Chemico-Biological Interactions 291 (2018) 95–102

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Biochemical and histological effects of sub-chronic exposure to atrazine in crayfish *Cherax destructor*

Alzbeta Stara, 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

ARTICLE INFO

Keywords:
Biochemical markers
Ecotoxicity
Histology
Invertebrate
Recovery
Triazine

ABSTRACT

Atrazine (ATR) is a triazine herbicide banned in the European Union. It remains one of the most widely used herbicides in other parts of the world. Considering the scarcity of data on its possible harm to the environment and to human health, we assessed sub-chronic effects of a 14-day exposure at the environmentally relevant concentration of 6.86 µg/L and at 10% of the 96hLC50 (1.21 mg/L) in crayfish *Cherax destructor* and their recovery in a 14-day period in ATR-free water. Indicators assessed were behavior; hemolymph biochemical profile; oxidative and antioxidant parameters in gill, hepatopancreas, and muscle; and histology of gill and hepatopancreas. Crayfish exposed to the environmental concentration showed significant differences ($P < 0.01$) from controls in biochemical parameters of hemolymph (lactate, alkaline phosphatase) and activity of superoxide dismutase, as well as in histology of gill tissue. The higher concentration led to low motor activity, differences in biochemical profile of hemolymph (lactate, alkaline phosphatase, ammonia, glucose), antioxidant biomarkers (superoxide dismutase, catalase, glutathione reductase, glutathione S-transferase, reduced glutathione), as well as gill and hepatopancreas histology. Some observed effects persisted after 14-days recovery in ATR-free water. The results provide evidence that environmental concentrations of ATR produce negative effects on freshwater crayfish.

1. Introduction

A growing human population demands an increase in agricultural production, which may require use of industrially manufactured substances, including pesticides. These substances are beneficial in some respects, but often adversely affect the environment and ultimately threaten human health. Pesticide runoff from agricultural land poses a significant hazard to aquatic ecosystems, interrupts the biological balance and produces toxic effects on non-target components of the aquatic biocenosis [1,2].

Triazines are compounds with herbicidal action discovered in early 1950s. Their use has rapidly expanded worldwide [3,4], generating serious concerns with respect to soil, surface, and groundwater contamination in recent decades [5]. Atrazine (ATR) [2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine] is one of the first triazine herbicides to be banned by the European Union, its use prohibited since 01 August 2005 under the European Commission Decision 2004/248/EC. Although ATR is a known water pollutant with potentially serious adverse health effects, it remained the second-most widely used pesticide in the United States in 2016 [6] and is one of the most widely used herbicides in Australia [7]. The US EPA is committed to assessing the impact of ATR on 15,000 endangered plant and animal species by the end of 2020, prior to enacting prohibition of the use of ATR [6].

Atrazine is soluble in water and can be transported over long distances [8]. Atrazine is slowly degraded by photolysis. It is resistant to decomposition by microorganisms and is therefore stable in soil and water. The half-life of ATR is variable, ranging from 14 days to 4 years in soil, and 6 months to several years in water [9]. It is currently detectable in the EU and Worlds waters in concentrations range from 0.005 µg/L to 227 µg/L [10–17].

Atrazine is reported to be slightly to highly toxic to aquatic organisms [18]. The 48hEC50 (48 h median effective concentration) values of ATR determined for cladocerans are 4.60 mg/L for *Ceriodaphnia dubia* [19], 12.37 mg/L for *Macrothrix flabelligera*, 14.30 mg/L for *Ceriodaphnia silvestrii*, and up to 50.41 mg/L in *Daphnia magna* [20]. Atrazine 96hLC50 (96 h median lethal concentration) in the copepod *Tigriopus brevicornis* is 0.124 mg/L [21]. Acute toxicity tests on freshwater decapods revealed 48hLC50 of 8.90 mg/L in *Palaemonetes argentinus* [22] and 96hLC50 of 12.10 mg/L in crayfish *Pacifastacus leniusculus* [23]. The 96hLC50 of ATR is 2.14 mg/L for *Cyprinus carpio*

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

https://doi.org/10.1016/j.cbi.2018.06.012
Received 9 January 2018; Received in revised form 7 June 2018; Accepted 12 June 2018
Available online 13 June 2018
0009-2797/ Published by Elsevier B.V.

[24], 5.6 mg/L for *Melanotenia fluviatilis* [25], 9.96 mg/L for *Psetta maxima* [26], 12–19 mg/L for subspecies of *Oncorhynchus* [27], 18.53 mg/L in *Rutilus frisii kutum* [28], and 42.38 mg/L in *Channa punctatus* [29].

Atrazine has potential to adversely affect aquatic organisms at environmental concentrations, including crayfish [30], frogs [17,31], and fish [17,24,32–34]. It is reported to damage DNA, causing genotoxicity, mutagenicity, immunosuppression, and immunotoxicity; impair reproduction, growth, and development; and accumulate in tissues. Atrazine is known for its endocrine disrupting and carcinogenic properties not only in crayfish [30], fish [34], and mammals [35], but also in humans [36–39].

Freshwater crayfish are prominent organisms in aquatic ecosystems, considered keystone species, ecosystem engineers, and suitable bioindicators. Negative effects on crayfish populations lead to changes in entire communities [40].

The objectives of this study were to evaluate the effects of ATR on the non-target crayfish *Cherax destructor* via biochemical analyses of hemolymph, assessment of oxidative stress and antioxidant biomarkers in tissues, and histology, and to assess recovery from exposure effects.

2. Materials and methods

2.1. Test chemical and crayfish

The tested substance atrazine (ATR) [2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine], of 98.9% purity, was obtained from Sigma Aldrich (Czech Republic).

Adult common yabby *Cherax destructor* were cultured at our laboratory at the Faculty of Fisheries and Protection of Waters in Vodňany, University of South Bohemia in České Budějovice, Czech Republic. Seventy-two crayfish, mean weight 5.69 ± 1.61 g and carapace length 25.00 ± 2.37 mm, were randomly divided into nine groups (n = 8) and placed in 50 L aquaria for seven days of acclimatization. Continuous aeration and a light cycle 12L:12D were provided. Crayfish were fed a commercial diet (Crabs Natural, Sera GmbH, Germany) at 1% of initial body weight per day.

2.2. Crayfish exposure, experimental design

Crayfish in the ATRenv group were exposed to ATR at 6.86 μg/L, the highest concentration detected in surface waters in the Czech Republic [13]. Group ATR10% was exposed to 1.21 mg/L, corresponding to 10% of the 96hLC50 determined by Velisek et al. [23]. A control group was held in fresh tap water only. The trial was conducted in triplicate. Crayfish were exposed to ATR for 14 days with daily exchange of the bath, followed by a 14-day recovery period in ATR-free water exchanged in the same manner.

Water samples were taken from the test aquaria 1 h after and immediately before renewing the test solutions, and actual concentrations of ATR were analyzed by liquid chromatography mass spectrometry (LC-MS) analysis. The mean concentration of ATR in the water was always within ± 5% of the intended concentration, water in control and recovery tanks was ATR-free. Water parameters were oxygen saturation 92.64 ± 7.36%, temperature 19.13 ± 0.25 °C, pH 8.19 ± 0.25, acid neutralization capacity $ANC_{4.5}$ 1.10 mmol/L, sum of Ca and Mg 8.91 mg/L, total ammonia 0.03 mg/L, NO_2^- 0.02 mg/L, NO_3^- 5.92 mg/L, PO_4^{3-} 0.02 mg/L, and COD_{Mn} 1.10 mg/L.

Mortality and changes in behavior were noted before the daily water exchange. Two specimens per aquarium were sampled for biochemical analysis and histology after 7 and 14 days of exposure and after 14 days recovery. Crayfish were anesthetized in ice, and hemolymph was collected from the ventral abdomen into a syringe containing 0.01 mL of sodium heparin (Heparin inj., Leciva, Prague, Czech Republic) per mL of hemolymph. Gills, hepatopancreas, and abdominal muscle were immediately dissected. Samples for assessment of biochemical parameters were stored at −80 °C for later analysis. Tissue samples for histology were fixed in 10% buffered formalin.

2.3. Biochemical hemolymph profile

Hemolymph was centrifuged for 10 min at 10,000 g. Plasma was removed immediately after centrifugation and kept at −80 °C until analysis. Measurement of biochemical hemolymph markers was conducted using the VETTEST 8008 analyzer (IDEXX Laboratories Inc., Maine, USA) as described by Kolarova and Velisek [41]. The biochemical profile of hemolymph included glucose (GLU), total protein (TP), albumin (ALB), total globulins (GLOB), ammonia (NH_3), triacylglycerols (TAG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), lactate (LACT), calcium (Ca), magnesium (Mg), inorganic phosphate (PHOS), creatinine (CREA), and alkaline phosphatase (ALP).

2.4. Oxidative stress and antioxidant biomarkers

Frozen tissue samples were weighed and homogenized (1:10, w/v) with a ball homogenizer (TissueLyser II QIAGEN) on ice using 50 mM potassium phosphate buffer (KH_2PO_4, containing 1 mM EDTA, pH 7.4) for assessment of superoxide dismutase (SOD) and catalase (CAT). For other parameters, phosphate buffer saline (PBS – 0.8% NaCl; 0.02% KCl; 0.29% $Na_2HPO_4.12H_2O$; and 0.02% KH_2PO_4 in deionized water, pH 7.2) was used.

The sample homogenate was centrifuged to obtain the post-mitochondrial supernatant at 30,000 g for 30 min at 4 °C for SOD and CAT and at 10,000 g for 15 min at 4 °C for other antioxidant parameters.

Lipid peroxidation was measured in the homogenate (no centrifugation) by the thiobarbituric acid method [42]. Thiobarbituric acid reacts with product of lipid peroxidation and generates colored thiobarbituric acid reactive species (TBARS) which was spectrophotometrically measured at 550 nm on spectrophotometer (Infinite M200, TECAN), and the concentrations (nmol TBARS/mg protein) were calculated according to the standard calibration generated with malondialdehyde.

The CAT (EC 1.11.1.6) activity was determined by spectrophotometric measurement of H_2O_2 breakdown at 240 nm on spectrophotometer, following the method of Beers and Sizer [43]. Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm based on inhibition of nitro-blue tetrazolium reduction and production of superoxides by NADH and phenazine methosulfate at neutral pH [44]. Glutathione reductase (GR) activity (EC 1.6.4.2) was assayed from the rate of NADPH oxidation determined spectrophotometrically at 340 nm on spectrophotometer [45]. The concentration of reduced glutathione (GSH) was assayed following Ellman [46] utilizing 5,5′-dithiobis-2-nitrobenzoic acid as a substrate, which interacts with -SH groups for the formation of the colored product determined at 420 nm on spectrophotometer. Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured spectrophotometrically at 340 nm absorbance using 50 mM 1-chloro-2,4-dinitrobenzene as a substrate, with the reaction initiated by the addition of 10 mM GSH [47]. Methods were modified according to Stara et al. [48] for use in crayfish.

Parameters were expressed as the volume of product formed per milligram protein. The protein concentrations were determined spectrophotometrically at 562 nm according to Bradford [49] with using bovine serum albumin as a standard for protein calibration.

2.5. Histology

Gill and hepatopancreas tissue was processed using routine histological procedures: dehydrated in ethanol series, cleared in xylene, embedded in paraffin, cut into 3–4 μm sections, stained with hematoxylin and eosin, examined by light microscopy, and photographed with a digital camera type E-600 Olympus BX51 (Japan). Pathology was