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The chronic effects of terbuthylazine-2-hydroxy on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*)

Dalibor Koutnik, Alzbeta Stara, Eliska Zuskova, Antonin Kouba, Josef Velisek *

University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology, Zatisi 728/II, 389 25 Vodnany, Czech Republic

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ABSTRACT

This study assessed the chronic effects of terbuthylazine-2-hydroxy (T2H), one of the main terbuthylazine degradation products, on early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*) by means of mortality, growth rate, early ontogeny, oxidative stress, antioxidant defence and histopathology. The crayfish were exposed to four concentrations of the tested substance as follows: 0.75 μg/l (environmental concentration), 75, 375 and 750 μg/l for 62 days. Concentrations over 75 μg/l caused lower weight compared to the control group. T2H at 750 μg/l caused delay in ontogenetic development. Levels of thiobarbituric acid reactive substances and total superoxide dismutase activity were significantly ($p < 0.01$) lower in groups exposed to 375 and 750 μg/l T2H. Crayfish in these treatments also showed alteration of tubular system including disintegration of tubular epithelium with complete loss of structure in some places of hepatopancreas and wall thinning up to disintegration of branchial filaments with focal infiltrations of hemocytes. In conclusion, chronic terbuthylazine-2-hydroxy exposure in concentrations up 75 μg/l (100 times higher than environmental concentration) affected growth, ontogenetic development, antioxidant system, caused oxidative stress and pathological changes in hepatopancreas of early life stages of marbled crayfish.

1. Introduction

During the last few decades there is a global problem with increasing concentrations of pollutants in both surface and ground waters [1]. Pesticide compounds cause physicochemical changes in water environment which may directly or indirectly lead to impacts on non-target aquatic organisms, as well as humans [1,2]. Some pesticides may accumulate in aquatic ecosystems and their degradation products can occasionally be more toxic than the parent compound [3].

Triazines are one of the most commonly found pesticides and their degradation products in water [1,4]. They inhibit photosynthesis [5]. Although most of them were banned in Europe in last years, these substances and their metabolites in residual concentrations are still present in water, soil, food and various components of the environment [6]. Currently in Europe, some triazine herbicides (atrazine and simazine) are considered hazardous for the ground water. As a result, they are ranked on the list of priority substances for testing in EU [7].

Terbuthylazine-2-hydroxy ($C_9H_{17}N_5O$; T2H) is the main metabolite of terbuthylazine, which has been used for control of weeds, aquatic plants and algae since 2006 as a substitute for the herbicide atrazine. The half-life of T2H is between 112 and 120 days in water at 20–25 °C, and 456 days at 10 °C [8]. The highest concentrations of terbuthylazine and T2H in rivers of the Czech Republic were found to be 2.9 and 0.75 μg/l respectively [6]. Masia et al. [9] report concentration of T2H in basin of Llobregat River (Catania, Spain) to be 9.24 μg/l.

Crayfish are important benthic invertebrates in the ecosystem, which are considered appropriate model organisms for assessment of environmental pollution [10,11]. There is a dearth of data on effects of triazine herbicides on crayfish following chronic exposure. Considering conservation status of European native crayfish [12], non-native and according to law non-protected species marbled crayfish (*Procambarus fallax* f. *virginalis*) were utilized as a model organism in this trial. The aim of the present study was to investigate chronic effects of long-term exposure to the T2H in a range of concentrations, including environmentally realistic exposure, on crayfish survival, growth, ontogeny, oxidative balance, antioxidant defence, and histology.

2. Materials and methods

2.1. Experimental animals

Eggs from a single marbled crayfish (*Procambarus fallax* f. *virginalis*) female (carapace length 28.94 mm, postorbital carapace length 22.69 mm, and weight 6.68 g) were gently stripped with tweezers from pleopods. The female originated from own laboratory culture.

* Corresponding author.
E-mail address: velisek@frov.jcu.cz (J. Velisek).

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Marbled crayfish is a fast growing organism that frequently reproduces via parthenogenesis with high fecundity, has an early maturation and low culture requirements making it a useful model organism [13]. It is also widely available in the pet trade and non-native in Europe [14,15].

2.2. Experimental protocol

Two hundred and ten eggs (mean weight of 2.18 mg) in X. stage of embryonic development (embryo with maxilla) were put into plastic macroplates where they were held separated as single individuals. The eggs were exposed to the T2H (Sigma Aldrich, Czech Republic, chemical purity 99.5%, CAS: 66753-07-9) concentrations while the water without tested substance served as a control. Forty-two individuals were tested per group and replicate. The tested T2H concentrations were as follows: 0.75 μg/l (environmentally relevant concentrations in Czech rivers – group 1 – E1), 75 μg/l (group 2 – E2), 375 μg/l (group 3 – E3), and 750 μg/l (group 4 – E4). Groups E1–E4 were used as contrast groups.

2.3. Water quality parameters

Aerated tap water was used, with the following parameters: dissolved oxygen > 75%, temperature 19.8–20.1 °C, pH 7.4–7.9, $ANC_{4.5}$ (acid neutralization capacity) 0.95 mmol/l, COD_{Mn} (chemical oxygen demand) 1.1 mg/l, total ammonia 0.05 mg/L, NO_3^- 4.10 mg/l, NO_2^- 0.04 mg/l, Cl^- 8.9 mg/l and sum of $Ca^{2+} + Mg^{2+}$ 1.0 mg/l. Temperature was measured hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). All T2H concentrations were checked daily by high performance liquid chromatography (HPLC). Water was chromatographed on a reverse phase HPLC column (Lichrosphere 100 RP_{18}, Vertex column, pore size 100 μm, particle size 5 μm, 250 mm × 5 mm ID) using solvent systems of methanol:water:ammonium:acetate 70:30:0.2 and 80:20:0.2 (v:v:v) isocratically, with a flow rate of 0.7 ml min^{-1}. Injection volume of samples was 100 μl per injection. UV detection was recorded at 230 nm. Column eluents (1 min fractions) were collected in scintillation vials using a fraction collector (LKB 2212 HeliRac; Amersham Pharmacia Biotech, Freiburg), dissolved in a scintillation cocktail and counted by LSC. Water samples were assayed using the method of Richter and Nagel [16]. Water samples were collected from all aquaria immediately before (24 h after application) and after renewing the test solutions (0 h). The values measured did not differ from the value stated for test purposes by >9%.

2.4. Test conditions

The macroplates were placed in a laboratory (open-air conditions) with the light regime (11:13 h light:dark). The exposure water for each treatment was renewed three times weekly. Water was gently drained from each chamber, and a new solution was slowly added. Survival was evaluated daily and dead individuals (eggs or juveniles) were removed. From the third development stage, juveniles were fed by freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii ad libitum once a day. During and at the end of the experiment, early development stages were observed to monitor development, occurrence of morphological anomalies and body weight of particular stages (always a day after moulting to allow at least partial calcification of the animal). Determination of developmental periods and stages followed Vogt et al. [17].

The toxicity test was ended after 62 days. At the end of the tests crayfish were sacrificed on ice anaesthesia, weighed, measured and stored for further processing. Weight to the nearest 0.1 mg, was measured using a Mettler-Toledo (Greifensee, Switzerland) analytical balance after removing excess water on a filter paper. The mean specific growth rate (SGR) for crayfish in each of the experimental groups was calculated for the period beginning at day 9 (the first sampling time) and ending at day 62 (end of the trial) using the method described by Kroupova et al. [18].

2.5. Oxidative stress and antioxidants biomarkers

At the end the trial surviving crayfish were immediately frozen and stored at −80 °C until the analysis. Frozen samples were homogenized (1:10, w/v) with an Ultra Turrax homogenizer (Ika Staufen, 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 the second centrifuged at 4 °C to obtain the post-mitochondrial supernatant for antioxidants parameters analyses – total superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). The TBARS method described by Lushchak et al. [19] was used to evaluate lipid peroxidation.

Total superoxide dismutase activity was determined spectrophotometrically by the method of Marklund and Marklund [20]. The catalase activity assay, using the spectrophotometric measurement of H_2O_2 breakdown at 240 nm, was performed following the method of Beers and Sizer [21]. Glutathione reductase activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [22]. Protein levels were estimated spectrophotometrically by the method of Bradford [23] using bovine serum albumin as a standard.

2.6. Histopathology examination

For histological investigations crayfish were fixed in neutral buffered 10% formalin at the end of the experiment. Later they were decalcified for 4 h (slow decalcifier DC1; containing formic acid and formaldehyde, Labonord SAS, Germany), embedded by using of tissue processor Histomaster 2052/1.5. Samples were circumfused with paraffin and sections from paraffin blocks were made on rotary microtome (4 μm), stained with hematoxylin-eosin (H&E) in automatic slide staining system (TISSUE-TEK DR 2000, SEKURA Mars, USA). The most visible tissues – hepatopancreas and gills were examined under the light microscope combined with camera system (MOTIC Wetzlar, Germany).

2.7. Statistical analysis

Data are expressed as mean ± SD. STATISTICA version 12.0 for Windows (StatSoft, Inc.) was used to perform the statistical analysis. To compare difference in means, two way analysis of variance (ANOVA). The significance levels for tests were $p < 0.05$ and $p < 0.01$.

Table 1
Mean body weight (mean ± SD), specific growth rate, inhibition of specific growth, and crayfish mortality on *early life stages of* marbled crayfish (*Procambarus fallax* f. *virginalis*) after terbuthylazine-2-hydroxy (T2H) exposure.

T2H (μg/l)	–	0.75	75	375	750
M_9 (mg)	5.93 ± 0.19	5.57 ± 0.39	5.61 ± 0.40	5.57 ± 0.45	5.70 ± 0.42
M_{62} (mg)	85.27 ± 14.48	78.06 ± 11.49	65.00 ± 12.32*	55.79 ± 9.11*	48.39 ± 6.97*
SGR (%)	5.00	4.98	4.58	4.33	4.01
I (%)	–	0.40	8.40	13.40	19.80
Cumulative mortality (%)	14.2	16.7	19.0	16.7	19.0

M_9, M_{62} = Mean body weight in selected group after 9 and 62 days exposure; SGR = mean specific growth rate in selected group; I = inhibition of specific growth in selected group; SD = standard deviation.

* Experimental groups significantly ($p < 0.01$, Tukey's test) different from the control group.