ORIGINAL ARTICLE

Oxidative stress parameters in early developmental stages of common carp (*Cyprinus carpio* L.) after subchronic exposure to terbuthylazine and metribuzin

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Abstract

OBJECTIVES: This work assessed the subchronic effects of the triazine compounds terbuthylazine and metribuzin on embryo-larval stages of common carp (*Cyprinus carpio* L.) using biotransformation enzyme and selected oxidative stress parameters.

DESIGN: Early developmental stages of fish, from embryo to larvae, were exposed to terbuthylazine at concentrations of 0.9, 160, 520, and 820 μg L^{-1}, and metribuzin at concentrations of 0.9, 4, 14, and 32 mg L^{-1} for 30 days and compared to the non-treated control group. For assessment of free radical defence the activities of glutathione S-transferase (GST) and glutathione reductase (GR) were measured; the lipid peroxidation (TBARS) level was determined as a marker of free radical damage.

RESULTS: Terbuthylazine had no significant effect ($p>0.05$) at any tested concentrations on GST activity. Terbuthylazine caused an increase ($p<0.05$) in GR activity at the concentration of 520 μg L^{-1}. We found a non-significant ($p>0.05$) effect of terbuthylazine exposure on TBARS level.

In metribuzin treated groups, the activity of GST was increased ($p<0.05$) in all experimental groups, with the highest at the concentration of 32 mg L^{-1}. GR activity was increased in the 0.9, 4, and 14 mg L^{-1} groups and the TBARS levels were the highest in the 0.9 mg L^{-1} group.

CONCLUSION: Our results suggest that terbuthylazine and metribuzin induced oxidative stress in embryo-larval stages of common carp. Increased activities of biotransformation enzymes or antioxidant defence enzymes were markers of free radical attack. There was low lipid peroxidation in early developed fish after triazine exposure.

Abbreviations:
GR - glutathione reductase
GSH - glutathione (reduced glutathione)
GSSG - disulfide form of glutathione (oxidized glutathione)
GST - glutathione S-transferase
MDA - malondialdehyde
NADPH - nicotinamide adenine dinucleotide phosphate (reduced form)
TBARS - thiobarbituric acid reactive substances

INTRODUCTION

In the last few decades environmental pollution has intensified due to increasing use of chemical substances in agriculture, industry and households (Valavanidis *et al.* 2006). Pesticides are significant pollutants and represent a serious threat to the ecosystem, especially aquatic environments. Fish are suitable organisms for the study of behavioral, biological or biochemical effects of pesticide exposure in the ecosystem (Powers 1989; van der Oost *et al.* 2003; Slaninova *et al.* 2009).

Terbuthylazine (N-2-tert-butyl-6-chloro-N-4-ethyl-1,3,5-triazin-2,4-diamine) is a symmetrical chloro-*s*-triazine selective systemic herbicide which belongs to the 1,3,5-triazines group. Metribuzin (4-amino-6-tert-butyl-4,5-dihydro-3-methythio-1,2,4-triazin-5-one) is an asymmetrical triazine selective systemic herbicide and belongs to the group of 1,2,4-triazinones.

The primary mode of triazine herbicide action on plants is the inhibition of the Hill reaction phase of photosynthesis by the blocking of electron transport. The absorption of triazine herbicides by the plant is mediated primarily by the roots but also by the leaves, and triazine herbicides are translocated in the xylem. After that, they are distributed through the plant. This enables triazine herbicide to be used in both pre- and post-emergence treatment (Roberts *et al.* 1998; Waxman 1998). The main process of metabolism in animals and plants is side-chain de-alkylation and oxidation to 2-hydroxy derivates (Roberts *et al.* 1998).

Terbuthylazine is an active compound of systematic herbicides which are used in sorghum, vines, maize, coffee, potatoes, citrus fruits, legumes, and forestry. Terbuthylazine is used as a substitute for the now banned atrazine and in combination with many other herbicides. Metribuzin is used as a broad spectrum herbicide for both pre- and post-emergence, mostly in crops including soybean, potatoes, tomatoes, asparagus, maize, cereals and sugar cane (Roberts *et al.* 1998; Tomlin 2003).

Triazine herbicides are a widely-used group of pesticides and therefore may contaminate aquatic ecosystems. According to the Czech State Phytosanitary Administration, about 126 000 kg of terbuthylazine and 5 700 kg of metribuzin were applied in the Czech Republic in 2011. The extensive use of triazine pesticides in agriculture results in environmental contamination.

Terbuthylazine was measured in rainwater in Denmark in 2000, mainly during the period of agricultural use, at concentrations above 0.1 $\mu g L^{-1}$ (Bossi *et al.* 2002). Levels of terbuthylazine were monitored in the River Po (Italy) for 3 years (1988–1991) and concentrations ranged between 0.0 and 0.3 $\mu g L^{-1}$ (Brambilla 1993). Hildebrandt *et al.* (2008) reported concentrations from 0.0 to 1.27 $\mu g\ L^{-1}$ in surface and groundwater in northern Spain. Terbuthylazine concentrations ranged from 0.01 to 0.02 $\mu g\ L^{-1}$ in the Kalamas River (Greece) with greater pesticide concentrations during the seasons of application (Lambropoulou *et al.* 2002). Kodes *et al.* (2010) reported triazine herbicides (inc. terbuthylazine and metribuzin) in Czech rivers. The highest environmental concentration of terbuthylazine in surface waters was 2.6 $\mu g\ L^{-1}$ in 2009. Environmental concentrations of metribuzin in water have been monitored by many authors and usually have low values <0.1 $\mu g\ L^{-1}$ (Laabs *et al.* 2002; Dores *et al.* 2006; Maloschik *et al.* 2007).

Toxicity studies of triazine pesticides on fish show that they may cause morphological, physiological and biochemical alterations (Saglio & Trijasse 1998; Arufe *et al.* 2004; Dezfuli *et al.* 2006; Nieves-Puigdoller *et al.* 2007; Mikulikova *et al.* 2011) but less is known about the specific effects of terbuthylazine or metribuzin on oxidative stress parameters in early developmental stages of fish. Pesticides, including triazines, induce and raise the level of oxidative stress in fish (Di Giulio & Hinton 2008; Haluzova *et al.* 2010). Oxidative stress is an effect of the imbalance between the production and elimination of free radicals (Davies 1995). The enzymes of antioxidant defence and the products of free radical damage have been investigated as biomarkers of oxidative stress (van der Oost *et al.* 2003).

The aim of the present study was to evaluate the effects of subchronic exposure of embryo-larval developmental stages of common carp (*Cyprinus carpio* L.) to terbuthylazine and metribuzin herbicides by using selected oxidative stress biomarkers.

MATERIAL AND METHODS

Experimental design

Aqueous testing solutions were prepared from commercial pesticides Click 500 SC (Oxon S.p.A.; Italy) and Sencor 70 WG (AgroBio Opava, s.r.o.; Czech Republic). The active compounds were terbuthylazine (Click 500 SC) at a concentration of 500 $g\ L^{-1}$ and metribuzin (Sencor 70 WG) at a concentration of 700 $g\ kg^{-1}$. Embryo–larval toxicity tests were conducted using a modified protocol according to the OECD 210 guidelines (Fish, early-life stage toxicity test) (OECD 1992). 100 fertilized eggs, 24 h post-fertilization, were randomly distributed into crystallization dishes, which contained terbuthylazine solutions at concentrations of 0.9, 160, 520, and 820 $\mu g\ L^{-1}$ and metribuzin solutions at concentrations of 0.9, 4, 14, and 32 $mg\ L^{-1}$; the last dish contained tap water free of terbuthylazine and metribuzin as a control dish. The experiment was

performed in triplicate. Tested concentrations of triazine herbicides were selected according to the literary sources and environmental concentration (the lowest tested concentration corresponded to the environmental concentration). For the test a semistatic method was used, with twice daily bath replacement. The temperature, pH, and oxygen saturation were recorded daily. During the test, the temperature ranged from 19 to 22 °C.; pH was 7.5–8.5, and the dissolved oxygen did not drop below 60%.

The beginning of the test was designated as Day 1 (24 h post-fertilization). Hatching began on Day 4 and was completed by Day 6; feeding started on Day 7 with freshly hatched *Artemia salina*, *ad libitum* twice a day before the bath was changed. After 30 days, when all larvae in the control groups reached the juvenile developmental stage, the test was ended. The experiment was 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).

Determination of terbuthylazine and metribuzin

Gas chromatography with ion trap mass spectrometry was used for the determination of terbuthylazine and metribuzin concentrations.

Fish sampling and homogenization

At the end of the experiment, the fish were killed (by CO_2), immediately frozen, and stored at –85 °C until analyses. Whole body samples were weighed and homogenised (1:10 w/v) using phosphate buffer (pH 7.2). The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and a second centrifuged (10,500 × g, 4 °C, 20 min) to obtain a supernatant fraction for the determination of glutathione S-transferase (GST) and glutathione reductase (GR) activities.

Measurement of biotransformation enzyme and oxidative stress parameters

The total catalytic concentration of glutathione *S*-transferase was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione (GSH) at 340 nm (Habig *et al.* 1974). The specific activity was expressed as the nmol of the formed product per min per mg of protein.

The catalytic concentration of GR was determined spectrophotometrically by measuring oxidation of the nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm, and the specific activity was expressed as the nmol of NADPH consumption per min per mg of protein (Carlberg & Mannervik 1975).

The protein concentration, for specific activities of GST or GR expression, was determined by Bicinchoninic Acid Protein Essay Kit (Sigma-Aldrich, St. Louis, MO, USA) and bovine serum albumin was used as a standard (Smith *et al.* 1985).

To check lipid peroxidation in the fish, malondialdehyde (MDA) was measured by the TBARS assay described by Lushchak *et al.* (2005) at 535 nm. The concentration was expressed as nmol per gram wet weight of tissue. All spectrophotometric measurements were performed using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc.).

Statistical analysis of data

STATISTICA 8.0 for Windows software (StatSoft, Czech Republic) was used to check the normality and homogeneity of the variances test. Data were subjected to parametric one-way ANOVA and the differences among test groups were assessed with the Tukey's HSD test with $p<0.05$ as the level of significance.

RESULTS

Mortality and concentration of terbuthylazine and metribuzin

Mortality was under 10% in all experimental and control groups. During the test, concentrations of terbuthylazine and metribuzin did not fall below 80% of the nominal concentration.

Effect of terbuthylazine and metribuzin on biotransformation enzyme

Non-significant differences ($p>0.05$) in GST activity were found in fish exposed to terbuthylazine at concen-

Tab. 1. Effect of subchronic exposure to terbuthylazine on glutathione S-transferase (GST) and glutathione reductase (GR) activities, and lipid peroxidation level (TBARS) in embryo-larval developed stages of common carp.

Parameters	Test group: Terbuthylazine (μg L^{-1})				
	control	0.9	160	520	820
GST (nmol min^{-1} mg $protein^{-1}$)	73.64±12.03	80.56±4.62	75.45±11.56	78.58±13.94	83.91±7.01
GR (nmol NADPH min^{-1} mg $protein^{-1}$)	17.88±2.43	17.91±1.95	19.98±2.95	**22.78±3.81***	21.98±3.48
TBARS (nmol g ww $tissue^{-1}$)	10.52±5.78	6.85±2.06	7.75±2.09	5.49±1.22	12.81±11.04

*Significant differences compared with control value ($p<0.05$). Data are means ± S.D., n=8.

trations of 0.9, 160, 520, and 820 μg L^{-1} compared to the control (Table 1).

Significant increases (p<0.05) in GST activity were found in fish exposed to metribuzin at all tested concentrations (0.9, 4, 14, and 32 mg L^{-1}) compared to the control (Table 2).

Effects of terbuthylazine and metribuzin on antioxidant enzyme

Compared to the control group, GR activity was significantly higher (p<0.05) only at a terbuthylazine concentration of 520 μg L^{-1}. Other concentrations had no significant effect (p>0.05) compared to the control (Table 1).

In metribuzin treated groups, the activities of GR were higher in the 0.9, 4, 14 mg L^{-1} groups compared to the control (p<0.05). There was non-significant difference in the 32 mg L^{-1} group compared to the control (Table 2).

Effect of terbuthylazine and metribuzin on lipid peroxidation

A decrease in TBARS level was found in fish exposed to terbuthylazine concentrations of 0.9, 160, and 520 μg L^{-1}. In contrast, the level was increased at a terbuthylazine concentration of 820 μg L^{-1}. But, there were non-significant (p>0.05) differences observed in TBARS levels after terbuthylazine exposure compared to the control group (Table 1).

Compared to the control group, TBARS levels were significantly (p<0.05) higher in fish exposed to metribuzin at 0.9 mg L^{-1} and in other tested groups were not significant difference compared to the control (Table 2).

DISCUSSION

Pesticide exposure may result in developmental alterations, including reproductive effects, embryotoxicity, and teratogenicity. The mechanism of many pesticide toxicity effects is directly linked with oxidative stress (Paskova *et al.* 2011). However, some studies report smaller effects on detoxifying systems (phase I biotransformation) in early juvenile fish exposed to pesticides (Modra *et al.* 2008; Plhalova *et al.* 2011) An important enzyme protecting the embryo and larvae against free radicals is glutathione *S*-transferase (phase II biotransformation) (Anguiano *et al.* 2001). The conjugation of pesticides with a reduced form of glutathione is catalyzed by glutathione-S-transferase. Another known function of GST is defence against oxidative damage and the oxidative products of DNA and lipids (Davies 1995; Di Giulio & Hinton 2008).

Our work showed that terbuthylazine at subchronic concentrations (0.9, 160, 520, and 820 μg L^{-1}) non-significantly increased GST activity in embryo-larval fish stages. Metribuzin exposure significantly raised GST activity in embryo-larval fish stages at all tested concentrations – up to 90% at 32 mg L^{-1} – compared to the control group. Our results concerning GST activity confirm the potential of metribuzin herbicides to affect detoxification enzymes in early life stages of fish, which can also have an influence on antioxidant defence systems. Such influence is clear with respect to metribuzin exposure. Such activity points to a clear level of xenobiotic biotransformation in early life stage fish development. Wiegand *et al.* (2001) reported an effect on the detoxification system of zebrafish (*Danio rerio*) by atrazine, in which a higher atrazine concentration (up to 5 mg L^{-1}) caused a decrease in GST activity. A study concerning chronic exposure of juvenile common carp to simazine reported no influence of simazine (at 0.06, 1, 2, and 4 μg L^{-1}) on GST activity during a 90-day period of exposure (Velisek *et al.* 2012). A study of GST activity changes in bluegill sunfish after exposure to atrazine reported that GST activity was significantly increased at concentrations of 6 and 9 mg L^{-1} compared to the control group (Elia *et al.* 2002). On the contrary, the study on juvenile common carp exposed to terbuthylazine reported no significant effect on GST activity under subchronic conditions (Mikulikova *et al.* 2011).

The transformation of the oxidized disulfide form of glutathione (GSSG) to the reduced form, catalysed by GR, is the way in which the GSH/GSSG ratio is maintained in organisms (Davies 1995; Di Giulio & Hinton 2008). Van der Oost *et al.* (2003) indicated GR as a potential oxidative stress parameter. We found a

Tab. 2. Effect of subchronic exposure to metribuzin on glutathione S-transferase (GST) and glutathione reductase (GR) activities, and lipid peroxidation level (TBARS) in embryo-larval developed stages of common carp.

Parameters	Test group: Metribuzin (mg L^{-1})				
	control	0.9	4	14	32
GST (nmol min^{-1} mg $protein^{-1}$)	73.64±12.03	**107.90±6.38***	**96.98±9.99***	**126.64±11.00***	**139.87±21.94***
GR (nmol NADPH min^{-1} mg $protein^{-1}$)	17.88±2.43	**24.99±2.99***	**23.31±3.07***	**25.94±2.25***	18.96±2.18
TBARS (nmol g ww $tissue^{-1}$)	10.52±5.78	**27.11±9.12***	9.72±4.17	9.20±4.97	16.62±9.37

*Significant differences compared with control value (p<0.05). Data are means ± S.D., n=8.

significantly higher increase in GR activity in embryo-larval fish stages at a terbuthylazine concentration of 520 μg L^{-1} and in metribuzin exposed groups at concentrations of 0.9, 4, 14 mg L^{-1} compared to control. However, the highest concentration of metribuzin (32 mg L^{-1}) had no effect on GR activity. This regression might be a sign of the breakdown of detoxifying systems in early development stages of fish organisms. Stara *et al.* (2012) observed the effect of simazine on oxidative stress and antioxidant responses in juvenile common carp after chronic exposure. There were no significant differences in values of GR activity in tissue of juvenile common carp at all tested concentrations (0.06, 2 and 4 mg L^{-1}) compared to control. Velisek *et al.* (2011) reported significantly lower GR activity in liver of juvenile common carp in all experimental groups after long-term terbutryn exposure (0.02, 0.2 and 2.0 μg L^{-1}) compared to the control group. Nwani *et al.* (2010) confirmed increased antioxidant activity, including GR, with increasing exposure to atrazine in freshwater fish (*Channa punctatus*).

Oxidative stress, which might be induced by pesticides, is a cause of polyunsaturated fatty acid oxidation. This process is well known as lipid peroxidation and has been investigated extensively. The degradation product, malondialdehyde, appears to be a potential biomarker of the lipid peroxidation level (van der Oost *et al.* 2003; Di Giulio & Hinton 2008; Slaninova *et al.* 2009). The most widely used method for determining MDA is the thiobarbituric acid test, and the TBARS level is commonly used for expression of the lipid peroxidation value (Lushchak *et al.* 2005). We obtained a non-significant increase in TBARS levels in embryo-larval fish stages exposed to the highest tested concentration of terbuthylazine (820 μg L^{-1}), but in other exposed groups the level of TBARS decreased non-significantly compared to control. Our results concerning TBARS levels after metribuzin exposure showed a significant increase in the 0.9 and a non significant increase in the 32 mg L^{-1} group; in the other groups (4 and 14 mg L^{-1}) there was no significant effect. We suppose that metribuzin herbicides initiate lipid peroxidation in embryo-larval developmental stages of fish. This affect was evident in some of the tested groups, but early-developed fish antioxidant defense was not altered enough to achieve a significant effect. Studies concerning the effects of triazine showed a rise in TBARS levels with a positive correlation to tested pesticide concentrations (Elia *et al.* 2002; Nwani *et al.* 2010, Xing *et al.* 2012). Oropesa *et al.* (2009) reported the effect of simazine exposure on juvenile common carp. MDA levels were significantly increased in fish from a simazine polluted reservoir, but after exposure during a laboratory toxicity test, MDA levels in fish remained at the control level. After exposure of juvenile common carp to simazine, Stara *et al.* (2012) found no significant differences in TBARS levels in fish tissues compared to the control group.

CONCLUSIONS

This work demonstrated the profound influence of terbuthylazine and metribuzin exposure on the biotransformation enzyme and oxidative stress markers of exposed embryo-larval stages of common carp. The triazine herbicides induced oxidative stress in early developmental stages of fish during a 30-day test and after this period we were able to use GST, GR and TBARS level as biomarkers of pesticide toxicity. The results of this work provide additional data on the subchronic exposure of embryo-larval fish stages to the triazine herbicides terbuthylazine and metribuzin. Triazine herbicides are an important group of pesticides with respect to environmental risk assessment.

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Potential Conflicts of Interest: *None disclosed.*

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