CZ). A certified standard of atrazine was purchased from Dr. Ehrenstorfer GmbH (Germany).

The detection limit (3σ) for atrazine was 0.04 μg.L–1. Expanded uncertainty was 6.3% on the condition that the coefficient of expansion was k=2.

**Statistical analysis**
Statistical analysis was conducted with Statistica 8.0 for Windows (StatSoft, Inc., Tulsa, OK). A normality test of all of the datasets obtained for the parameters investigated was conducted with the Shapiro–Wilk test. An analysis of variance (ANOVA) was used to evaluate results of oxidative stress parameters. When significant differences were found, a conservative Tukey’s test was conducted as a post hoc test to determine differences among groups. Data of cumulative mortality, morphometric and condition parameters, and hatching did not show normal distribution, and data were subjected to a Kruskal-Wallis ANOVA. Significant differences among pairs of groups were evaluated using the multi-sample rank sums test.

**RESULTS**

**Hatching**
Hatching began on day 3 in all groups. On the first day of hatching, treatment groups showed significantly higher (p<0.05) hatching rates compared to the control group (8%) in this order: 30% (100 μg.L–1), 28% (30 μg.L–1), 26% (0.3 μg.L–1), and 20% (300 μg.L–1). Hatching was completed on day 6.

**Mortality rate**
Cumulative mortality in the control group and all exposed groups was similar, below 23%.
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Early ontogeny

The developmental stages observed at the sampling times in all tested concentrations and controls did not show significant differences in early ontogeny.

Morphological anomalies

The control and environmental concentration showed morphological anomalies in fewer than 5% of specimens. Anomalies (edema, yolk sac deformity, axial and/or lateral curvature of the spine – lordosis, scoliosis, and shortened body) were observed in approximately 25% of the fish in the 30 μg.L⁻¹, 100 μg.L⁻¹, and 300 μg.L⁻¹ group on day 6.

Histopathology

No lesions of skin, gill, or liver were observed in any group.

Morphometric and condition characteristics

Atrazine exposure did not significantly affect morphometric or condition characteristics of early life stages of common carp. Significant differences appeared only in TL (mm) after 6 and 12 days of atrazine exposure (Table 1).

Oxidative stress

Glutathione reductase

The highest activity of GR was found in the control and environmental concentration. Significantly lower (p<0.05) activity of GR was observed in groups exposed to 30, 100 and 300 μg.L⁻¹ compared to the group exposed at 0.3 μg.L⁻¹, but no significant differences were found compared to the control (Table 2).

Catalase

The slight increase in CAT activity was observed at all tested concentrations compared to the control group, with significant difference (p<0.05) seen only at the environmental concentration of 0.3 μg.L⁻¹ (Table 2).

Superoxide dismutase

Compared to the control group, slightly increased SOD activity was detected at 0.3 μg.L⁻¹ and 30 μg.L⁻¹ atrazine, with higher activity (p<0.05) at 0.3 μg.L⁻¹. Atrazine at 300 μg.L⁻¹ showed a significant (p<0.05) reduction in SOD activity compared to the control group (Table 2).

Glutathione S-transferase

GST activity was significantly increased (p<0.05) in larvae exposed to atrazine at 0.3 μg.L⁻¹ compared to the control. The lowest level (p<0.05) of GST activity was observed in groups exposed to 100 and 300 μg.L⁻¹ compared to the group exposed at 0.3 and 30 μg.L⁻¹, but no significant differences were found compared to the control group (Table 2).

Lipid peroxidation – TBARS

The slight increase in TBARS values compared to the control group was observed with atrazine at 100 and 300 μg.L⁻¹ (Table 2). A technical problem precluded measuring TBARS levels in the 0.3 and 30 μg.L⁻¹ concentrations.

Glutathione peroxidase

A significant increase (p<0.05) in GPx activity compared to the control was found in all atrazine exposed groups (Figure 1).
DISCUSSION

Acute toxicity tests on various species of fish have determined 48 h LC$_{50}$ values for atrazine ranging from 10 to 100 mg.L$^{-1}$ (Svobodova et al. 1987). Wiegand et al. (2000) found 48 h LC$_{50}$ in early life stages of zebrafish (Danio rerio) to be 36.8 mg.L$^{-1}$. There are currently no reports of acute poisoning of fish by pesticides with the active ingredient of atrazine (Svobodova et al. 2011).

Atrazine and its metabolites remain in the aquatic environment, as these chemical substances are persistent in surface and groundwaters (Sehonova et al. 2012). In our study, the embryo-larval toxicity test used an environmentally relevant concentration of atrazine, 0.3 μg.L$^{-1}$, and concentrations of 30, 100, and 300 μg.L$^{-1}$. After 33 days of exposure, the range of concentrations did not differ in effects on the early developmental stages, mortality, morphometric and condition characteristics, and histopathology of common carp. Scahill (2008) obtained similar results, observing that atrazine (20–250 μg.L$^{-1}$) did not significantly affect timing of development, muscle contractions, embryo length, yolk diameter, or mortality of embryonic fathead minnows (Pimephales promelas) and the African clawed frog (Xenopus laevis).

At day 6 of exposure, the carp embryos showed morphological anomalies at atrazine concentrations of 30, 100, and 300 μg.L$^{-1}$ (edema, yolk sac deformity, lordosis, scoliosis, and body shortening). Similarly, Scahill (2008) described abnormal spine curvature, abnormal eye, and lack of pigmentation in atrazine exposed frogs and fish.

Our study found atrazine to have significant effects on biotransformation and oxidative enzymes and defense systems at 0.3 μg.L$^{-1}$. Atrazine and its metabolites remain in the organism and are associated with damage such as increased superoxide radical formation (Xing et al. 2012a). The oxygen free radicals and other reactive oxygen species potentially cause oxidative damage to membrane lipids, DNA, and proteins and induce changes in antioxidant enzyme levels (Halliwell & Gutteridge 1999; Avci et al. 2005; Valavanidis et al. 2006; Di Giulio & Meyer 2008; Slaninova et al. 2009; Hostovsky et al. 2012).

The present study showed significantly higher ($p<0.05$) activity of SOD, CAT, and GST in larvae exposed to 0.3 μg.L$^{-1}$ atrazine compared to the control and slightly increased activity of these enzymes at 30 μg.L$^{-1}$ atrazine (Table 2). Blahova et al. (2013) documented a slight increase of SOD and GST activity in zebrafish after subchronic exposure of atrazine in the environmental concentration of 0.3 μg.L$^{-1}$. Xing et al. (2012a) detected increase of SOD and CAT with atrazine at 4.28 μg.L$^{-1}$ in common carp after 40 days exposure, and Jin et al. (2010) reported an increase ($p<0.05$) of SOD and CAT activity in ovary of zebrafish after 14 day exposure to atrazine at 10 μg.L$^{-1}$ compared to a control group. Like other organisms, fish can combat elevated levels of ROS in their systems with protective ROS-scavenging enzymes such as SOD and CAT, which convert superoxide anions (O$_{2}^{-}$) to H$_{2}$O$_{2}$ and then to H$_{2}$O and O$_{2}$. Thus, it is possible that an increase in the activity of these enzymes contributes to the elimination of ROS induced by atrazine exposure from the cell (Jin...
et al. 2010). However, our results showed that a higher concentration of atrazine used in our experiment (100 μg L⁻¹) was associated with decreased activity of SOD, CAT, and GST. Similar results have been obtained in other studies, suggesting that organisms suffered serious damage due to weakening of their antioxidant capacity (Wiegang et al. 2001; Xing et al. 2012a, b; Blahova et al. 2013).

Glutathione reductase plays an important role in cell antioxidant protection in catalyzing the regeneration of glutathione. A decrease in this enzyme activity may result in glutathione depletion and a reduction in antioxidant defense (Stepanova et al. 2013). Higher GR activity was found only at 0.3 μg L⁻¹, while other concentrations showed a reduction in GR activity. Significant differences (p<0.05) were found only between environmental levels 0.3 μg L⁻¹ and other concentrations (Table 2). Blahova et al. (2013) observed lower GR activity in zebrafish exposed to atrazine at 90 μg L⁻¹ compared to concentrations of 0.3 μg L⁻¹ and 30 μg L⁻¹. Conversely, Nwani et al. (2010) reported GR activity unchanged in the liver of Clarias punctatus following exposure to atrazine at 4.24, 5.30, and 10.60 mg L⁻¹.

Activity of GPx converts H₂O₂ or other lipid peroxides to water or hydroxyl lipids, and, in the process, glutathione is converted to oxidized glutathione. A significant increase in GPx activity compared to the control was found in fish in all groups exposed to atrazine (Figure 1). Xing et al. (2012a) obtained similar results when, after a 40 day exposure of common carp to atrazine, GPx activity was highest at concentrations 4.28 and 42.8 μg L⁻¹.

Lipid peroxidation determined by the formation of TBARS is one of the commonly used markers of oxidative stress. The TBARS level in the group exposed to 100 and 300 μg L⁻¹ was slightly increased over than of the control group (Table 2). Our results are in agreement with Nwani et al. (2010) and Blahova et al. (2013), who confirmed an elevated level of lipid peroxidation in liver of zebrafish exposed to atrazine at 90 μg L⁻¹.

Exposure to atrazine demonstrated a significant effect on biotransformation and oxidative defense enzymes of early life stages of common carp, and, based on these results, LOEC = 0.3 μg L⁻¹ was determined. The determined LOEC value is equal to atrazine environmental concentration in the Czech Republic (0.3 μg L⁻¹).

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