Comparison of Terbutryn Acute Toxicity to *Danio rerio* and *Poecilia reticulata*

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Abstract

The aim of the present study was to determine and compare acute toxicity of terbutryn in *Danio rerio* and *Poecilia reticulata,* and in two different developmental stages of *D. rerio* – embryonic and juvenile. Acute toxicity tests were performed according to OECD methodology. The LC50 values were assessed by probit analysis using EKO-TOX 5.2 programme. The 96hLC50 terbutryn mean value of 5 tests was 2.85 ± 0.75 mg·l^{-1} for the juvenile stage of *P. reticulata* and 5.71 ± 0.46 mg·l^{-1} for the juvenile stage of *D. rerio*. For the embryonic stage of *D. rerio* the 144hLC50 terbutryn mean value of 6 tests was estimated as 8.04 ± 1.05 mg·l^{-1}. The study proved significantly higher ($p < 0.01$) sensitivity of the juvenile stage of *D. rerio* to terbutryn compared to the embryonic stage of *D. rerio* and significantly higher ($p < 0.01$) sensitivity of the juvenile stage of *P. reticulata* to terbutryn compared to the juvenile stage of *D. rerio. herbicides.* This study documented differences in sensitivity of several fish species and different developmental stages of fish to one of triazine.

Embryonic stage, juvenile stage, zebrafish, guppy, LC50

Terbutryn (2-(*tert*-butylamino)-4-(ethylamino)-6-(methylthio)-*s*-triazine) belongs to substituted symmetrical triazines. Triazines form a group of similar 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 (Muir 1980; Moretti et al. 2002; Arufe et al. 2004). Triazine herbicides are divided into two groups, asymmetrical triazines or triazinones, such as metribuzin, and symmetrical triazines. The most commercially used symmetrical triazines are simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon and terbutryn (Stevens et al. 2001). Herbicidal activity of triazines was first discovered in 1952 by J. R. Geigy in Switzerland (Roberts et al. 1998).

Terbutryn has been used worldwide for the control of most annual grasses and broadleaf weeds in many crops including winter cereals, potatoes, legumes, maize, sugarcane and citrus. Terbutryn is a selective systemic herbicide which acts as a photosynthesis inhibitor in the xylem and accumulates in the apical meristems. In plants it is metabolised by oxidation to 2-hydroxy derivates and by side-chain de-alkylation. Terbutryn is also used in the aquatic environment as an aquatic herbicide for the control of algae, submerged and free-floating weeds and may affect many non-target organisms there (Muir 1980; Roberts et al. 1998; Arufe et al. 2004). The application of terbutryn has been banned in many countries because of its the potential to bioaccumulate in organisms, but it can be still detected in the water environment (Rioboo et al. 2007). The preparations containing terbutryn have not been registered in the Czech Republic since 2005, however, terbutryn can be still detected in the environment. Its highest environmental concentration was found in the Czech rivers and according to the Czech Hydrometeorological Institute report it was 0.02 μg·l^{-1} in 2008.

The acute toxicity of chemical substances depends on many factors, such as species,

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age, body weight, feeding conditions, intermediary metabolism rate, temperature, salinity, concentration of oxygen dissolved in the water, etc. (Mayer, Jr. and Ellersieck 1988). Median lethal concentration (LC50) values of organic chemicals in different fish species can often differ by factors of 10 or more and toxicity testing with only one fish species may be inadequate to classify chemicals belonging to these classes for their environmental impact (Geyer et al. 1993; Gallo et al. 1995). *Danio rerio* and *Poecilia reticulata* belong among fish species recommended by OECD Guidelines for Testing of Chemicals and to species frequently used in aquatic toxicology (Svobodová and Vykusová 1991; Gallo et al. 1995; Kovrižnych and Urbančíková 2001; Hill et al. 2005; Jurčíková et al. 2007; Voslářová et al. 2008; Mikula et al. 2009; Plhalová et al. 2009).

The aim of the present study was to determine and to compare the mean lethal concentration of terbutryn in embryonic and juvenile developmental stages of *D. rerio* and juvenile developmental stages of *P. reticulata*.

Materials and Methods

Experimental fish

Tests of terbutryn toxicity were performed on the aquarium fishes *D. rerio* and *P. reticulata*. Experimental procedures were in compliance with the 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). All fish were held for a minimum of 7 days in the aquaria for the acclimatization period during which they were fed with commercial fish pellets. Food was withheld 24 h preceding start of the test.

Acute toxicity test

The acute toxicity tests on juvenile stage of *D. rerio* and *P. reticulata* were performed according to the method OECD No. 203 Fish, Acute Toxicity Test in the series of five tests. We dissolved terbutryn by the addition of dimethyl sulphoxide (DMSO) as terbutryn exhibits low water solubility. This stock solution was applied to particular test vessels, the highest DMSO concentration in test vessels being 0.1%. In each test series we used five ascending concentrations of the tested substance (0.5; 2.0; 3.5; 5.0; 7.5 $mg \cdot l^{-1}$) for *P. reticulata* and (4.0; 5.0; 6.0; 7.0; 8.0 $mg \cdot l^{-1}$) for *D. rerio* and two control groups (one control group in the dilution water only and another control group in the dilution water with the solvent – DMSO in the amount of 0.1%). Ten juvenile fish (aged 2–3 months, randomly selected from the stock population) were placed in each test vessels. We used semi-static test procedure (test solutions were replaced at 24-h intervals). During the tests, the condition of fish was checked and the number of dead fish was recorded for different concentrations every 24 h as well as the water temperature, pH and the oxygen saturation of water. Water temperature in the tests ranged 23 ± 2 °C. Dissolved oxygen concentrations were above 60% (62.5–89.9%), the pH was between 7.87 and 8.36. No fish from control groups died during any of the tests. The duration of these tests was 96 h for each of them.

Embryonic toxicity test

Toxicity tests in the embryonic stage of *D. rerio* were performed according to the method OECD No. 212 Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages in the series of six tests. In each test series we used five ascending concentrations of the tested substance (7.5; 10; 12.5; 15; 17.5 $mg \cdot l^{-1}$) and two controls (one control in the dilution water only and the other one in the dilution water and solvent - DMSO at the amount of 0.1%). Twenty fertilized eggs were placed in Petri dishes. The eggs were placed in Petri dishes within 8 h at the latest after the fertilization. The tests were terminated after hatching and the absorption of the yolk sack in the control dish (144 h after initiation). We used the semi-static test procedure, the solutions were replaced at 24-h intervals. During the tests, numbers of dead embryos at individual concentrations were recorded. The mortality rate of the control embryos did not exceed 20%. Water temperature in the tests ranged 25 ± 1 °C.

The results and statistical analysis

The results of acute toxicity tests (the number of dead fish in individual test concentrations) on embryonic and juvenile stages were processed by the probit analysis (EKO-TOX 5.2 programme) to determine the LC50 terbutryn values (for embryonic stage 144hLC50 and for juvenile stage 96hLC50). The significance of the difference between LC50 values for embryonic and juvenile stages of *D. rerio* and juvenile stage of *P. reticulata* was calculated using the non-parametric Mann-Whitney test and Unistat 5.1 software.

Water quality indicators

The basic physical and chemical properties of the dilution water used in toxicity tests were: COD_{Mn} (chemical oxygen demand) 1.4–1.9 $mg \cdot l^{-1}$; total ammonia below the limit of detection; NO_3^- 24.5–31.4 $mg \cdot l^{-1}$; NO_2^- below the limit of determination; Cl^- 18.9–19.1 $mg \cdot l^{-1}$; Σ Ca ± Mg 14 $mg \cdot l^{-1}$.

Determination of terbutryn

Gas chromatography with ion trap mass spectrometry (GC/IT-MS) was used for the determination of terbutryn.

Preparation of the unfiltered sample was based on the simple liquid-liquid extraction into hexan. Separation, identification and quantification of terbutryn were based on the GC/IT-MS method described by Djozan and Ebrahimi (2008). Gas chromatograph Varian 450-GC with Varian 220-MS ion trap mass spectrometer and VF-5ms (30 m × 0.25 mm) column were used for the separation of terbutryn. Detection limit (3σ) of terbutryn was 0.01 μg·l^{-1}. Expanded uncertainty was 6.5% on the condition that coefficient of expansion was k = 2. In all toxicity tests, the concentration of terbutryn after 24 h was above 80% of the dosed initial concentration.

Results

The 144hLC50 terbutryn mean value for embryonic stage of *D. rerio* was estimated as 8.04 ± 1.05 mg·l^{-1} (ranging from 6.71 to 8.91 mg·l^{-1}). The mean cumulative mortality of *D. rerio* during embryonic toxicity tests is showed in Fig. 1. For the juvenile stage of *D. rerio* the 96hLC50 mean value was 5.71 ± 0.46 mg·l^{-1} (ranging from 5.20 to 6.13 mg·l^{-1}). The mean cumulative mortality of *D. rerio* in particular concentrations of acute toxicity tests is presented in Fig. 2. Results of this study showed significantly higher ($p < 0.01$) sensitivity of juvenile fish to terbutryn compared to the embryonic stages of *D. rerio*.

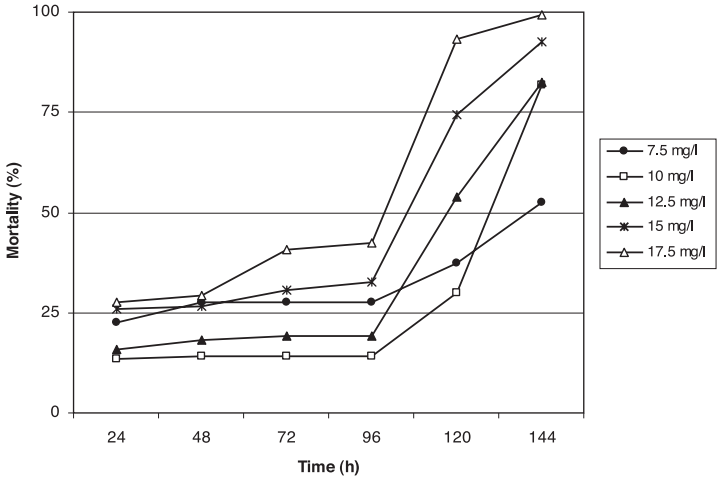

Fig. 1. Mean cumulative mortality of *D. rerio* during embryonic toxicity tests

The 96hLC50 terbutryn mean value for the juvenile stage of *Poecilia reticulata* was estimated as 2.85 ± 0.75 mg·l^{-1} (ranging from 2.07 to 3.93 mg·l^{-1}). The mean cumulative mortality of *P. reticulata* at particular concentrations in acute toxicity tests is presented in Fig. 2.

The comparison of terbutryn LC50 mean values for *D. rerio* and *P. reticulata* showed significantly higher ($p < 0.01$) sensitivity of *P. reticulata* compared to *D. rerio*.

Discussion

This study showed that the juvenile stages *of D. rerio* are more sensitive to terbutryn than the embryonic stages of *D. rerio*. Majority of studies show that sensitivities of the embryonic and juvenile stages of *D. rerio* are comparable, however, in several cases, juvenile fish have been found to be more sensitive depending on the substance tested. Newly hatched larvae constitute a particularly critical and sensitive life stage, because at hatching the embryos lose their protective membrane and are fully exposed to potential toxicants (Arufe et al. 2004). On the other hand, Kovrižnych and Urbančíková (2001)

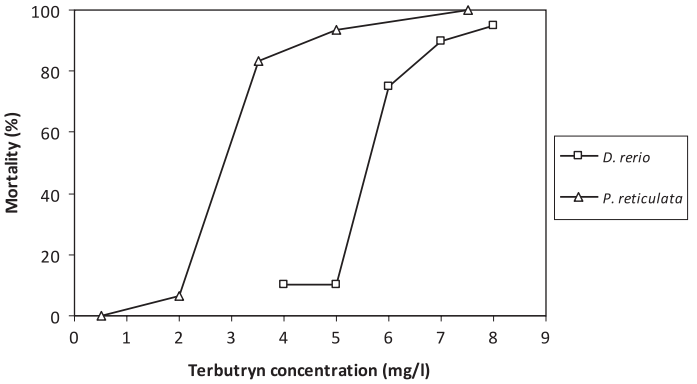

Fig. 2. Mean cumulative mortality of *D. rerio* and *P. reticulata* at particular concentrations in acute toxicity tests

tested eight chemical substances (acetochlor, acrylamide, benzene, colchicine, diethylene glycol, diethylnitrosamine, methanol and Triton X-100) and their toxicity data showed that the results of tested substances were comparable in 7 of 8 chemicals for both life stages of *D. rerio* (adult fish and embryos on eggs). Pištěková et al. (2006) and Mácová et al. (2008) stated very diverse susceptibility to chemical substances. These authors reported that juvenile fish are more sensitive than embryonic stages of *D. rerio*. Pištěková et al. (2006) studied the effect of age of *D. rerio* on nitrite toxicity. Mácová et al. (2008) found higher sensitivity in juvenile fish of *D. rerio* to 2-phenoxyethanol, but acute toxicity values of clove oil for juvenile and embryonic stages were comparable.

This study proved that *P. reticulata* is more sensitive to terbutryn than *D. rerio*. The results of the present and other studies confirm that acute toxicity depends not only on age but also on the species studied (Table 1). Gallo et al. (1995) tested two carbamates, aldicarb and carbaryl, and found that toxicity of both these carbamates was higher in *P. reticulata* than in *D. rerio*. Doleželová et al. (2008) observed comparable sensitivity of *D. rerio* and *P. reticulata* to silver nitrate. But *P. reticulata* showed significantly higher ($p < 0.05$) tolerance to sodium chloride than *D. rerio,* whereas *D. rerio* showed significantly higher ($p < 0.05$) tolerance to formaldehyde than *P. reticulata.* The acute toxicity of potassium permanganate was comparable for both fish species (Doleželová et al. 2009). Svobodová and Vykusová (1991) did

Table 1. Comparison of LC50 *D. rerio* and *P. reticulata* to different chemical substances

Substance	LC50		Authors
	Danio rerio	*Poecilia reticulata*	
Aldicarb	52.9 µmol·l^{-1*}	3.5 µmol·l^{-1*}	Gallo et al. (1995)
Carbaryl	46.0 µmol·l^{-1*}	12.5 µmol·l^{-1*}	
Silver nitrate	15.00 ± 0.52 µg·l^{-1*}	17.14 ± 5.43 µg·l^{-1*}	Doleželová et al. (2008)
Sodium chloride	10.39 ± 0.12 g·l^{-1*}	21.69 ± 0.92g·l^{-1*}	Doleželová et al. (2009)
Formaldehyde	0.12 ± 0.003 ml·l^{-1*}	0.1 ± 0.003 ml·l^{-1*}	
Potassium permanganate	1.25 ± 0.15 mg·l^{-1*}	1.43 ± 0.05 mg·l^{-1*}	
p-nitrofenol	105.0 ± 1.48 mg·l^{-1**}	138.4 ± 8.54 mg·l^{-1**}	Svobodová and Vykusová (1991)
Zinc sulfate	87.6 ± 15.06 mg·l^{-1**}	61.0 ± 4.77 mg·l^{-1**}	
Potassium dichromate	25.7 ± 0.77 mg·l^{-1**}	25.2 ± 0.70 mg·l^{-1**}	

*96hLC50
**48hLC50

not find any significant difference between the sensitivity of *D. rerio* and *P. reticulata* to the chemical compound which was commonly used as a standard in toxicity testing – p-nitrofenol. Significantly higher sensitivity to zinc sulphate was recorded in *P. reticulata*, by contrast *D. rerio* was more sensitive to potassium dichromate than *P. reticulata*.

Bathe et al. (1973), Kidd and James (1991) assessed terbutryn 96hLC50 values for *Cyprinus carpio* as 4 $mg \cdot l^{-1}$ and the same LC50 value was found for *Lepomis macrochirus*. 96hLC50 values for *Onchorhynchus mykiss* was 3 $mg \cdot l^{-1}$. Arufe et al. (2004) stated the 72hLC50 of a commercial herbicide containing terbutryn (59.4%) and triasulphuron (0.6%) to be 1.41 $mg \cdot l^{-1}$ for yolk-sack larvae of *Sparus aurata,* whereas the LC50 values for triasulphuron are well above these values, reported to be > 100 $mg \cdot l^{-1}$ for the species *L. macrochirus* and *O. mykiss,* indicating a significantly lower acute toxicity (Office of Pesticide Programs 2000). The comparison of our LC50 terbutryn values for *D. rerio* and *P. reticulata* with the LC50 terbutryn values in other fish species showed that the results are practically conformable.

Porovnání akutní toxicity terbutrynu pro *Danio rerio* a *Poecilia reticulata*

Cílem předložené práce bylo stanovit a porovnat akutní toxicitu terbutrynu pro juvenilní stádia *Poecilia reticulata* a *Danio rerio* a pro dvě rozdílná vývojová stádia *Danio rerio* –embryonální a juvenilní. Testy akutní toxicity byly provedeny dle metodiky OECD. Hodnoty LC50 byly stanoveny probitovou analýzou pomocí programu EKO-TOX 5.2. Průměrná hodnota 96hLC50 terbutrynu pro 5 opakování testů byla 2,85 ± 0,75 $mg \cdot l^{-1}$ u juvenilního stádia *P. reticulata* a 5,71 ± 0,46 $mg \cdot l^{-1}$ pro juvenilní stádium *D. rerio*. Pro embryonální stádium *D. rerio* byla průměrná hodnota 144hLC50 terbutrynu pro 6 opakování testů 8,04 ± 1,05 $mg \cdot l^{-1}$. Porovnáním hodnot akutní toxicity terbutrynu pro embryonální a juvenilní stádia *D. rerio* byla zjištěna významně vyšší citlivost juvenilního stádia *D. rerio* ($p < 0,01$) a významně vyšší ($p < 0,01$) citlivost juvenilního stádia *P. reticulata* ve srovnání s juvenilním stádiem *D. rerio*.

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