The effects of subchronic exposure to terbuthylazine on zebrafish

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Abstract

OBJECTIVES: Terbuthylazine belongs to the group of symmetrical triazine herbicides used extensively in agriculture and non-agricultural sites, primarily to control broadleaf and some grassy weeds that have become ubiquitous contaminants of the environment. The aim of this study was to assess the impact of terbuthylazine in surface waters on fish under experimental conditions. The effects of subchronic exposure to terbuthylazine on fish growth and the development of histopathological changes in selected organs (gill, kidney, liver), and on the activity of some biochemical parameters – glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) and on the concentration of thiobarbituric acid reactive substances (TBARS) in Danio rerio were investigated during a 28 day toxicity test.

METHODS: Juvenile growth tests were performed on *Danio rerio* according to OECD guideline No. 215. Fish at the age of 30 days were exposed for 28 days to a range of sublethal concentrations of terbuthylazine (0.55 – environmental concentration, 150, 400, 700 and 1000 $\mu g\,L^{-1}$).

RESULTS: There were no significant differences ($p<0.05$) between the specific growth rates (r) of the test groups and control group. Histopathological examination revealed pathological changes only in the liver in fish exposed to terbuthylazine at concentrations of 700 and 1000 $\mu g\,L^{-1}$. Significant differences ($p<0.05$) in activities of biochemical markers were found in GST (400, 700 and 1000 $\mu g\,L^{-1}$), GR (700 and 1000 $\mu g\,L^{-1}$), significant differences ($p<0.05$) of TBARS concentration were found at 1000 $\mu g\,L^{-1}$.

CONCLUSIONS: Based on the results of growth rate, the histopathological examination of selected organs, and the results of biochemical parameters, it was found that the environmental concentration of terbuthylazine did not have any effects on juvenile *D. rerio*. The values of NOEC and LOEC for terbuthylazine for juvenile D. rerio were 150 $\mu g\,L^{-1}$ and 400 $\mu g\,L^{-1}$.

ORIGINAL ARTICLE

Abbreviations:

GST	- glutathione S-transferase
GR	- glutathione reductase
GPx	- glutathione peroxidase
GC/IT-MS	- gas chromatography with ion trap mass spectrometry
HE	- haematoxylin and eosin
NOEC	- no observed effect concentration
LOEC	- lowest observed effect concentration
OECD	- Organization for Economic Cooperation and Development
TBARS	- thiobarbituric acid reactive substances

INTRODUCTION

Terbuthylazine and other triazines and their metabolites are some of the most widespread contaminants of the aquatic environment (Carabias-Martínez *et al.* 2003; Castillo *et al.* 2003; Rodriguez-Mozaz *et al.* 2004; Noppe *et al.* 2007; Otto *et al.* 2007; Quednow & Pütmann 2007). Terbuthylazine (6-chloro-N-(1,1-dimethylethyl)-N-ethyl-1,3,5-triazine-2,4-diamine) has substituted the use of atrazine in maize crops, atrazine being banned in many countries. This herbicide is also used in sorghum, vines, citrus fruits, coffee, potatoes, and legumes, as well as in forestry for pre- or post-emergence. It is a selective systemic herbicide and acts as a photosynthesis inhibitor. It is absorbed by leaves and roots, translocated acropetally in the xylem and accumulates in the apical meristem. The water solubility of terbuthylazine is 8.5 mg L^{-1} at 20 °C and is stable in neutral, weakly acidic and weakly alkaline media (Roberts *et al.* 1998; Langenbach *et al.* 2001; Otto *et al.* 2007).

Terbuthylazine is present in the aquatic environment. According to data of the Czech Hydrometeorogical Institute terbuthylazine was present in about 50% of water samples taken between the years 2005 and 2009. The highest environmental concentrations of terbuthylazine in surface water reached 0.1 μg L^{-1} in 2005 and 2.8 μg L^{-1} in 2006. During 2009 nearly 11 tons of terbuthylazine were applied in the Czech Republic (Kodes *et al.* 2010).

The presence of terbuthylazine and its metabolite desethylterbuthylazine in surface water, ground water and drinking water has also been frequently detected in other European countries (de Barreda *et al.* 1991; Brambilla *et al.* 1993; Tsipi & Hiskia 1996; Castillo *et al.* 2003; Otto *et al.* 2007; Noppe *et al.* 2007; Hildebrandt *et al.* 2008; Fava *et al.* 2010).

Castillo *et al.* (2003) found terbuthylazine in 90% of well water samples in a citrus area of Spain (the highest concentration was 0.285 μg L^{-1}). In another citrus area in Spain an even higher terbuthylazine concentration (1.8 μg L^{-1}) was detected in well water (de Barreda *et al.* 1991). Hildebrandt *et al.* 2008 also reported higher concentrations of terbuthylazine in water in Spain. The highest concentration in surface water was 0.24 μg L^{-1} and in ground water, 1.24 μg L^{-1}.

In Italy, Brambilla *et al.* (1993) monitored some triazines herbicides in the River Po over a three-year period (1989–1991) and reported that the highest concentration of terbuthylazine was 0.3 μg L^{-1}. Likewise, Otto *et al.* (2007) found terbuthylazine in the aquifer in north-eastern Italy, but the highest concentration of terbuthylazine (0.04 μg L^{-1}) was below the EU drinking water limit (0.1 μg L^{-1}).

The aim of this study was to investigate the long-term effects of subchronic exposure to environmental and other sublethal concentrations of terbuthylazine on growth and the formation of histopathological changes in selected organs (gills, kidney, liver) in juvenile *Danio rerio*, as well as on the activity or concentration of some biochemical markers (GST, GR, GPx, TBARS), over a 28-day exposure period, and to make an estimation of the LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) values of terbuthylazine.

MATERIAL AND METHODS

Experimental fish

Tests of terbuthylazine toxicity were performed on *D. rerio*, which is one of the model organisms most commonly used in toxicity tests to determine the effects of chemicals and pharmaceuticals on fish in the aquatic environment (Oliveira *et al.* 2009; Segner 2011; Modra *et al.* 2011; Praskova *et al.* 2011; Sisman 2011). Experimental procedures were 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).

The subchronic toxicity test

The tests were performed on *D. rerio* at the age of 30 days, according to OECD guideline No. 215 Fish, Juvenile Growth Test. Aqueous testing solutions were prepared from Click 500 SC produced by SIPCAM S.p.A. The active compound was terbuthylazine at a concentration of 500 g L^{-1}.

The fish were randomly distributed into 30 liter glass aquaria, 40 specimens per each. The experiment was conducted in a flow-through system and the volume of each test solution was replaced twice a day. The fish were exposed to a range of terbuthylazine concentrations (0.55 – environmental concentration, 150, 400, 700 and 1000 μg L^{-1}) for 28 days. Each test on a terbuthylazine-treated group was performed in duplicate. The average initial weight of fish used in the experiment was 0.025 ± 0.011 g and the average initial total length of fish was 15.78 ± 1.85 mm. The fish were fed with dried *Artemia salina* without nutshells to the amount of 8% of their body weight per day. The food ration was based on initial fish weights and was recalculated after 14 days. At the end of the tests, the fish were euthanized by carbon dioxide, weighed, and their tank-average specific growth rates determined. Food was withheld from the fish 24 h prior to weighing.

Tank-average specific growth rates were calculated using the following formula according to OECD No. 215:

$$r = \frac{\overline{\log_e W_2} - \overline{\log_e W_1}}{t_2 - t_1} * 100$$

r – tank-average specific growth rate,

W_1, W_2 – weights of a particular fish at times t_1 and t_2 respectively,

$\overline{\log_e W_1}$ – average of the logarithms of the values W_1 for the fish in the tank at the start of the study period,

$\overline{\log_e W_2}$ – average of the logarithms of the values W_2 for the fish in the tank at the end of the study period,

t_1, t_2 – time (days) at the start and end of the study period.

During the tests, living conditions were checked at 24-hour intervals and the number of dead fish was recorded in each concentration. The mean values for water quality were: temperature 25±1 °C, oxygen saturation above 60% (ranging from 79% to 96%), and pH from 7.99 to 8.36. The basic chemical parameters of dilution water used were: COD_{Mn} (chemical oxygen demand) 1.1–1.3 mg L^{-1}, total ammonia below the limit of determination (<0.04 mg L^{-1}), NO_3^- 14.8–17.5 mg L^{-1}, NO_2^- below the limit of determination (<0.02 mg L^{-1}), Cl^- 18.6–19.7 mg L^{-1}, Σ Ca±Mg 12.8 mmol L^{-1}.

Determination of terbuthylazine concentrations

Gas chromatography with ion trap mass spectrometry (GC/IT-MS) was used for the measurement of terbuthylazine. Sample preparation was based on simple liquid-liquid extraction into hexane.

The separation, identification, and quantification of terbuthylazine were based on the GC/IT-MS method. A Varian 450-GC gas chromatograph (Varian Inc., USA) and VF-5 ms (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 methods described by Perreau and Einhorn (2006). All solvents were GC/MS-grade purity (Chromservis, s.r.o., CZ). Certified standard terbuthylazine was purchased from Dr. Ehrenstorfer GmbH (Germany).

The detection limit (3σ) of terbuthylazine was 0.01 µg L^{-1}. Expanded uncertainty was 6.0% conditional on a coefficient of expansion of k=2.

Histopathological examination

The fish (10 specimens from each concentration) were prepared for histopathological examination (on selected organs – gill, kidney, liver), fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 µm, and stained with haematoxylin and eosin (HE). Five sections from each fish were examined at different levels.

Fish sampling and homogenization

At the end of the test, the fish were euthanized by carbon dioxide, 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), glutathione peroxidase (GPx), and glutathione reductase (GR).

Measurement of biochemical parameters

The total catalytic concentration of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione 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 NADPH oxidation at 340 nm (Carlberg *et al.* 1975). The catalytic concentration of GPx was calculated from the amount of NADPH oxidation by the reaction with GR at 340 nm (Flohe & Gunzler 1984). The specific activity of GR and GPx was expressed as the nmol of NADPH consumption per min per mg of protein.

Protein concentrations were determined by a Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as the standard (Smith *et al.* 1985).

To check lipid peroxidation, malondialdehyde was measured by the TBARS method as described by Lushchak *et al.* (2005) at 535 nm. The concentration was expressed as nmol per gram wet weight of tissue.

Statistical analysis and estimation of NOEC and LOEC

Data on tank-average fish specific growth rates and oxidative stress markers were tested for normal distribution using the Shapiro–Wilk test. After testing for homogeneity of variance across groups (Levene test), an analysis of variance (one-way ANOVA) was used. The differences between test groups with different concentrations and the control group were assessed using the Dunnett`s test with $p<0.05$ chosen as the level of significance. In the case of malondialdehyde, data did not have a normal distribution and so the nonparametric Median test was used.

The estimation of the LOEC and NOEC was based on the identification of the lowest concentration at which these differences in specific growth rate and biochemical markers were (were not) significant at a 0.05 probability level, and, further, on the results of

histopathological examination and the assessment of changes in fish behaviour.

RESULTS

Mortality and growth rate

In all terbuthylazine-exposed groups and in the control group, mortality did not exceed 5% during the 28-day experimental period (mortality was 0% in the control group). Fish in all treated groups and control showed normal feeding behaviour during the experiment.

The initial body weights were not significantly different between groups, and at the end of the trial, body weights in all terbuthylazine-exposed tanks were compared to the control group. No significant differences ($p>0.05$) in the specific growth rate of fish from particular terbuthylazine concentrations in comparison with the control group were found.

Histopathological examination

Histopathological examination revealed similar pathological lesions in pesticide-exposed fish at the concentrations of 700 μg L^{-1} and 1000 μg L^{-1} of terbuthylazine. Morphological changes were observed only in the liver tissue of examined fish and were represented by moderate dystrophic lesions of the hepatocytes. There were morphological signs of initial cell injury represented by hydropic to vacuolar degeneration of hepatocytes and the dilatation of capillaries (Figure 1). Affected liver tissues were histopathologically compared with tissue sections from the control group. Tissues and organs of fish exposed to terbuthylazine at the concentrations of 0.55 μg L^{-1}, 150 μg L^{-1} and 400 μg L^{-1} exhibited no pathomorphological changes.

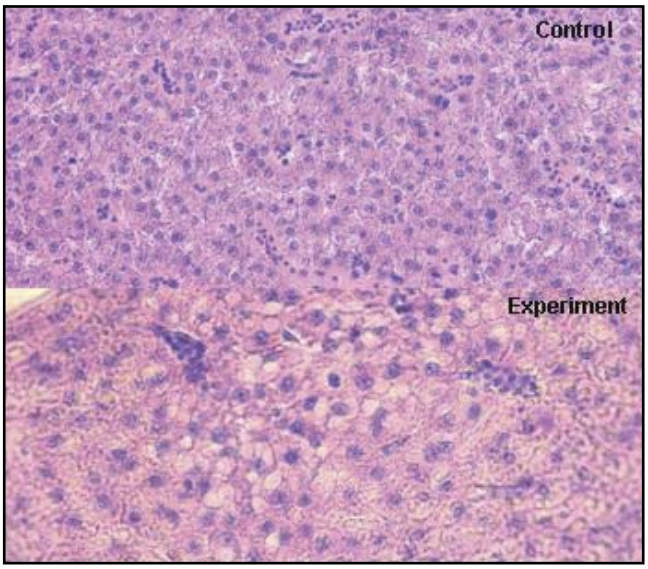

Fig. 1. Dystrophic lesions and hydropic to vacuolar degeneration of hepatocytes in the liver of *D. rerio* exposed to terbuthylazine concentrations of 700 and 1000 μg L^{-1} for 28 days (HE, 400x).

Biochemical parameters

Compared to the control group, GST activity was elevated in all experimental groups; significantly higher ($p<0.05$) GST activity was found in the groups exposed to 400, 700 and 1000 μg L^{-1} of terbuthylazine (Figure 2).

Non-significant lower GR activity was seen in fish exposed to terbuthylazine at 0.55, 150 and 400 μg L^{-1} compared to the control. Significantly lower ($p<0.05$) GR activity was found in the groups exposed to 700 and 1000 μg L^{-1} of terbuthylazine compared to the control group (Figure 3).

Higher GPx activity was found at 0.55 μg L^{-1} of terbuthylazine compared to the control group. At other terbuthylazine concentrations the GPx activity was lower than the control group (Figure 4), but none of these differences were significant ($p>0.05$).

Significantly lower ($p<0.05$) concentrations of TBARS were observed at the highest concentration of terbuthylazine (1000 μg L^{-1}) (Figure 5). Lower concentrations of TBARS were also observed at 150 and 700 μg L^{-1} of terbuthylazine compared to the control group, but these differences were not significant.

Validity of the tests

Our tests met all conditions required by the OECD – mortality in the control groups was below 10%, the final weight of control fish was higher than 150% of the initial weight, the dissolved oxygen concentrations were at least 60%, the water temperature did not differ by more than ±1 °C among test aquariums, and test substance concentrations were above 80% of the measured initial concentration.

DISCUSSION

Due to the presence of triazines and their metabolites in the aquatic environment it is important to study the influence of these substances on fish to determine their long-term effects. In our study we focused on the effects of subchronic exposure to the environmental concentration and other sublethal concentrations of terbuthylazine on juvenile *D. rerio*.

The concentrations of terbuthylazine used in our test (0.55 to 1000 μg L^{-1}) did not have any effect on fish growth, mortality, or fish behaviour. However, Stepanova *et al.* (2012), who tested the toxic effects of terbuthylazine (0.9 to 820 μg L^{-1}) on embryonic and larval stages of common carp (*Cyprinus carpio*), observed inhibition of growth at 520 and 820 μg L^{-1}. In addition, these concentrations were also associated with a delay in development compared to the control and the other experimental groups.

Our results showed histopathological changes in liver only at the two highest concentrations of terbuthylazine (700 and 1000 μg L^{-1}); other organs, gills and kidney were without histopathological changes in all treated groups. Likewise, Stepanova *et al.* (2012) found histopathological changes in liver in fish exposed only

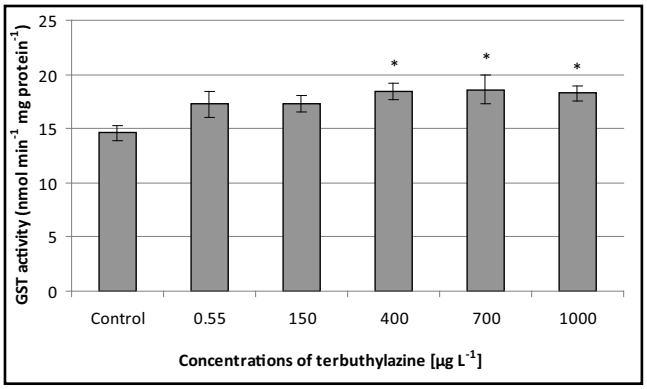

Fig. 2. Comparison of glutathione *S*-transferase (GST) activity (mean ± SD) in *D. rerio* for control and tested terbuthylazine concentrations (*p<0.05).

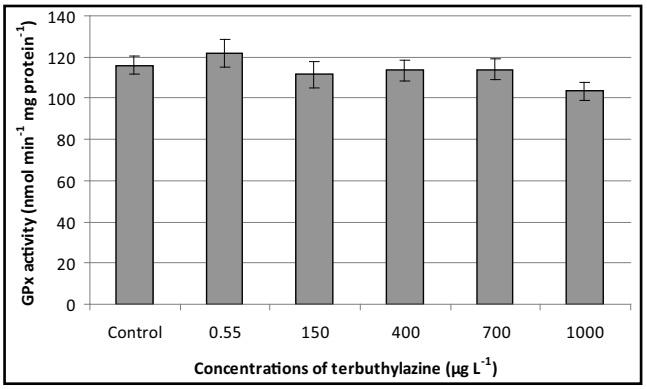

Fig. 4. Comparison of glutathione peroxidase (GPx) activity (mean ± SD) in *D. rerio* for control and tested terbuthylazine concentrations (*p<0.05).

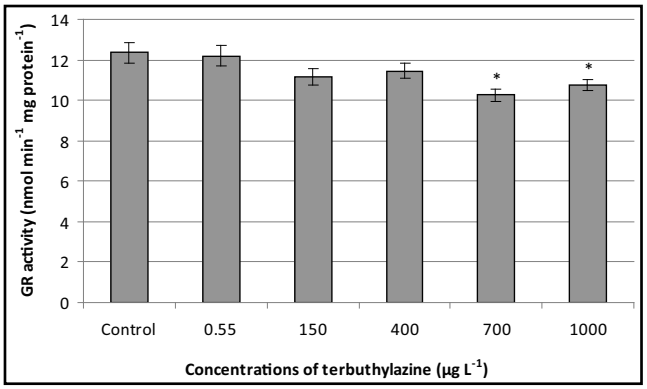

Fig. 3. Comparison of glutathione reductase (GR) activity (mean ± SD) in *D. rerio* for control and tested terbuthylazine concentrations (*p<0.05).

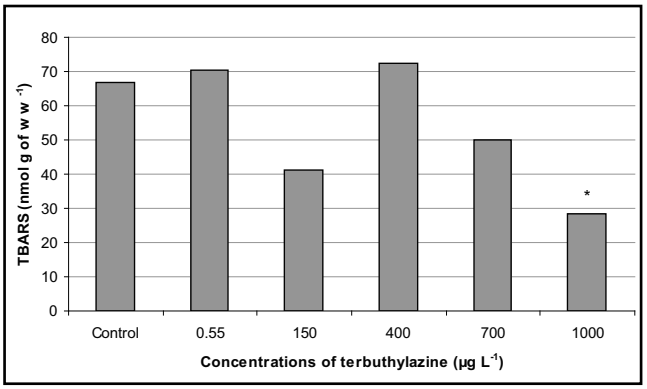

Fig. 5. Comparison of levels of thiobarbituric acid reactive substance (TBARS) activity (median) in *D. rerio* for control and tested terbuthylazine concentrations (*p<0.05).

to the highest concentration (820 µg L^{-1}) after a 30-day test. As in our study, histopathological examination did not reveal any changes in other tissue or organs of fish. Nevertheless, Mikulikova *et al.* (2011) found changes in the gills of *C. carpio* at a lower concentration of terbuthylazine (550 µg L^{-1}) after 91 days of exposure. They described vacuolisation and desquamation of respiratory epithelium and venostasis.

Similar changes have also been discovered in fish after exposure to other triazines – simazine, terbutryn and atrazine. Oropesa-Jimenez *et al.* (2005) and Oropesa *et al.* (2009a) described pathological changes in the gills, liver and kidney of common carp exposed to sublethal simazine concentrations. In *D. rerio*, exposure to simazine (60 µg L^{-1}) caused changes not only in gills (hypertrophy, hyperplasia and desquamation of epithelial cells and multiple foci of coagulation necrosis) and liver (dystrophic lesions and swelling and hydropic vacuolar degeneration of hepatocytes), but also in kidney (coagulation of apical part of cytoplasm of epithelial cells of the renal tubules) (Plhalova *et al.* 2011). Velisek *et al.* (2009) reported steatosis, hyperaemia, and necrosis in liver of common carp caused by simazine concentrations of 4.0 µg L^{-1} and 20 µg L^{-1}, and massive coagulation necrosis and the degradation of physiological cell structure were identified at 50 µg L^{-1}. Likewise, Velisek *et al.* (2010) detected histopathological changes in liver and caudal kidney of *C. carpio* at sublethal terbutryn concentrations. Fischer-Scherl *et al.* (1991), Braunbeck *et al.* (1992), Oulmi *et al.* (1995), and Jin *et al.* (2012) described histological alterations in liver or kidney of fish exposed to sublethal concentrations of atrazine.

We also determined biochemical parameters in fish whole body homogenate, because some triazines can induce oxidative stress in fish (Slaninova *et al.* 2009). Significantly higher (p<0.05) GST activity, which is used as an indicator of the detoxification metabolism (Wiegand *et al.* 2001), was found at 400, 700 and 1 000 µg L^{-1} of terbuthylazine. An increase in GST activity was also observed after atrazine exposure (Elia *et al.* 2002).

Significantly lower (p<0.05) GR activity in fish was recorded at 700 and 1000 µg L^{-1} of terbuthylazine, which was also reported by Velisek *et al.* (2011). However, Stara *et al.* (2012) did not discover any differences in common carp tissue. The activity of GPx in all test

groups was not significantly different compared to the control. Similarly Velisek *et al.* (2011) did not observe differences in GPx activity in fish tissue after terbutryn exposure. A decrease in this was found only in gills at 2.0 μg L^{-1}. Stara *et al.* (2012) reported lower GPx activity in liver caused by simazine.

We found no significant differences ($p<0.05$) in TBARS levels at all test concentrations of terbuthylazine except the highest concentration (1 000 μg L^{-1}). Velisek *et al.* (2011) reported a significant increase in TBARS levels in brain and liver of common carp exposed to sublethal concentrations of terbutryn (0 .02; 0.2 and 2 μg L^{-1}). Likewise, Elia *et al.* (2002) described an increase in TBARS levels in fish liver exposed to atrazine, but it was after acute exposure with higher concentrations (6 and 9 mg L^{-1}). Nevertheless, Oropesa *et al.* (2009b) and similarly Stara *et al.* (2012) mentioned no significant differences in TBARS levels compared to control in tissues of fish exposed to low simazine concentrations. The results of biochemical parameters showed that exposure to some terbuthylazine concentrations caused activation of the detoxification metabolism and compartments of antioxidant defence.

In the light of the above findings, it was concluded that the lowest test concentration (the environmental concentration) of terbuthylazine, 0.55 μg L^{-1}, has no effect on fish behaviour or mortality, growth, the development of histopathological changes in selected organs, on the activity or concentration of biochemical parameters (GST, GR, GPx, TBARS) in *D. rerio*. In the present study, the values of terbuthylazine NOEC and LOEC for juvenile *D. rerio* were found to be 150 μg L^{-1} and 400 μg L^{-1}, respectively.

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Potential Conflicts of Interest: *None disclosed.*

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