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Evaluation of hematological parameters, oxidative stress and DNA damage in the cichlid *Australoheros facetus* exposed to the fungicide azoxystrobin

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ABSTRACT

Azoxystrobin (AZX) is a broad-spectrum systemic fungicide massively used worldwide. Its mode of action consists in the inhibition of mitochondrial respiration decreasing the synthesis of ATP and leading to oxidative stress in the target fungus. However, whether this effect occurs in non target organisms has been scarcely studied. The objectives of this work were (1) to evaluate biomarkers of oxidative stress, hematological, physiological and of genotoxicity in the native cichlid fish *Australoheros facetus* exposed to environmentally relevant concentrations of AZX and (2) to compare these biomarkers in different developmental stages using juvenile and adult fish (n = 6) exposed during 48 h. The exposure concentrations were 0 (negative control, C (−)), 0.05, 0.5, 5 and 50 μg/L AZX of the commercial formulation AMISTAR®. Blood was drawn to evaluate hematology, and DNA damage through the comet assay (CA) and micronucleus test (MN). Genotoxicity was observed by mean of both biomarkers in juvenile and adult fish at 50 μg/L AZX. Samples of liver and gills were used to determine antioxidant enzymes activity, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents. In juvenile fish inhibition of superoxide dismutase (SOD) was observed in liver at 0.05, 5 and 50 μg/L AZX and in gills at 5 and 50 μg/L AZX. Glutathione- S- transferases (GST) activity increased in gills at all AZX concentrations tested. In adult fish, increase of hepatic catalase (CAT) activity at 0.5 and 50 μg/L AZX and MDA content at 50 μg/L AZX were observed. In gills only H_2O_2 content showed changes at 50 μg/L AZX. The sensitivity showed by gills constitutes the first report about AZX toxicity in this organ. All these negative effects were observed in the range of realistic AZX concentrations, which warns of the possible consequences that it may have on the health of aquatic biota. Differences between juvenile and adult fish demonstrate the relevance of considering the developmental stage on the evaluation of biomarkers.

1. Introduction

Currently, global concern is based on the indiscriminate and random use of broad spectrum biocides that cause environmental pollution and toxicity to non-target organisms like fishes and invertebrates, ultimately leading to an imbalance of the entire ecosystem (JanakiDevi et al., 2013). Particularly, the use of agrochemicals in Argentina increased from 73 to 236 million kg per year in the last years (De Gerónimo et al., 2014). Fungicides are a complex chemical group of compounds used in crops against fungal infection, and after herbicides they are among the most important groups quantitatively in some parts of the world (Olsvik et al., 2010). One of the most used groups of fungicides in the world is the strobilurins, and its possible toxicity is beginning to be studied in aquatic species.

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After its launching in Europe in 1996, Amistar®, whose active component is azoxystrobin (AZX), became one of the fungicide world leaders in less than three years (Tomlin, 2000; Syngenta, 2004). The mode of action (MOA) of AZX is the inhibition of mitochondrial respiration via a blockade of the electron transfer between cytochrome *b* and cytochrome c1, which results in oxidative stress in the target fungus (Bartlett et al., 2002; Han et al., 2016). Previous studies showed that AZX is highly toxic to fishes and invertebrates from freshwater ecosystems, affecting the mitochondrial respiration and the mechanisms that control cell proliferation and growth in fish (Olsvik et al., 2010). Azoxystrobin concentrations detected in different parts of the world ranged from 0.3 μg/L in streams to >1 μg/L in water samples from agricultural regions in Sweden (Han et al., 2016). Furthermore, a maximum of 29.7 μg/L have been reported in a lotic water body from an agricultural setting in Germany (Berenzen et al., 2005). In Argentina, AZX concentrations in freshwater ecosystems ranged from 0.01 to 0.06 μg/L (Corcoran et al., 2020).

Australoheros facetus is a native cichlid from Argentina, Paraguay, Uruguay and Brazil, representative of freshwater ecosystems (Casciotta et al., 2005; Rican et al., 2006; Rosso, 2006). This species is easy to identify, abundant in vegetated pond and streams, easy to rear and reproduce in the laboratory, is located at the top of the trophic chains and has demonstrated to be sensitive to multiple pollutants (Bulus Rosini et al., 2004; Torres-Bugarin et al., 2007; Crupkin et al., 2013, 2018; Iturburu et al., 2018).

Several cellular processes in the aquatic organisms can be negatively affected by pesticides, and they can be detected using a battery of biomarkers. Most organisms have developed a complex antioxidant system that protects cell membranes and organelles from the toxic effects of reactive oxygen species (ROS) generated by oxidative stress, being the main enzyme systems superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Nagarani et al., 2011). To measure the response to environmental stressors, some changes in blood composition as hematocrit (Ht), hemoglobin (Hb) content, red and white cells counts (RBC and WBC, respectively) and plasma biochemistry are widely used to assess the overall condition of fish (Maisano et al., 2013; Bachetta et al., 2014; Parrino et al., 2018).

DNA damage evaluation in aquatic organisms has been highlighted because genotoxic effects could lead to the initiation of carcinogenicity when somatic cells are targeted, and to inheritable mutations and reproduction defects when germ cells are affected. These events can lead to reproductive impairment and subsequent changes in population dynamics (Belfiore and Anderson, 2001; Bony et al., 2008, 2010). The evaluation of genotoxicity in fish exposed to pesticides has been classically done by using the micronucleous (MN) test as a reliable tool, but the recent use of the Comet Assay (CA) results one of the most sensitive and versatile methods (Winter et al., 2004; Iturburu et al., 2018).

Several environmental factors (e.g. temperature, salinity) or characteristics intrinsic to organism (e.g. size, sex) turn out to be confounding factors in the interpretation of the toxicity of xenobiotics. One of the factors to take into account when studying different biomarkers is the developmental stage of the fish. Rudneva et al. (2010) demonstrated age-dependent responses of antioxidant enzymes in several fish species. In *A.facetus* exposed to sublethal concentrations of the insecticide endosulfan GST and CAT activities for example showed a size-dependent variation (Crupkin, 2013).

Based on the aforementioned, we established the following objectives (1) to evaluate biomarkers of oxidative stress, hematological, physiological and of genotoxicity in the native cichlid fish *A. facetus* exposed to environmentally relevant concentrations of AZX and (2) to compare these biomarkers in different developmental stages using juvenile and adult fish.

2. Materials and methods

2.1. Reagents and test species

The commercial formulation of AZX used for bioassays was Amistar® (Bayer, 250 g/L). Other reagents for chromatographic, biochemical and microscopy determinations were purchased from Sigma Aldrich® and Biopack® and they were of the highest purity available. Environmentally relevant physical–chemical properties of azoxystrobin are a water solubility of 6 mg/L at 20 °C, an octanol–water coefficient (log Kow) of 2.5 (Bartlett et al., 2002), and a half-life in aquatic environments of 15–28 days (Tomlin, 2000).

Juvenile and adult specimens were collected in a non-anthropized freshwater body of the General Pueyrredon municipality (Buenos Aires Province, Argentina).The rearing and feeding conditions were similar to the experimental conditions. The fish were acclimated for 2 months in 140 L aquariums under constant aeration system and with light-dark cycles (12/12). Fish were fed with Shullet Pellets containing the following formulated in percentages: Minimum of crude protein: 45%, Minimum of total lipids: 2%, Maximum of crude fiber: 3%, Calcium min: 1.95, max 2.99%, Phosphorus: min: 1, max: 1.4%. Fish were fed until the beginning of the trials, since they were fasting during the trials.

2.2. Bioassays design

Two concentration-response bioassays (A, B) were performed in order to establish possible effects of different concentrations of AZX on two size-groups (corresponding to juvenile and adult fish) of *A. facetus*. Healthy specimens with approximately the same size were selected for each bioassays: A, adult fish: total length (mean ± SD): 7.95 ± 1.4 cm and total weight (mean ± SD): 13.2 ± 6.4 g; B, juvenile fish: total length (mean ± SD): 3.53 ± 0.59 cm and total weight (mean ± SD): 1.06 ± 0.49 g.

Tested concentrations of AZX were: 0.05, 0.5, 5 and 50 μg/L, during an exposure period of 48 h. A negative control (C (−)) of tap water was included. AZX concentrations were established taking into account reported values from Argentina (Corcoran et al., 2020) and worldwide freshwater ecosystems, in order to assess environmentally realistic scenarios of short term exposure. We have designed acute bioassays because fungicides like AZX reach the aquatic ecosystems by drift or run off, showing short-term concentrations pulses in surface water after spraying and rain events that take place during the crop season.

A stock solution of 1 g/L AZX was prepared diluting 40 μL of the commercial formulation in 9.96 mL of water, and serial dilutions were made starting from it. Afterwards, appropriate exposure mediums we prepared by diluting different amounts of solutions in tap water. The bioassays were conducted in glass tanks containing six fish (n = 6) per treatment in tap water. The experimental room was illuminated with fluorescent lamps with 12: 12 (light: dark) periods. Bioassays were carried out under the following conditions: temperature 16.4 ± 0.88 °C, pH 8.9 ± 0.28. Ten minutes after the bioassays beginning and at the end of the same, aliquots of exposure media were sampled from all the aquaria for the analytical determination of AZX. Once the exposure time finished, fish length and weight were recorded, and blood, liver and gills samples were obtained. Animals handling, samples extraction and euthanasia method were carried out according to the protocols

approved by the Animal Ethical Committee at the National University of Mar del Plata (CICUAL/UNMDP, OCA Nº 146/15, 387/17, 411/19). Blood samples were extracted by puncturing the tail vein with a heparinized syringe and a 21G x 1 ½ "or 27G x ½" Terumo brand needle depending on the fish size, duration 2 min maximum from capture. Then, immediately, euthanasia by cervical dislocation was carried out. Anesthetic or cold was not used because both can modify the enzymatic responses.

2.3. AZX quantification

Water samples (6 ml) were diluted with 4 ml acetonitrile. 25 µl of 5 ppm sulfachlorpiridazine (SIGMA) in acetonitrile was added as internal standard. About 1 ml of the dilution was filtered and transferred to a chromatography vial and refrigerated until use. Chromatographic conditions: A Waters Aquity H Class UPLC was used for the chromatographic run, associated with a Waters XEVO TQ-Micro (Triple Quadrupole Mass Detector). Movil Phase C consisted in: 95:5 Methanol: Water, 10 mM Ammonium formiate; Movil phase D Consisted in: Methanol, 10 mM Ammonium Formiate. Gradient conditions were 0,5 min 100% C, 7 min, 100% D; 11 min, 100% D, 11,1 min 100% C; 13,5 min 100% C. UPLC flow was 0,3 ml/ml. Injection was with an automatic injector. Mass Quadrupole conditions were:

After the run was completed, AZX was quantified with the chromatograph (TargetLynx_XS, MassLynx) software. An analyte recovery percentage between 82 and 110% and a coefficient of variation less than 12% were obtained (Fig. 1 Suppl. Data). To evaluate the method performance, calibration curves at concentrations of 10, 25, 50, 75, 100 ng/L were constructed. Injections of the standards were repeated at concentrations of 50 ng/L in matrix to assure system stability. The limits of detection and quantification were 10 ng/L (signal-to-noise ratio > 10).

2.4. Biomarker of energy reserves

Glycogen content in liver was evaluated in order to assess AZX possible effects on the energetic reserves of *A. facetus*. Its determination was evaluated according Schmitt and Santos (1993), quantifying glucose equivalents after glycogen hydrolysis. Briefly, liver samples were heated (100 °C, 4 min) to later be incubated in acetate buffer (pH 4.8), with and without the presence of α-amiloglucosidase (0.2 mg/mL) during 2.5 h at 55 °C. After incubation, samples were centrifuged (2000 g, 30 min). Free glucose was quantified using a commercial kit (Wiener Lab AA), and glycogen content was calculated for spectrophotometer at 505 nm (Biotek EPOCH) as the difference between samples with/without α-amiloglucosidase, and results were expressed as glucose mg/tissue g.

Compound	Precursor Ion	MRM 1	Collision Energy (V)	MRM 2	Collision Energy (V)	Retention time (min)
Azoxystrobin	404.3	329	30	371.9	12	7.14

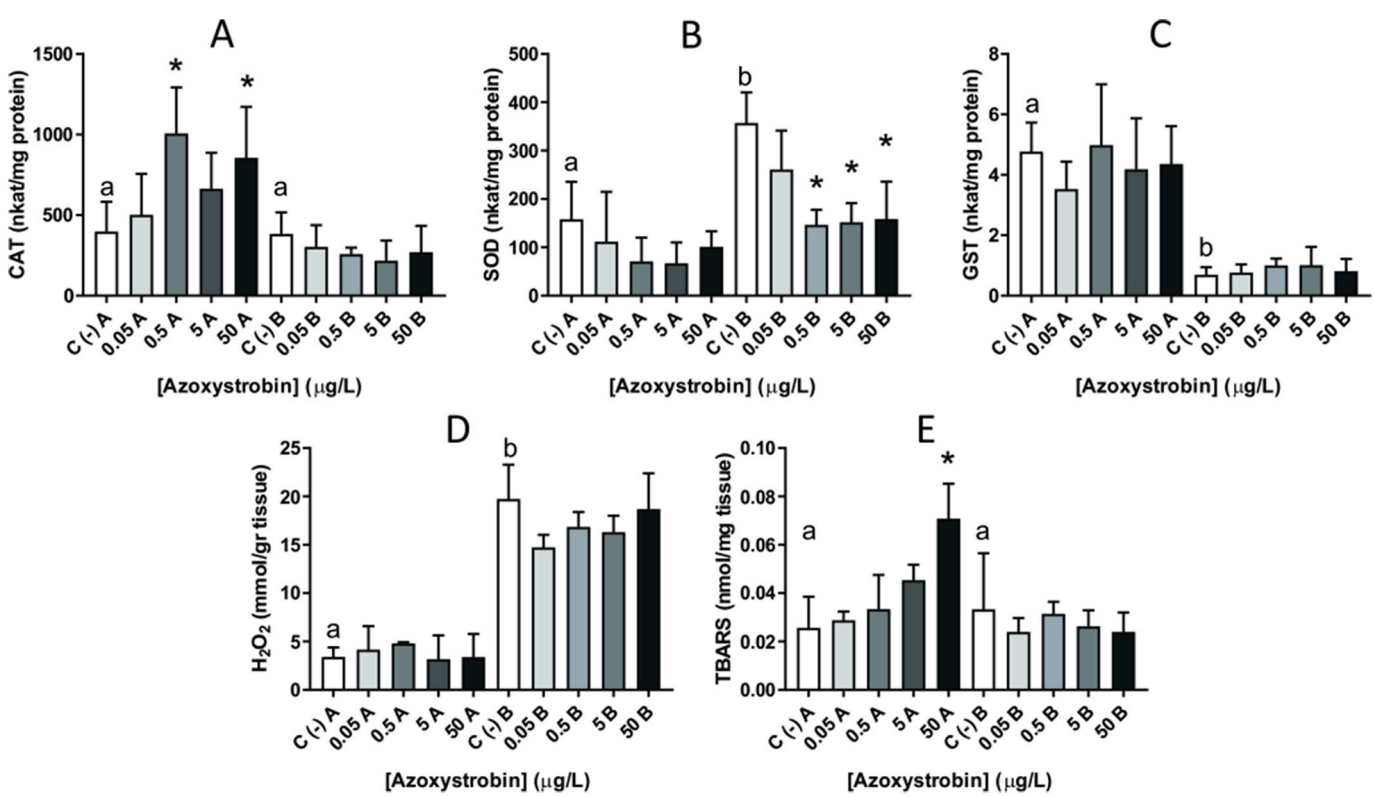

Fig. 1. Enzyme activities (nkat/mg protein), hydrogen peroxide (mmol/mg FW) and thiobarbituric acid reactive substances (nmol/mg FW) contents (mean ± SD) in liver of adults and juveniles of the freshwater fish Australoheros facetus exposed to azoxystrobin (AZX). A: Catalase activity (CAT), B: Superoxide dismutase activity (SOD), C: Glutathione- S- Transferase activity (GST), D: Hydrogen Peroxide (H2O2) content, E: Tiobarbituric acid reactive substances (TBARS) content. In the X axe: A: adult fishes, B: juvenile fishes. Asterisk (*) indicates significant difference with their respective controls (p value < 0.05). Different lowercase letters indicate differences in basal levels between adult and juvenile fish.

2.5. Hematological biomarkers

Blood samples were extracted by punction of the caudal vessel, using heparinized syringes. RBC counts were performed in a Neubauer chamber by microscopy (OLYMPUS CX31RTSF), using physiological solution for dilutions. Hematocrit values were determined using capillary tubes which were centrifuged at 1400 g for 10 min according to Jawad et al. (2004). Concentration of Hb was measured by the cyanomethemoglobin method at wavelength of 546 nm by spectrophotometry (Houston, 1990). Glucose levels in whole blood were by the monitoring system ACCU-CHEK Performa® (Roche). All these biomarkers were only analyzed in adult fishes because the volume of blood from the juvenile ones was not enough.

2.6. Oxidative stress biomarkers

2.6.1. Extract preparation and enzymes activities measurement

In order to evaluate oxidative stress enzymes activities, liver and gills homogenates were obtained according Wiegand et al. (2000) with modifications set up by Cazenave et al. (2006). Enzymatic activities were determined by triplicate spectrophotometrically: activity of soluble GST was determined according to Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate, CAT according to Claiborne (1985) and SOD activity was assayed by the inhibition of nitro blue tetrazolium (NBT) reduction (Scebba et al., 1998). The total protein content for each sample was assessed spectrophotometrically by the Bradford (1976) method, using bovine serum albumin solution as standard. The enzymatic activities were reported in nanokatals per milligram of protein (nkat/mg prot), being 1 kat the conversion of 1 mol of substrate per second.

2.6.2. Non-enzymatic parameters

Concentration of H_2O_2 was quantified in liver and gills, by the FOX1 method in the same extracts used for enzymatic activities quantification, following the methodology proposed by Bellincampi et al. (2000). The content of H_2O_2 was calculated based on a standard curve.

Lipid peroxidation was determined as a biomarker of oxidative damage in liver and gills. It was carried out by measuring the formation of thiobarbituric acid reactive substances (TBARs), according to the procedures of Oakes and Van Der Kraak (2003) and using spectrophotometry (532 nm). TBARs concentration was expressed as nanomoles per mg of fresh tissue ($\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

2.7. Genotoxicity biomarkers

2.7.1. DNA fragmentation

The alkaline CA was performed using erythrocytes from blood samples, following the methodology of Singh et al. (1988) with modifications for *A. facetus*, described by Iturburu et al. (2018). Briefly, after blood extraction, the sample was diluted in PBS solution (1:40). An aliquot of cell dillutions (two slides per fish) were mixed with 1% low melting point (LMP) agarose, and this mixture was added to slides previously coated with 1% normal melting point (NMP) agarose. After LMP layer gelling, the slides were submerged in fresh cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 10% DMSO and 0.1% Tritón-X, pH 10), and left at 4 °C overnight. After lysis process, slides were washed and excess liquid dabbed off. Slides were placed in an electrophoresis tank for DNA undwinding in freshly made alkaline solution (300 mM NaOH, 1 mM EDTA; pH > 13) during 10 min. Electrophoresis was performed for 10 min, at 24 V, 300 mA (0.70 V/cm). All of the steps were carried out under conditions of minimal illumination and low temperature (on ice). Later, the slides were neutralized (0.4 M Tris–HCl, pH 7.5) and dehydrated with ethanol. Finally, slides were randomly coded for 'blind' analysis, stained with DAPI (Sigma Aldrich, 20 μg/mL) and comet images were obtained from each sample under fluorescent microscopy (Leica DM2500). One hundred randomly selected cells (50 from each of two replicated slides) were scored. DNA damage index (DI) score in CA was obtained from cells visually classified into four classes plus the class 0 which refers to the undamaged nucleoids (Simoniello et al., 2009). The classification was performed according to tail size and intensity. Damage index was calculated as: DI = n1+2.n2+3.n3+4.n4, where n1, n2, n3 and n4 are the number of cells in each class of damage, respectively. A positive control was developed for juvenile fish, consisting in the addition of 50 μM H_2O_2 *in vitro* during 10 min. For adult fish previous studies have shown its sensitivity (Iturburu et al., 2018).

2.7.2. MN and nuclear abnormalities

For microscopic quantification of MN and nuclear abnormalities (NA), two smears per fish were prepared with obtained blood samples. Smears were fixed in methanol (100%, 15 min) and stained with Giemsa solution (15%, 15 min). Two thousand erythrocytes, 1000 per slide, were analyzed from each animal under 1000X magnification. Coded and randomized slides were scored using a blind review by a single observer. Only cells with intact cell and nuclear membranes were scored. MN and NA were recorded according to the criteria of Carrasco et al. (1990) and both were expressed as number per 1000 cells (‰). Included NA were notched nuclei (nuclei with invaginations in their envelopments) and buds (nuclei with evaginations which included lobed and blebbed forms).

2.8. Calculations and statistics

Normality and homogeneity of variances were verified by Shapiro–Wilk and Levene tests, respectively. For same biomarkers, a one-way ANOVA test was performed followed by the post hoc LSD test. In the cases where the assumption of homogeneity of variance was not meet, non-parametric Kruskal–Wallis or Mann–Whitney U tests were applied. The significance level was 5%.

3. Results

3.1. AZX in the exposure medium

Analytical measurement of proposed nominal concentrations of AZX in the assayed treatments showed the stability of the fungicide in the exposure medium after 48 h at the bioassay conditions (Table 1).

3.2. Energy reserves

Hepatic glycogen content was studied as an energy reserve biomarker, and it did not show differences when fish were exposed to different concentrations of AZX ($p > 0.05$, Table 2).

3.3. Hematological parameters

The results in adult fish showed that sublethal AZX concentrations did not produce changes in any of the hematological parameters studied ($p > 0.05$, Table 2).

Table 1
Nominal and measured concentrations of azoxystrobin (AZX) in the experimental solutions 10 min and 48 h after exposure.

Nominal Concentration (μg/L)	Measured Concentration (μg/L) 10 min	48 h
Control	< DL	<DL
0.05	< DL	<DL
0.5	0.47 ± 0.01	0.50 ± 0.02
5	5.28 ± 0.41	5.34 ± 0,26
50	48.04 ± 0.37	48.78 ± 0.74

*Value reported as < DL is below the detection limit (DL) of the method: 10 ng/L.

Table 2
Hematological parameters and hepatic glycogen content in adults of *Australoheros facetus* exposed at sublethal concentrations of azoxystrobin during 48 h.

Parameter	Control (−)	0.05 μg/L	0.5 μg/L	5 μg/L	50 μg/L
RBC (10^6/μL)	1.64 ± 0.36	1.07 ± 0.64	1.07 ± 0.51	1.64 ± 0.47	0.93 ± 0.46
Ht (%)	18.01 ± 6.32	20.28 ± 4.3	17.52 ± 5.15	23.62 ± 6.78	17.88 ± 1.83
Hb (g/dL)	3.61 ± 0.65	3,61 ± 0.20	3.41 ± 1.50	4.00 ± 1.70	4.01 ± 2.42
Glucose (g/L)	39.75 ± 9.67	27 ± 12.30	34.33 ± 3.66	29.33 ± 4.03	28.00 ± 6.60
Glycogen (mg/g tissue)	27.13 ± 7.59	35.32 ± 3.74	33.47 ± 10.00	24.94 ± 8.39	27.06 ± 5.76

The values are expressed as mean ± standard deviation.

3.4. Oxidative stress

Oxidative stress biomarkers in liver of fish exposed to AZX are shown in Fig. 1. A comparison of basal values (control) in the biomarkers between juvenile and adult fish showed some differences. Thus, GST activity was higher in adults than in juveniles, while SOD activity and H_2O_2 content showed the inverse pattern ($p < 0.05$, Fig. 1).

On the other hand, in adult fish exposed to 0.5 and 50 μg/L AZX CAT activity increased 153 and 115% respectively ($p < 0.05$, Fig. 1A). At 50 μg/L AZX an increase of TBARS content was also observed, being of 176% ($p < 0.05$, Fig. 1E) while activities of SOD and GST as well as H_2O_2 content did not show changes ($p > 0.05$, Fig. 1B, C, D). In juvenile fish, hepatic SOD activity decreased at 0.5, 5 and 50 μg/L AZX 59, 58 and 56% respectively ($p < 0.05$, Fig. 1B) while the other biomarkers did not show changes in comparison to control ($p > 0.05$, Fig. 1A, C, D, E).

Similarly to the case of liver, the comparison of basal levels of oxidative stress biomarkers between juvenile and adult fish in gills showed that GST activity was higher in adults than in juvenile, while SOD activity decreased in adults with respect to juveniles ($p < 0.05$, Fig. 2). In adult fish exposed to AZX, H_2O_2 content increased at the highest concentration tested, 296% with respect to control ($p < 0.05$, Fig. 2D) while the other biomarkers did not show changes in comparison to control ($p > 0.05$, Fig. 2A, B, C, E). In juvenile fish, a decrease of SOD at 5 and 50 μg/L AZX (93 and 100% with respect to control); and an increase of GST activity at 0.05, 0.5, 5 and 50 μg/L AZX (184, 197, 141 and 181 with respect to control) ($p < 0.05$, Fig. 2B and C) were observed.

3.5. Genotoxicity

While basal levels of MN where similar between juvenile and adult fish ($p > 0.05$, Fig. 3A), total NA and DI values were higher in adult fish ($p < 0.05$, Fig. 3B). On the other hand, *in vitro* C (+) for CA showed an increased DI respect to the C (−) ($p < 0.05$, Fig. 3C).

The three biomarkers employed to explore a possible DNA damage elicited by AZX on blood cells of *A. facetus* evidenced this effect (Fig. 4). The highest concentration of AZX (50 μg/L) increased MN frequency and DI both in juvenile and adult fish ($p < 0.05$, Fig. 3A and C) as well as total- and notched- NA in juveniles ($p < 0.05$, Fig. 3B and Table 1 Suppl. Data). Otherwise, total NA in adult fish showed a trend to increase but it was not significant ($p > 0.05$, Fig. 3B).

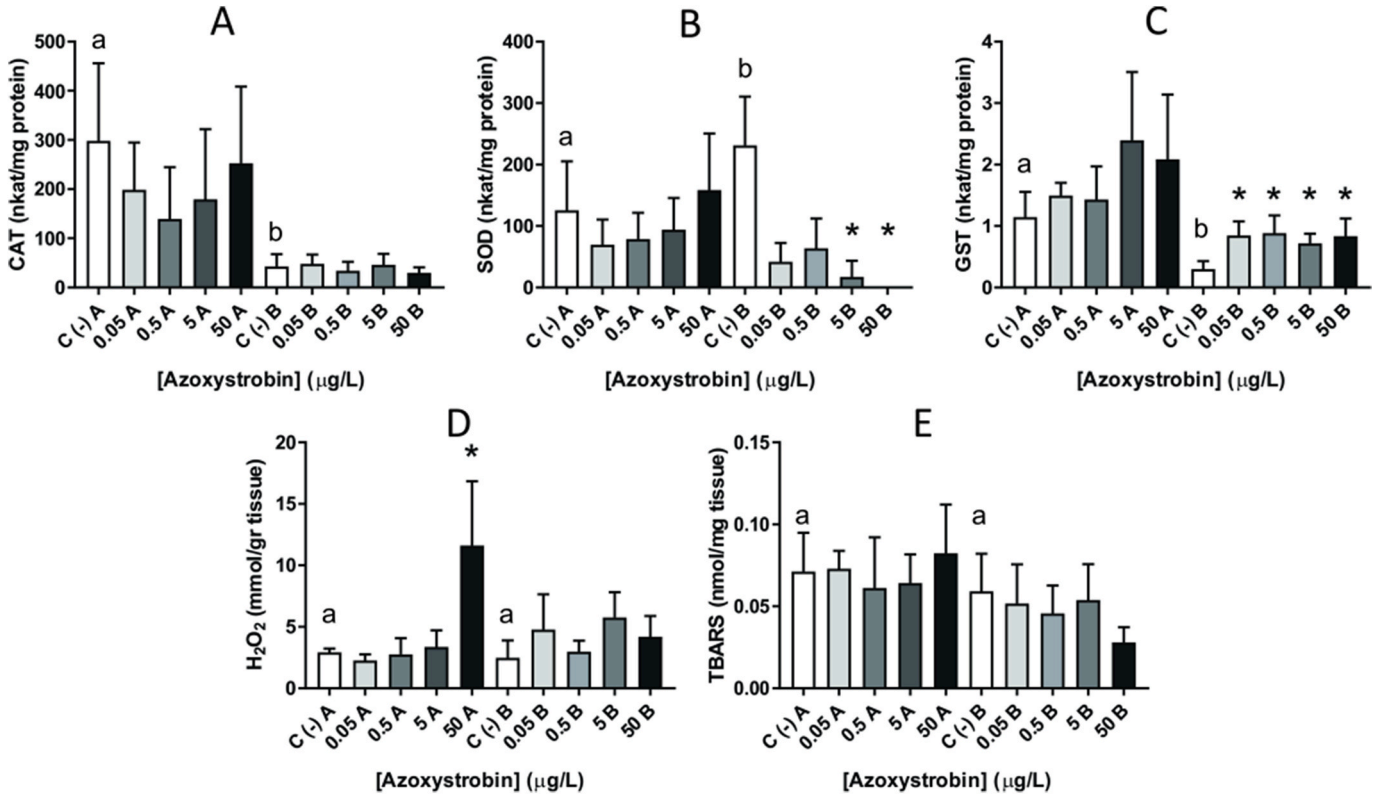

Fig. 2. Enzyme activities (nkat/mg protein), hydrogen peroxide (mmol/mg FW) and thiobarbituric acid reactive substances (nmol/mg FW) contents (mean ± SD) in gills of adults and juveniles of the freshwater fish Australoheros facetus exposed to azoxystrobin (AZX). A: Catalase activity (CAT), B: Superoxide dismutase activity (SOD), C: Glutathione- S- Transferase activity (GST), D: Hydrogen Peroxide (H_2O_2) content, E: Tiobarbituric acid reactive substances (TBARS) content. In the X axe: A: adult fishes, B: juvenile fishes. Asterisk (*) indicates significant difference with their respective controls (p value < 0.05). Different lowercase letters indicate differences in basal levels between adult and juvenile fish.

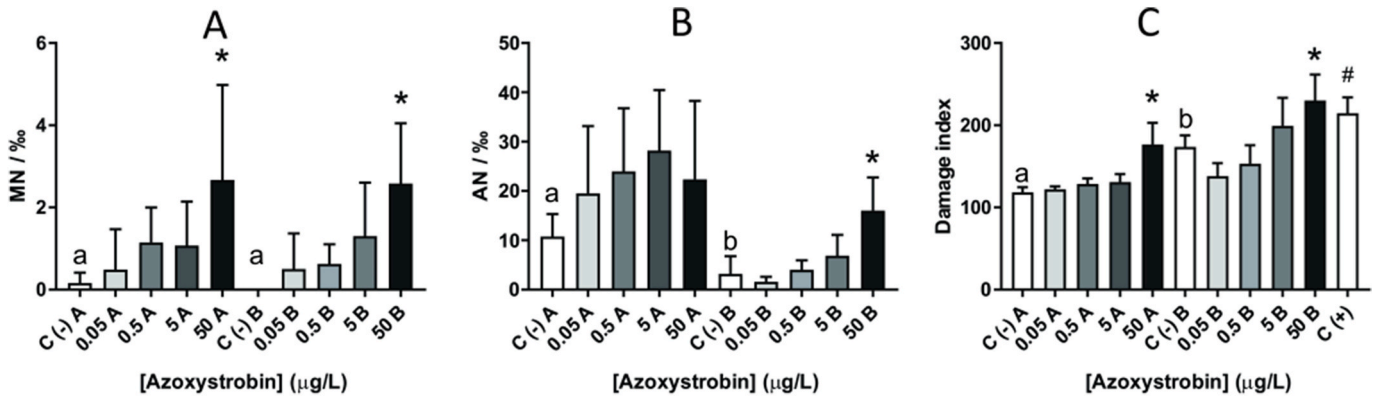

Fig. 3. Micronucleus (MN), total nuclear abnormalities (NA) frequencies and Damage Index (DI) (mean ± SD) in erythrocytes of adults and juveniles of the freshwater fish *Australoheros facetus* (n = 6) exposed to azoxystrobin. In the X axe A: adult fishes, B juvenile fishes. Asterisk (*) indicates significant difference with their respective controls (Co−) (p value < 0.05). Positive Control (C+): H_2O_2 50 μM. Different lowercase letters indicate differences in basal levels between adult and juvenile fish.

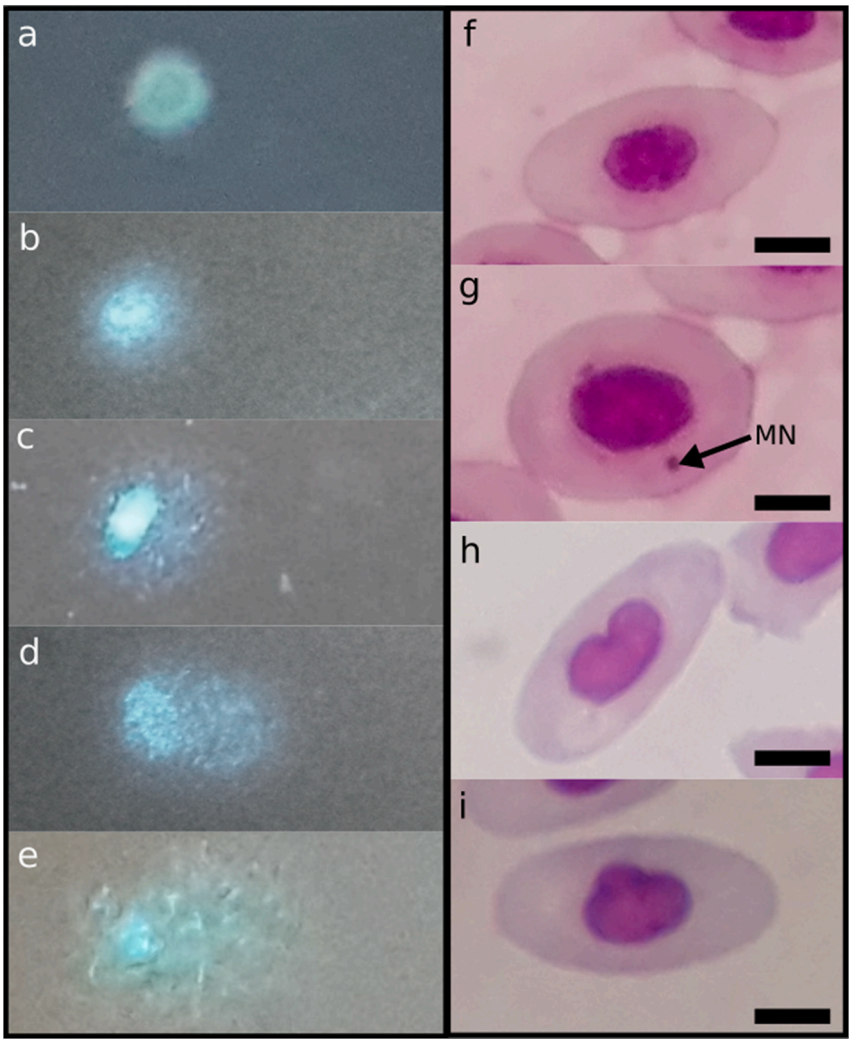

Fig. 4. Photomicrographs of peripheral erythrocytes of *Australoheros facetus* exposed to azoxystrobin (AZX), showing Comet Assay images (a–e) a: undamaged nucleoids, b: damage class 1, c: damage class 2, d: damage class 3, e: damage class 4; and cytogenetic abnormalities (f–i) f: normal nuclei, g: micronuclei (MN), h: notched nuclei, i: bud nuclei. Black bar size: 5 μm.

4. Discussion

The evaluation of a battery of biomarkers allowed us to detect two main effects, oxidative stress and DNA damage in *A. facetus*. Changes in antioxidant enzyme systems were observed, mainly in gills of juvenile fish even at the lower concentrations studied being plausible to be found in environment. On the other hand, the concentration of 50 μg/L AZX was genotoxic for both ontogenic stages (juvenile and adult fish), evidenced by both the MN and the CA.

The stability observed in the concentration of AZX for 48 h coincides with data evaluated in natural water bodies (Tomlin, 2000) and for different pH ranges (Singh, 2010).

4.1. Energy reserves and hematology

Taking into account the mechanism of action of AZX which block the electron transfer and consequently conduce to an ATP deficit and anaerobic glycolysis, changes in energy reserves such as increase in blood glucose levels and a decrease in liver glycogen content would be expected. These types of biomarkers generally show significant variations because of the metabolic costs associated with detoxification of damage repair but they are evident usually in organisms near to death (Campbell et al., 2003). In *A. facetus* no changes were observed after 48 h, probably due to this short term exposure and to the low concentrations used. These factors could also explain the hematological response observed. Other bioassays of acute exposure (96 h) have shown a significant increase in this kind of parameters including hematocrit and glucose at 352 μg/L AZX (Olsvik et al., 2010). These authors found a positive correlation between the insulin-like growth factor binding protein 1 (IGFBP1) liver transcripts and plasma glucose levels and concluded that AZX exposure mediated a hormone response through the glucocorticoid system (primary stress response). This mechanism is important for the understanding of AZX toxicity but it seems to take place at higher concentrations than those reported in the literature for freshwater ecosystems.

4.2. Oxidative stress

Oxidative stress in liver of adult fish exposed to AZX was evidenced by the significant increase of CAT activity and a peak of TBARS. CAT and SOD are two important components of the antioxidant defense system that scavenge the superoxide anions and hydrogen peroxide to protect the organism from oxidative stress (Han et al., 2016). Therefore, in *A. facetus*, CAT activity would keep stable H_20_2 levels but it would not be enough to avoid the oxidative damage observed at the higher concentration tested. Similarly, in juvenile grass carp *Ctenopharyngodon idella* exposed at 50–250 μg/L AZX during 48 h an increased CAT activity was observed (Liu et al., 2013). This behavior was also reported for CAT activity as well as for CAT expression in zebrafish subchronically exposed (7–21 days) to a range 10–100 μg/L AZX (Han et al., 2016; Cao et al., 2018). Concomitantly to the increase of CAT activity, the lipid peroxidation observed in *A. facetus* was previously reported in zebrafish exposed to 250 μg/L after 7 days (Jia et al., 2018). The results of H_2O_2 content suggest that other ROS like hydroxyl radicals ($OH^{\cdot}$) would participate in the oxidative damage observed in the liver (Han et al., 2016).

Taking into account that gills are in direct contact with waterborne pollutants, and considering the generation of excess ROS attributable to the MOA of this fungicide, a strong response of this organ was expected. In adult fish only a significant change of H_2O_2 content at 50 μg/L AZX was detected while in juveniles SOD activity was overwhelming inhibited in liver and gills even at the environmentally relevant lower concentrations tested. This lost of catalytic capacity can be due to the excess of radicals superoxide anions, as it was observed in *D. rerio* larvae in the range 0.1–100 μg/L AZX after 48 h (Jiang et al., 2018) and in the range 1–100 μg/L AZX for male adults after 7 days of exposure (Han et al., 2016). Then, because of the lack of activity of SOD in *A. facetus*, levels of H_2O_2 and CAT activity remained similar to control. The response of SOD is controversial since Cao et al. (2018) showed increased SOD activity from day 8 in adults zebrafish at 200 μg/L AZX and Han et al. (2016) reported its inhibition in zebrafish males from day 7 and its increase in females exposed to 1, 10 and 100 μg/L AZX. We hypothesized that the lack of response of SOD in adults of *A. facetus* could be due on one hand to the short exposure period used in our bioassay, and on the other hand to its low basal activity in comparison to juveniles. The activity of GST increased at all concentrations tested in gills of juveniles showing its antioxidant role as suggested Han et al. (2016) for *D. rerio* and Liu et al. (2015) for the green algae *Chlorella vulgaris*. In this sense, GST activity could conjugate glutathione to inactivate highly reactive aldehydes produced from lipid peroxyl radicals (West and Marnett, 2006) leading to maintenance of the basal levels of lipid peroxidation observed in gills of *A. facetus*. The potential role of GST in the bioatransformation of AZX would be discarded since in animals AZX is detoxifies through the conjugation with mercapturic acid instead of with glutathione (GSH) (Balba et al., 2007).

4.3. Genotoxicity

Previous assays in *A. facetus* have shown the feasibility of detecting DNA damage in its red cells when the fish were exposed *in vivo* to a known mutagenic agent as, for example, methyl metane-sulfonate (Crupkin et al., 2013; Iturburu et al., 2017), or *in vitro* to H_2O_2 (Iturburu et al., 2018). This species is also sensitive to xenobiotics like the organochlorine endosulfan (Crupkin et al., 2013) and the neonicotinoid imidacloprid (Iturburu et al., 2018). In the present study *A. facetus* demonstrate again to be a reliable model to evaluate genotoxic effects of other xenobiotics. Our results evidenced genotoxicity at 50 μg/L AZX in the acute exposure, a concentration previously demonstrated to cause DNA damage in fish, but at chronic and subchronic exposure times. Bony et al. (2010) detected DNA damage in liver and sperm cell of *D. rerio* (via CA) and in blood cells (using MN) from day 7 of exposure at 0.48 μg/L AZX. However, Han et al. (2016) have found genotoxicity in liver cells of both *D. rerio* males and females from 10 μg/L AZX at day 7 of exposure. Regarding other fish species, Bony et al. (2008) observed that specimens of brown trout (*Salmo trutta fario*) exposed at field conditions in cages located in a river with high levels of AZX showed an increased score of the CA in red cells. The same authors found that adult fishes of European topminnow (*Phoxinus phoxinus*) exposed in semi-field conditions to sediments with a mixture of diuron and AZX also showed an increase of DNA damage. Few reports have been presented about the genotoxicity of AZX in species which could inhabit the same ecosystems than *A. facetus*, with the only exception of studies in aquatic macrophytes. Pérez et al. (2019) found that after 48 h of exposure at 0.1 μg/L AZX, root cells of *Bidens laevis* showed an increase of the chromosomal aberration frequencies. Moreover, Garanzini and Menone (2015) observed DNA damage in the macrophyte *Myriophyllum quitense* exposed 24 h exposure at 100 μg/L AZX.

The sensitivity of the genotoxicity biomarkers has been discussed in several studies (Bolognesi and Cirillo, 2014). However, the discussion about the ability of the CA and the MN techniques to detect DNA damage is still open. In our case, both CA and MN test evidenced the genotoxic effect at the same AZX concentration, showing the same sensitivity. The total NA frequency increased in juvenile fish but not in adults could be due to the higher sensitivity common in early development stages. It is noteworthy that this response was only observed at the higher AZX concentration tested and for notched nuclei, a category of NA that is not yet clearly associated to genotoxicity in the literature. However, Bolognesi et al. (2006) and Bolognesi and Hayashi (2011) have previously associated notched nuclei to cytotoxicity.

4.4. General comparison of juvenile and adult fish

The comparison of biomarkers between juvenile and adult fish showed differences for oxidative stress but no for genotoxicity. In this sense, contrary to the high sensitivity expected in juveniles, both adults and juveniles showed similar sensitivity in terms of DNA damage.

4.4.1. Basal levels

Few studies have compared the antioxidant capacity during the growth of the fish.

Rudneva et al. (2010) showed different types of age-dependent responses of antioxidant enzymes including: 1. Enzymatic activity did not change with age; 2. enzymatic activity decreased with age and 3. enzymatic activity increased with age or varied unclearly. Considering the fish size as a factor that modulates physiologically basal levels of enzyme activity and that is able to influence the potential toxicity of AZX, we have been found the three behaviors described. For example, in liver of *A. facetus* basal CAT activity showed the pattern number 1 as it was previously reported in red blood cells of the fish *Neogobius melanostomus* (Rudneva et al., 2010) and in brain of albino rats (Yargicoglu et al., 1999). Basal SOD activity in liver and gills of *A. facetus* followed the type number 2, a behavior that was showed by Rudneva et al. (2010) in the fish species *Scorpaena porcus, Neogobius melanostomus*, *Mullus barbatus ponticus* and by Otto and Moon (1996) in *Oncorhynchus mykiss* and *Ameiurus melas*. This trend of decrease of enzymatic activity in erythrocytes and liver of old fish as compared with younger ones were associated with the accumulation of oxidative products and decrease of its defense abilities (Rudneva et al., 2010). Finally, basal GST activity in liver and gills of *A. facetus* followed the type number 3. Similar results showed the fishes *N. melanostomus* and *Spicara* (Rudneva et al., 2010) and in previous studies from our laboratory in *A. facetus* (Crupkin, 2013). In this case GST activity could be explained by its physiological role in biotransformation. In spite of the above mentioned examples, all these patterns can be found for all of the antioxidant enzyme systems and result specie-specific.

4.4.2. Response to AZX

In general terms, the oxidative stress biomarker responses were observed in adults at the higher AZX tested, while all the responses detected in juveniles took place at low AZX concentrations, showing the higher sensitivity of young fish. Age and maturation could influence the responses of the biomarkers as observed in this work. Adult fish of *A. facetus* exposed to AZX showed significant responses at 50 μg/L for CAT and TBARS, but juvenile fish did not show changes in these biomarkers.

In comparison to adult fish, in juveniles of *A. facetus* the enzymes showed other behavior. Only the gills of the juvenile fish evidenced a significant increase activity of GST at all AZX concentration tested and significant inhibition of SOD activity. These effects indicate an oxidative disturbance, since this tissue is the first to contact waterborne chemical compounds. Oxidative damage in gills could impair important physiology processes in the tissue, including osmoregulation, gas exchange, and excretion, and decrease the fitness of the fish (Baldisserotto, 2013; dos Santos et al., 2016). The sensitivity showed by gills in juveniles of *A. facetus* constitutes, as far as we know, the first report about AZX toxicity in this organ and should be taken into account.

5. Conclusion

Based on the results obtained in the present work, we can conclude that AZX cause oxidative stress in the freshwater fish *A. facetus* even at low concentrations found in realistic scenarios. In this sense, this negative effect was evident at concentration below the value 3.3 μg/L AZX defined by EFSA as a regulatory acceptable concentration (RAC) (Rodriguez et al., 2013). Genotoxicity, on the other hand, was also an important effect observed but at high environmentally relevant levels. While this effect occurred at a concentration above the risk value, it should not be underestimated because it is well known that DNA damage contributes to chromosome instability, inflammation, and precedes illnesses like cancer among other chronic ones (Guo et al., 2019).

Differences between juvenile and adult fish indicate that a strict selection of fishes that should belong to the same developmental stage is recommended, for bioassays as well as in biomonitoring studies in which biomarkers will be analyzed.

Author contributions

Andrea C. Crupkin- Conceptualization, Investigation, Formal analysis, Methodology, Writing - original draft (+Review), Visualization. Ariana B. Fulvi- Investigation, Methodology, Formal analysis, Visualization. Fernando G. Iturburu- Investigation, Methodology, Formal analysis, Writing Original Draft, Visualization. Sandra Medici- Investigation, Methodology. Julieta Mendieta- Investigation, Methodology. Ana M. Panzeri- Investigation, Methodology. Mirta L. Menone- Conceptualization, Investigation, Writing - original draft (+Review), Visualization, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111286.

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