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# The herbicide atrazine affects sperm quality and the expression of antioxidant and spermatogenesis genes in zebrafish testes

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ABSTRACT

The herbicide atrazine (ATZ) is used worldwide in the control of annual grasses and broad-leaved weeds. The present study evaluated sperm quality parameters in zebrafish *Danio rerio* after 11-day exposure to nominal ATZ concentrations of 2, 10, and 100 μg $L^{-1}$. All ATZ concentrations caused a decrease in motility, mitochondrial functionality, and membrane integrity, as measured using conventional microscopy or fluorescence microscopy with specific probes. The DNA integrity of sperm was not affected. The levels of expression of genes related to spermatogenesis, antioxidant defenses, and DNA repair were also investigated using RT-qPCR. The ATZ caused transcriptional repression of the spermatogenesis-related genes *SRD5A2* and *CFTR*, the antioxidant defense genes *SOD2* and *GPX4B*, and the DNA repair gene *XPC*. This is the first study to show that environmentally relevant concentrations of ATZ significantly affect the sperm quality in fish, possibly resulting in reduced fertility rates. In addition, we showed that the repression of genes related to spermatogenesis and cellular defense could be part of the mechanisms involved in the ATZ toxicity in the testes of male fish.

## 1. Introduction

Atrazine (ATZ) (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is considered one of the most widely used herbicides worldwide and has been extensively employed to control annual grasses and broad-leaved weeds (Graymore et al., 2001). Several studies report that ATZ could act on non-target organisms in the aquatic environment (Rohr and McCoy, 2010) affecting reproduction (Hayes et al., 2003; Suzawa and Ingraham, 2008; Papoulias et al., 2014). Although it have been shown that ATZ affect sperm in mammals, as it was reported using in vitro and in vivo experiments (Betancourt et al., 2006; Abarikwu et al., 2010), the effects in the sperm of fish exposed to ATZ have not been investigated so far. In this context, the sperm quality is considered a crucial parameter that contributes to male reproductive success (Santolaria et al., 2015) and investigating this parameter could allow inferring about the effects of ATZ in the reproductive success of fish in the aquatic environment.

The herbicide ATZ had a profound influence on oxidative stress markers (Jin et al., 2010) and increased DNA damage in zebrafish (Zhu et al., 2011). As a strategy of cellular defense against the harmful effects produced by oxidative stress, the fish would increase transcription of antioxidant enzymes, such as superoxide dismutase (*SOD*) and glutathione peroxidase (*GPX*), as well as DNA repair system enzymes, such as DNA damage recognition and repair factor (*XPC*).The effect of ATZ on the expression of these genes in the testes has not been investigated so far, and it would be important to understand how the ATZ act on zebrafish gonad of male fish.

Analysis of genes related to sperm quality can be considered as predictors in terms of the ability to produce healthy offspring (Robles et al., 2017). For example, Hering et al. (2014) identified genes encoding key enzymes of spermatogenesis or related to the basic characteristics of semen that are significantly associated with sperm motility, such as steroid 5-α reductase α-polypeptide 2 (*SRD5A2*) and cystic fibrosis trans-membrane conductance regulator (*CFTR*). Based on that, the evaluation of these genes may help to understand the effect and mechanisms involved in the effects of ATZ on gonadal physiology of male fish.

The aim of this study was to evaluate the effects of ATZ (2, 10 and 100 μg $L^{-1}$) on sperm quality of male zebrafish (*Danio rerio*), a suitable animal model for toxicological studies (Segner, 2009). The concentrations of 2 and 10 μg $L^{-1}$ ATZ have been found in surface waters in different regions of the world (e.g., Sousa et al., 2016; Davies et al.,

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1994) and thus it is urgent to investigate if those environmentally relevant concentrations represent some risk for fish populations. The effects of ATZ were assessed by evaluating the sperm parameters: motility, mitochondrial functionality, cell membrane integrity and DNA integrity. In addition, we evaluated the effects in the transcription of genes involved in spermatogenesis, antioxidant defenses and DNA repair, in order to test the possible involvement of these genes in the ATZ reproductive effects in the gonad of male fish.

## 2. Material and methods

### 2.1. Animals and treatment

Sixty-four mature fish *Danio rerio* (3.23 ± 0.292 cm length) were obtained from a commercial distributor and kept for one month in tanks containing dechlorinated and aerated water at 28 ± 2 °C, pH 7.0, photoperiod of 12 h light: 12 h dark. Fish were fed with commercial Tetracolor™ Tropical Granules (Tetra) twice a day, with 10% of food per body weight per day. After acclimation, the fish were randomly distributed in four 16 L aquariums (n = 16 fish per aquarium). The ATZ (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions, and added to three aquariums, that received the final nominal concentrations of 2, 10, and 100 μg $L^{-1}$ ATZ for 11 days. The final concentration of 0.005% DMSO was maintained in all four aquariums, including the control group, which did not receive ATZ. The water used in the experiment was previously filtered with Hydronix UDF-10 and CB-25-1005 filters to remove residues of chloride and chemicals. The concentration of 2 μg $L^{-1}$ ATZ corresponds to the maximum level allowed by the Brazilian regulator (CONAMA, 2005) and concentrations around10 μg $L^{-1}$ ATZ have been found in surface waters worldwide (Sousa et al., 2016; Davies et al., 1994). The sub lethal ATZ concentration of 100 μg $L^{-1}$ has been used in previous experiments in toxicology to study molecular mechanisms involved in the effects of ATZ in zebrafish (e.g. Jin et al., 2010; Dong et al., 2009). The water in the four aquariums was completely replaced every 24 h.

Chemical analysis was performed in the water of the tanks that were sampled in duplicate on days 0, 1, 10, and 11 of the experiment, just before the renewal of the water. Water samples of 1 mL were filtered using polyethersulfone syringe filters (0.45 μm) and analyzed for ATZ concentration by liquid chromatography-tandem mass spectrometry (LC–MS/MS), in accordance with the work conditions of Demoliner et al. (2010). At the end of the exposure period, the animals were anesthetized with a 150 mg $L^{-1}$ tricaine methanesulfonate (MS-222) solution (Sigma, St Louis, MO, USA) for 2.5 min, euthanized by cervical transaction and the testes were dissected. The male gonad (i.e. the testes) in zebrafish are long, white, paired organs. Since this organ is paired, we separated the right testis to do the sperm quality test, and the left testis was separated for gene expression evaluation. While dissecting fish we note that some fish were females, and then, those fish were not used in the experiment. The total of male fish collected in the control, 2 μg $L^{-1}$ ATZ, 10 μg $L^{-1}$ ATZ, and 100 μg $L^{-1}$ ATZ groups was n = 9, n = 7, n = 9 and n = 13, respectively. These procedures were approved by the Ethics Committee on Animal Use (CEUA) at FURG.

### 2.2. Sperm parameters

The testis that was excised from individual male fish was placed into tubes containing 100 μL of Beltsville Thawing Solution (BTS) for subsequent analysis. The tubes were shaken to release spermatozoa. Sperm was released by gently and repeatedly disrupting the spermatozeugmatas with a 10-μL pipette tip. Of this sperm suspension, 200 sperm cells were counted and evaluated with an epifluorescent microscope at 400× magnification (Olympus BX 51) to determine sperm motility, motility period, mitochondrial functionality, and membrane and DNA integrity.

For the evaluation of sperm motility, a 10-μL sperm sample was diluted in a 40-μL working solution in an isotonic saline solution, including 1.7 mM-formaldehyde, 20 μM carboxyfluorescein diacetate (CFDA), and 7.3 μM propidium iodide (IP). Sperm with green fluorescence were considered viable, because their metabolic activity allowed carboxyfluorescein diacetate to accumulate in their cytoplasm, whereas those with heads with either red or red and green fluorescence were classified as non-viable (Harrison and Vickers, 1990). The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or red and green fluorescence). The sperm motility period was expressed as the percentage of progressive motile spermatozoa 10 s after activation, and the motility period was comprised of the time (in seconds) between sperm activation and the absence of progressive movement (straight line movement).

Mitochondrial functionality was evaluated after incubation of a 10-μL sperm sample with a 40-μL rhodamine 123 solution (13 μM) at 20 °C for 10 min. Sperm with positive rhodamine staining (green fluorescence) were considered as having functional mitochondria. Conversely, nonfunctional mitochondria were characterized by negative rhodamine staining (sperm with no fluorescence) (He and Woods, 2004). The rate of mitochondrial functionality was determined by the proportion of sperm emitting green fluorescence compared with total sperm (green or no fluorescence).

Membrane integrity of the sperm was examined following the methodology of Harrison and Vickers (1990). Briefly, 5 μL of sample were diluted in 20 μL of saline solution with 1.7 mM formaldehyde, 20 μM carboxyfluorescein diacetate (CFDA), and 7.3 μM propidium iodide (PI). When the spermatozoa membrane was intact, CFDA accumulation occurred. After the hydrolysis of CFDA, carboxyfluorescein was generated along with a corresponding green fluorescence. Sperm with damaged membranes incorporated PI and emitted a red or red and green fluorescence. The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or red and green).

Sperm DNA integrity was evaluated after placing a 45-μL sperm sample in 50-μL TNE (0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.2). After 30 s, 200 μL of Triton solution 1× were added and, 30 s later, 50 μL of acridine orange were added (2 mg/mL in deionized $H_2O$). Evaluation was performed after 5 min, without exceeding 1 min of slide exposure. Sperm with green fluorescence were considered as having intact DNA, whereas those with red or orange fluorescence were considered as having denatured DNA (Bencharif et al., 2010). The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red, or orange fluorescence).

### 2.3. Gene expression analysis

The testis excised from fish were preserved in RNAlater® (Ambion) for 24 h at room temperature and then stored at −80 °C. Total RNA was isolated from tissue using TRIzol reagent (Invitrogen), following the manufacturer's recommendations. The quality of RNA was evaluated via 1% agarose gel electrophoresis to confirm RNA integrity. The total RNA was treated with DNAse (Invitrogen™) to avoid contamination with genomic DNA. Total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA) and a mix of oligo-dT and random hexamer primers. First-strand cDNA was amplified by real-time PCR (qPCR) using gene-specific primers (Table 1), which were designed from sequences available in the National Center for Biotechnology Information Support Center, NCBI (https://www.ncbi.nlm.nih.gov/) (Table 1), using the Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/). The qPCR analyses were performed in duplicate with the GoTaq qPCR Master Mix Kit (Promega, Madison, WI, USA) and real-time PCR System 7300 (Applied Biosystems) using the following program: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and