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Embryo-toxic effects of atrazine environmental concentrations on the crustacean *Daphnia magna*

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Abstract

Atrazine is one of the most used pesticides all over the world and it is frequently detected in surface water. The aim of this study was to evaluate the effects promoted by environmental concentrations of atrazine on the embryo development of *Daphnia magna*. All the concentrations induced a percentage of embryo abnormalities higher than 78%. The profile of embryo abnormalities included effects in both stages of embryo development (gastrulation and organogenesis). At the lowest concentration (1 µg L^{-1}) we noted the appearance of 20% of arrested eggs. This effect increased with the concentrations, which indicate the increment of the effects severity. Therefore, the maximum allowable concentration proposed for atrazine by the environmental quality standards (2 µg L^{-1}) induces high toxic effects to the exposure embryos.

Keywords: *Daphnia magna*; Atrazine; Embryo development

1. Introduction

The use of herbicides is increasing worldwide and there is a considerable potential risk that water bodies be contaminated with herbicides through spray drift, runoff and direct overspray. Thus it is important to assess the adverse impacts that these chemicals may have on non-target organisms in the aquatic ecosystem [1].

Atrazine is one of the most used pesticides all over the world and frequently detected in surface waters, due to its water solubility (33 mg L^{-1}, 22 ^{o}C) and moderate ability to be adsorbed by soils [2]. Atrazine was detected in water, in spring-summer months, immediately after its crops application [3, 4]. Atrazine may reach values above the maximum admissible concentration (MAC) according to Portuguese Legislation, in surface waters [5]. Furthermore, we cannot forget, that atrazine physico-chemicals properties allow its accumulation in the phytoplankton, invertebrates and fish species. This process may induce chronic toxic effects to the aquatic species [2, 6]. Though Solomon et al. [7], concluded that atrazine does not pose a significant threat to the aquatic environment; they cautioned that when atrazine is retained in small standing-water reservoirs or has repeated inputs to a reservoir, significant damage could occur.

This herbicide is in the list of hazardous substances or priority pollutants reported by European Water Framework Directive [8]. The pollutants on this list have been selected due to their environmental relevance and high toxicity in the different ecosystems.

Acute and chronic toxicity of atrazine in freshwater invertebrates is well documented [6, 9-11]. However, information on the toxic effects of atrazine environmental concentrations on cladocerans embryonic developmental stages is not clearly reported till date.

Baird et al. [12], showed that daphnids embryos had a higher sensitivity to several toxicants than later stages of the life-cycle. Nevertheless, until now, only a few investigations have been undertaken to determine the effects of environmental pollutants on the daphnid developmental stages [13-15]. Furthermore, such tests were found to be more cost-effective than whole-life cycle tests, with higher sensitivity and shorter exposure periods [16].

The purpose of this study was to determine the toxic effects of environmental concentrations of atrazine on embryo development of *D. magna.*

2. Experimental

2.1. Test chemicals and chemicals analyses

Atrazine PESTANAL® ($C_8H_{14}ClN_5$; CAS No. 1912-24-9; 97.4% purity) was obtained from Riedel-de-Häen Laborchemikalien GmbH. Stock solutions were prepared with dimethyl sulfoxide (DMSO), obtained from Merck® (>99% purity), as a carrier solvent. Working stock solutions were prepared immediately prior to the test using MilliQ® water (resistivity >18 MΩcm). In all experiments the maximum amount of DMSO added was 0.01% (v/v). Prior to the development of toxicity tests, the solution of DMSO in MilliQ® water was analysed to check the absence of toxic effects for the organisms used. The 0.01% (v/v) DMSO solution did not show toxic effects and these results were supported by other authors [17]. A DMSO-negative control was included in all experiments.

Duplicate water samples of freshly and 1-day old prepared test solutions were collected to measure the concentrations of the pesticide. The chemical analysis of atrazine was conducted by gas chromatography-mass spectrometry (GC-MS) (VARIAN 3400 GC with Saturn II MSD) according to DIN EN ISO 6468 [18], with a limit of quantification of 0.01 µg L^{-1}. Measured concentrations were within 10% of nominal concentrations. Thus, calculated exposure levels were based on nominal concentrations.

2.2. Animal cultures

Daphnids were obtained from continuous cultures maintained in the laboratory in 800 mL of ASTM hard water [19], enriched with the organic additive Marine "25" (Pann Britannica Industries Ltd, Waltham Abbey, U.K), an extract from the algae *Ascophyllum nodosum* [20], at a concentration of 4.0 mL L^{-1}. The culture medium was renewed three times per week and enriched with organic additive. Animal density was fifteen animals per 800 mL. Daphnids were fed with algae (*Pseudokirchneriella subcapitata*)with a density of $3.0x10^5$ cells mL^{-1} *Daphnia*$^{-1}$ (an equivalent carbon content of 2.65 mg C mL^{-1}), and maintained with a photoperiod of 16 h light: 8 h dark at a light intensity of 100 to 1000 Lx, at a temperature of 20 ± 1 ºC. *D. magna* eggs were obtained from females of the laboratory cultures, only the eggs from the third to fifth broods were used in embryo exposure test.

2.3. Assessment of direct embryo exposure to atrazine

Just after the release of the third brood, females were isolated from culture and observed until the passage of the new embryos from the ovaries to the brood chamber. This event was considered to represent time zero of embryos development. Eight hours after time zero, females were placed under a dissecting microscope and embryos were removed by introducing a small pipette with ASTM hard water in the brood chamber to create a slow flow, pushing the embryos to the microscope slide [16]. After being washed, embryos were placed in individual wells of tissue culture plates with 1 ml of control solvent with a concentration of 0.01% (v/v) (ASTM + DMSO) or with atrazine, at the desired concentration (1.0, 2.5, 5, 10, 20 µg L^{-1}), with eighteen replicas per concentration. Embryos were incubated at a constant temperature (20 ± 1ºC) with a photoperiod of 16 h light: 8 h dark. Embryo development was observed at 24, 48 and 96 h. The percentage of embryos that exhibited development abnormalities was determined when the development of the control embryos was completed. For the analysis of embryo toxicity, we followed the six stages of embryonic development described by LeBlanc et al. [13].

2.4. Statistical analysis

Prior to statistical analysis, data with values ranging from 0 to 100%, such as embryo abnormalities were transformed using the arcsine square root transformation. As abnormal developments were absent in the controls, we used the Fisher´s Exact Probability Test with Holm correction for significant differences from zero [21]. This analysis was performed using STATISTIC 6.0 Software ™ Inc., PA, US, 2001.

3. Results and Discussion

The toxic effects of atrazine on freshwater cladocerans have been largely documented, but most of the studies were devoted to acute and chronic toxicity to juvenile or adult *D. magna* in 48 h and 21 d toxicity experiments. The results of the present study confirm that daphnid eggs test could be an attractive cost-effective test for cladocerans, as reported by other authors [14, 15], and that embryos developed normally when they were removed from the brood chamber of the parental organisms at the exact time (eight hours after time zero) and could be incubated in culture media.

The exposure of embryos to 1.0 µg L^{-1} of atrazine significantly increased the development of embryo abnormalities to a proportion of 78±0.43%, while the maximum proportion of abnormal development reached

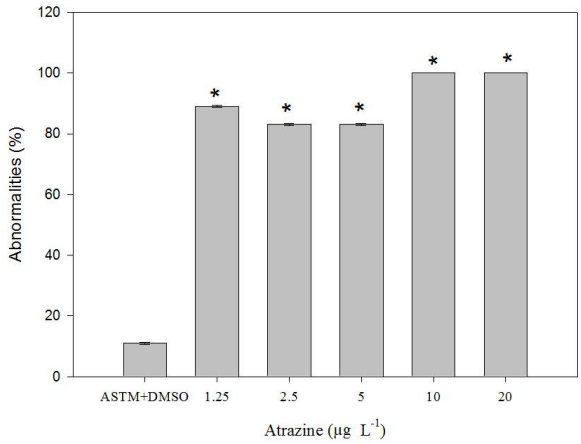

Figure 1. Proportion of developmental abnormalities resulting from embryo exposure to concentrations of atrazine (mean ± SD; n=12). ASTM + DMSO were used as control. Asterisks indicate a significant difference from the control ($p<0.05$, Fisher Exact Test with Holm correction for multiple testing).

100% at the concentration of 10 µg L^{-1} (Figure 1). Performing binominal tests for the null hypothesis for all tested concentrations, the proportions of abnormalities were significantly larger than 0%. The atrazine concentrations which induced acute and chronic toxic effects in *D. magna* were 48 h – EC_{50} of 35.5 mg L^{-1} [22], and 0.2 mg L^{-1} (for the decrease of offspring number per female) [9, 23], respectively. Further the literature reported values of no observable effect concentration (NOEC) between 140 and 200 µg L^{-1} [24, 25]. In the present study we observed embryo toxic effects from the lower concentration (1.0 µg L^{-1}). Hence, the present results demonstrated that the embryo development bioassay was more sensitive than the acute and chronic tests generally used. Similar results were reported for 4-nonyphenol [26], for 3,4 – dichloroaniline [16], and for chlorpyrifos [15]. Moreover, it should be emphasized that even at the NOEC values reported atrazine may be harmful for survival and development of *D. magna* embryos.

Atrazine induced the appearance of embryonic abnormalities in both phases of development, gastrulation (stage 2) and organogenesis (stage 3-6), namely characterized by early development arrest, underdeveloped second antennae and curved or inexistent shell spine (Figure 2 (b-d)). The profile of the development abnormalities effects showed that the embryos with early developmental arrests

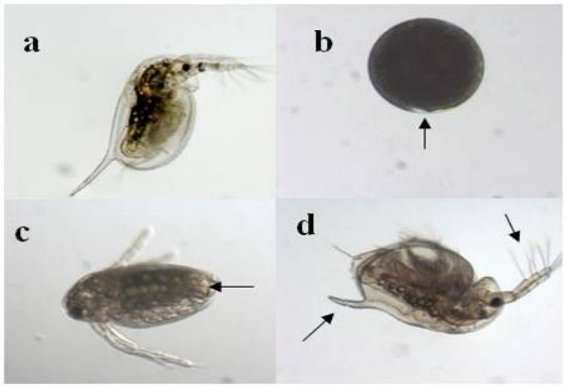

Figure 2. Optical microscope photography of development abnormalities resulting from embryo exposure to concentrations of atrazine. **a**: normal (control) neonatal daphnid at stage 6; **b**-neonatal daphnid that had undergone development arrest during early stages of embryo maturation: note a presence of an eye (a stage 4 characteristic) along with a spherical embryo (a stage 1 characteristic); **c**: neonatal daphnid in a stage 5 of the embryonic development, without a shell spine; **d**: neonate daphnid with development abnormalities at the second antennae and shell spine (arrows), the morphological areas that most commonly exhibit abnormalities. All the neonatal daphnids with abnormalities have the same age as the control **a**.

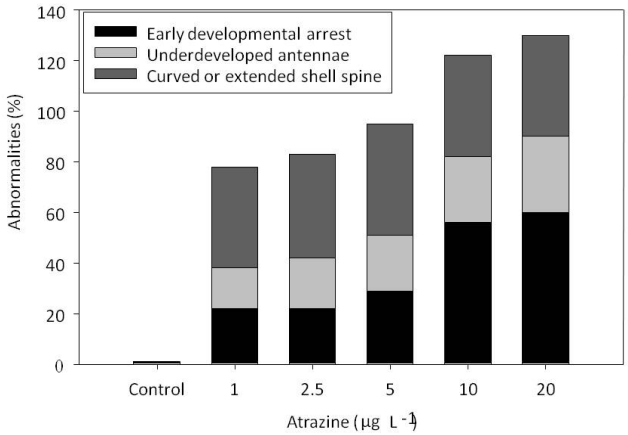

Figure 3. Profile of developmental abnormalities resulting from exposure of embryos to the concentrations of atrazine.

increased with the concentration. Moreover at the higher concentrations tested (10 and 20 µg L^{-1}) this type of abnormalities (early development arrest) was higher than the abnormalities observed at the late stages of development (Figure 3). Thus, the induction of high percentages of embryos with early developmental arrest as a consequence of atrazine activity may promote a decline in the population of *D. magna* worldwide.

Furthermore, some of the present concentrations used in this study were in the same range of values observed in European surface waters (0.55-5.5 µg L^{-1}) [4]. So by the present results, this fact suggests that the exposure to this herbicide can be a potential risk to the embryonic development of freshwater crustacean species in these aquatic ecosystems. Furthermore, recently the European Water Framework Directive 2000/60/EC [8], proposed the environmental quality standards (EQS), in surface water, for hazardous substances [27]. Hence, the EQS proposed for atrazine were: (i) the annual average quality standard concentration (AA) of 0.6 µg L^{-1}, which should be established at a level providing protection against long term exposure, and (ii) the maximum allowable concentration (MAC) of 2.0 µg L^{-1}, which should provide the protection of the aquatic ecosystem against short term exposure. In this context, it is interesting to notice that the MAC concentration established in the EQS is two times higher than the concentration that promoted embryo toxic effects in *D. magna*, in the present study.

4. Conclusions

The results of this study demonstrate that although atrazine does not represent an environmental risk for juveniles and adults crustaceans can constitute a risk to the embryos of these species since the severe abnormalities were observed at environmental concentrations detected in the aquatic ecosystems.

Our findings also underline the importance of the development of a standardized embryo assay-system for the rapid screening of hazardous compounds. Furthermore, the results obtained in this kind of bioassay, should be taken into account when establishing the guideline values for hazard compounds.

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