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Cypermethrin induction of DNA damage and oxidative stress in zebrafish gill cells

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ABSTRACT

Cypermethrin (CYP) is a synthetic pyrethroid insecticide, used to control pests in domestic, industrial and agricultural environments. According to recent reports, it is one of the most common contaminants in freshwater aquatic systems. The aim of this study was to evaluate its potential genotoxic effect and the activation of the superoxide dismutase (SOD) and catalase (CAT) systems of adult zebrafish gill cells after in vivo exposure. The comet assay (CA) demonstrated that gill cells are sensitive to DNA damage after in vitro exposure to hydrogen peroxide (H_2O_2), showing a dose-dependent response. We also found an increase in DNA damage of gill cells following a dose- and time-dependent treatment with CYP. Moreover, it was verified that SOD and CAT activities significantly increased after exposure to 0.6 µg/L CYP, both during six and nine days. The same treatment caused a significant up-regulation of the mRNA levels of *Mn-sod* and *cat* genes. These data indicate that CYP causes gill cell's DNA damage and oxidative stress, modifying the activities of the enzymes responsible for maintaining ROS balance, as well as in their corresponding gene expression levels.

1. Introduction

Due to rapid and profound changes in agricultural practices, the application of large amounts of pesticides to control pests and the use of synthetic fertilizers to increase crop yields, freshwater resources are polluted and becoming undrinkable, thus affecting both the terrestrial and aquatic fauna, and decreasing its value for irrigation. Synthetic pyrethroids have been largely used by farmers due to their lower mammalian toxicity when compared to organophosphorus and organochlorine pesticides (Kannan et al., 2014). Unfortunately, application of these synthetic pyrethrin derivatives is highly toxic to non-target organisms as bees, frogs, freshwater fish and other aquatic organisms even at very low concentrations (Begum, 2005; Oudou et al., 2004). Among these, fish are highly sensitive to pyrethroid pesticides due to their neurotoxic effects and lethality produced at concentrations much lower than the corresponding values for mammals and birds (Koprucu and Aydin, 2004).

Cypermethrin, alpha-cyano-3-phenoxybenzyl ester of 2, 2-dimethyl-3-(2,2-dichlorovinyl) cyclopropane carboxylic acid, a widely used Type II pyrethroid pesticide, is one of the most common contaminants in freshwater aquatic systems (Carriquiriborde et al., 2007; Marino and Ronco, 2005), reaching levels up to 3 g/L in surface water (Jaensson et al., 2007).

The gills are perhaps the fish organs most directly exposed to the aqueous medium, and therefore, targets on which a straighter incidence of the presence of CYP would be expected (Wendelaar Bonga, 1997). Due to their lipophilicity, pyrethroids easily permeate through the membrane, contributing to fish sensitivity to aqueous pyrethroid exposures (Mishra et al., 2005). Therefore, gill cells are supposed to be very suitable for the evaluation of genotoxic effects of water contaminants (Sharma et al., 2007).

Reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are continuously formed in oxygen consuming organisms (Jin et al., 2010). Exposure to xenobiotics or toxic chemical pollutants may produce an imbalance between endogenous and exogenous ROS, and can subsequently induce a decrease in antioxidant defense or cause oxidative damage outright in organisms (Valavanidis et al., 2006). Despite the potential danger of ROS, cells

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exhibit a variety of defense mechanisms to neutralize the harmful effects of free radicals. The antioxidant defense system includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST) and other low molecular weight scavengers such as glutathione (GSH) (Droge, 2002). For several years, measurements of antioxidative enzyme activities in fish have been used to assess the oxidative damage caused by chemicals in aquatic ecosystems (Zhang et al., 2009; Stara et al., 2013; Taju et al., 2014; Arslan et al., 2017).

The in vivo exposure of zebrafish to CYP, to environmentally relevant concentrations, promotes differential activation of antioxidant defense systems against superoxide production and can cause DNA damage of the gill cells. In the present work, a study combining DNA damage, activity and gene expression of antioxidant enzymes was used to elucidate the potential mechanism of CYP toxicity on gill cells of an adult zebrafish in vivo bioassay.

2. Material and methods

2.1. Chemicals

Cypermethrin, commercial formulation SHERPA®, 25% CYP as active ingredient, was used. The stock solution was prepared by dissolving this formulation in 0.1% acetone (Cicarelli®, BA, Argentina). The stock solution (2.4 μg/L CYP) was stored in dark at 4 °C. H_2O_2 was purchased from Cicarelli®, (BA, Argentina). RPMI-1640 medium was purchased from HyClone (Thermo Scientific, Logan, UT). Dimethyl sulphoxide (DMSO), low-melting-point agarose (LMPA), acridine orange, and other reagents for CA, as well as general laboratory chemicals were provided by Sigma-Aldrich® (St. Louis, MO).

2.2. Animals

Eight-month-old zebrafish from our laboratory stock (produced by brood fish) were used in all the experiments. Fish were acclimatized for 48 h in glass tanks with dechlorinated tap water at 26.5 ± 1 °C, with a photoperiod of 14 h light/10 h dark (Westerfield, 2000). The physicochemical parameters of water quality are shown in Table 1. They were fed twice daily with flake food (TetraMin®, Germany) and once daily with brine shrimp. Fish were sacrificed by cooling in ice, and gills were extracted and processed as described below for CA studies and oxidative stress determination. This study was approved by the Institutional Animal Care and Use Committee of the National University of Entre Rios and the Italian University Institute of Rosario (Rosario, Argentina) (Animal Study Protocol N° 028/12).

2.3. In vivo CYP bioassay

In vivo experiments were carried out by triplicate of n = 15 fish per treatment (each of the 5 fish). Zebrafish were exposed to the CYP at concentrations of 0.3 μg/L or 0.6 μg/L for 3, 6, 9 or 12 days, in water containing 0.0125% and 0.025% acetone (v/v) respectively, with daily changes of the solution. Control animals for 0.3 μg/L were exposed to water containing 0.0125% acetone (v/v), whereas for 0.6 μg/L treatment the concentration was 0.025% acetone (v/v). The CYP concentrations were based on reported concentrations in aquatic environmental systems (Jergentz et al., 2005; Jin et al., 2011). Under normal environmental temperatures and pH, CYP is stable to hydrolysis, (half-life > 50 days), and to photodegradation, (half-life > 100 days) (EXTOXNET, 2018).

Table 1
Bioassay water quality.

pH	Hardness (mg/L $CaCO_3$)	Conductivity (μS/cm^2)	Oxygen concentration (mg/L)
6.8–7.5	50–100	410–440	5.5–6.2

2.4. Epithelial gill cells extraction

Gills from both sides of each specimen were excised, filaments sliced from arches, and washed two times with Ca^{2+} and Mg^{2+}-free PBS to remove blood cells. Following, samples were dipped in tubes containing 1 mL of PBS and 1 mL 0.05 trypsin, and softly dissociated for approximately 2–3 min. The supernatant containing the isolated cells was transferred to a second tube with 3 mL of PBS and centrifuged at 45*g* for 5 min (Hayashi et al., 1998). After removing most of the supernatant, 50 μL of the cell suspension were diluted with 950 μL of RPMI for in vitro exposure, or directly used for CA application in the case of in vivo exposure.

2.5. Comet assay

Isolated gill cells were washed with 25 μL PBS, and gently disrupted with micropipette tips. Gill cells suspensions were diluted with RPMI-1640, 1:19 (v/v) for in vitro exposure or used directly for the CA after in vivo exposure to CYP.

Before running comet assay, cell viability was determined. Briefly, suspensions were mixed with a working solution of acridine orange and ethidium bromide (EB) 1:1, prepared in Ca^{2+} and Mg^{2+}-free PBS, smeared on slides and examined under a fluorescence microscope (40 ×). One hundred cells were randomly counted per sample, and the percentage of viable cells was determined (Mercille and Massie, 1994). Gill cells from in vitro and in vivo exposures were used for CA as follows: 50 μL of cell suspensions were mixed with 200 μL 1% LMPA, two slides per sample were prepared and immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1% Triton X-100, 10% DMSO; pH = 10) for 24 h. Next, slides were incubated in fresh alkaline solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13) for 10 min, electrophoresed 10 min at 300 mA and 25 V (0.70 V/cm) and later neutralized (0.4 M trizma base, pH = 7.5). Finally, samples were dehydrated in ethanol and left to dry (Simoniello et al., 2009). Lysis, unwinding, and electrophoresis were conducted at 4 °C and the preparations were kept in the dark to avoid damage caused by direct light exposure on DNA. All samples were coded for 'blind' analysis, stained with EB (2 μg/mL), and comet images of 100 randomly selected nucleoids (50 from each of two replicated slides) were scored from each sample under a fluorescence microscope. Comet images were analyzed and registered using a monochromatic, refrigerated Apogee CCD camera (Andor, Belfast, UK), coupled to an epifluorescence Olympus BX50 microscope (Olympus, Japan). Nucleoids were classified into five classes according to tail size and intensity (from no damage: class 0, to maximum damage: class 4), resulting in a single DNA damage score (Damage Index, DI) for each animal or sample. Comet cell profiles with small or nonexistent head or extremely prominent and diffuse tail were not scored, since these could be apoptotic or necrotic cells (Fairbairn et al., 1995). The DI was calculated as follows:

DI = n1 + 2n2 + 3n3 + 4n4 where: n1, n2, n3 and n4 are the numbers of nucleoids in classes 1, 2, 3 and 4, respectively (Dusinska and Collins, 2008; Gedik et al., 1992).

Twenty zebrafish were used to determine DNA damage basal values and to standardize the assay conditions using H_2O_2 as known genotoxic agent. Immediately after the acclimation period, fish were sacrificed, and gill cells obtained as previously described. Half of the samples were used for basal value determinations and the remaining for the standardization method, as follows: cell suspensions were made to 1:19 (v/v) with RPMI and then exposed to H_2O_2 (2.5; 5.0 and 10.0 μM) for 10 min at 25 °C (Simoniello et al., 2009; de Miranda Cabral Gontijo et al., 2003; Paravani et al., 2018). Control assays were carried out in water. At the end of exposure, tubes were centrifuged at 400*g* for 10 min and cells used for the CA.

2.6. Enzymatic activity assay

Gill were homogenized in 50 µL PBS and centrifuged. The supernatants were collected and used to determine SOD and CAT activities. SOD activity was measured with a spectrophotometer at 550 nm (Misra and Fridovich, 1976). The reaction mixture contained 50 mM sodium carbonate dissolved in 50 mM K, Na-phosphate buffer (pH = 7.8, at 25 °C); 0.1 mM EDTA; 0.1 mM xanthine and 25 µM nitro blue tetrazolium chloride. The activity of SOD was calculated according to a standard curve and expressed as U/mg protein (Zhang et al., 2004). CAT activity was determined by recording the absorbance of the generated stable chromophore at 405 nm due to H_2O_2 consumption, according to Aebi's method (Aebi, 1984).

2.7. Gene expression analysis

Total-RNA was isolated from gills using the GeneJET RNA Purification Kit (Thermo Scientific®) following the manufacturer's instructions. The ratio of absorbance at 260 nm–280 nm was used to verify the quality of the RNA in each sample. Subsequently, RNAs were denatured at 65 °C for 15 min. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®) was used for cDNA synthesis. RT products were used directly for polymerase chain reaction (PCR), using an Ivema T-18 thermo-cycler (Llavallol, BA, Argentina) with the following program: denaturation, 10 min, 95 °C; 30 cycles of 1 min at 95 °C, 90 s at 50 °C, and 90 s at 72 °C. Oligonucleotide primers (Jin et al., 2010) were used to detect the expression of β-actin, *Mn-sod* (Genbank AY 195857), and *cat* (Genbank AF 170069), as can be seen in Table 2. The mRNA expression levels were standardized, using *β-actin* transcripts (Genbank AF057040). The *Mn-sod* and *cat* mRNA levels were expressed regarding to the *β-actin* mRNA (Jin et al., 2008; Jin et al., 2011; Paravani et al., 2018). The mRNAs levels were established by co-amplification of each interest gene with *β-actin* mRNA, using the RNA extracted from gill cells as template (treatment and control fish), and visualized in ethidium bromide-stained 2.5% agarose gel. The cDNA bands were visualized by a UV transilluminator (Spectroline TE-312S, Spectronics Corporation, Westbury, NY) and registered using a digital camera. Bands intensity of each row, internal control and target gene, were quantified by ImageJ software and intensity ratios were calculated. The control group value was considered as 1, whereas the treatment group values were analyzed regarding to control and plotted on a bar diagram.

2.8. Statistical analysis

The statistical analysis was performed using SPSS 17.0 software (SPSS Inc. Chicago, USA). The normal distribution of the data was analyzed with the Shapiro-Wilk's test and the homogeneity of variances between groups was verified by the Levene's test. The in vitro assays were analyzed using a one-way ANOVA test, followed by Dunnett's test. The DNA damage index (DI) between control and CYP treated fish was statistically analyzed using two-way ANOVA, followed by Tukey's post hoc test. The two concentrations and the four exposure times were considered grouping variables. The gene expression levels were expressed as mean ± standard error (SE). The differences of $p \leq 0.05$ were considered statistically significant whereas $p \leq 0.01$ were considered highly significant.

Table 3
Damage Index (DI) values in gill cells of zebrafish after in vitro exposure to H_2O_2. Values significantly different from the control are indicated by asterisks (** $p < 0.01$).

	DI (mean ± SE)
Control	171.2 ± 1.8
Treatment (µM H_2O_2)	
2.5	208,4 ± 6.1**
5.0	257.3 ± 5.2**
10.0	271.7 ± 8.3**

3. Results

3.1. In vitro H_2O_2 assay

Cell viability obtained by fluorescent DNA-binging dye method was in the range of 90 – 95% for all samples. The DI of the in vitro assay shows significant dose-dependent increase values in samples exposed to H_2O_2, at all concentrations tested (Table 3) compared to the untreated control.

3.2. In vivo CYP bioassay

No fish mortality was verified in any of the in vivo experimental treatments performed. Gill cells from fish exposed to 0.3 µg/L CYP exhibited very significant differences in DI at all times of exposure, regarding to the respective controls (Supplementary Fig. S1). The analysis of two-way ANOVA yielded a significant interaction (Table 4, Supplementary Material). Additionally, the comparison between groups, at different times, shows very significant differences, except between 9 versus 12 days, where there are just significant differences and 6 regarding to 9 days where there are statistically no significant differences (Fig. 1). In terms of percentages, the group exposed for 3 days showed an increase of 47.86%, being of 67.37% at 6 days; 67.48% at 9 days and, finally, there was an increase of 71.97% at 12 days, compared with to their respective controls.

On the other hand, the gill cells of fish exposed to 0.6 µg/L CYP, showed higher DI values regarding to the respective controls, with highly significant differences in all cases (Fig. 1). From the analysis between the groups, it was observed that fish exposed for 12 days showed very significant difference compared with all groups ($p < 0.01$), except for fish exposed for 9 days, where the difference was less significant ($p < 0.05$). Similar results were observed between fish exposed to 6 days and those exposed to 9 days. While no significant differences were observed during the comparison between fish exposed at 3 and 6 days. In terms of percentage, the gill cells showed an increase in DI values, also after the 3 days of exposure, following a time-dependent behavior regarding to the controls: 3 days 67.66%; 6 days 76.58%; 9 days 83.10% and 12 days 90.02%.

Table 2
Sequences of primer pairs used in the PCR reactions.

Target gene	Accession no.	Primer sequences (from 5´ to 3´)	Product length (bp)
Mn-Sod	AY195857	F: 5′-CCGGACTATGTTAAGGCCATCT-3′ R: 5′-ACACTCGGTTGCTCTCTTTTCTCT-3′	123
Cat	AF170069	F: 5′-AGGGCAACTGGGATCTTACA-3′ R: 5′-TTTATGGGACCAGACCTTGG-3′	499
β-actin	AF057040	F: 5′-ATGGATGAGGAAATCGCTGCC-3′ R: 5′-CTCCCTGATGTCTGGGTCGTC-3′	106

Table 4
Two-way ANOVA analysis using exposition time and CYP concentration, as grouping variables (Tukey Test).

Concentration within 3 d			
Comparison	**Diff of Means**	**P**	**P < 0,05**
0.6 µg/L vs. Control	104,533	< 0001	Yes
0.6 µg/L vs. 0.3 µg/L	30,467	< 0001	Yes
0.3 µg/L vs. Control	74,067	< 0001	Yes
Concentration within 6 d			
Comparison	**Diff of Means**	**P**	**P < 0,05**
0.6 µg/L vs. Control	115,933	< 0001	Yes
0.6 µg/L vs. 0.3 µg/L	14,267	< 0001	Yes
0.3 µg/L vs. Control	101,667	< 0001	Yes
Concentration within 9 d			
Comparison	**Diff of Means**	**P**	**P < 0,05**
0.6 µg/L vs. Control	128,267	< 0001	Yes
0.6 µg/L vs. 0.3 µg/L	24,467	< 0001	Yes
0.3 µg/L vs. Control	103,800	< 0001	Yes
Concentration within 12 d			
Comparison	**Diff of Means**	**P**	**P < 0,05**
0.6 µg/L vs. Control	142,333	< 0001	Yes
0.6 µg/L vs. 0.3 µg/L	28,533	< 0001	Yes
0.3 µg/L vs. Control	113,800	< 0001	Yes
Time within 0.3 µg/L CYP			
Comparison	**Diff of Means**	**P**	**P < 0,05**
12 d vs. 3 d	43,667	< 0001	Yes
12 d vs. 6 d	18,067	< 0001	Yes
12 d vs. 9 d	12,800	< 0001	Yes
9 d vs. 3 d	30,867	< 0001	Yes
9 d vs. 6 d	5267	0032	Yes
6 d vs. 3 d	25,600	< 0001	Yes
Time within 0.6 µg/L CYP			
Comparison	**Diff of Means**	**P**	**P < 0,05**
12 d vs. 3 d	41,733	< 0001	Yes
12 d vs. 6 d	32,333	< 0001	Yes
12 d vs. 9 d	16,867	< 0001	Yes
9 d vs. 3 d	24,867	< 0001	Yes
9 d vs. 6 d	15,467	< 0001	Yes
6 d vs. 3 d	9400	< 0001	Yes

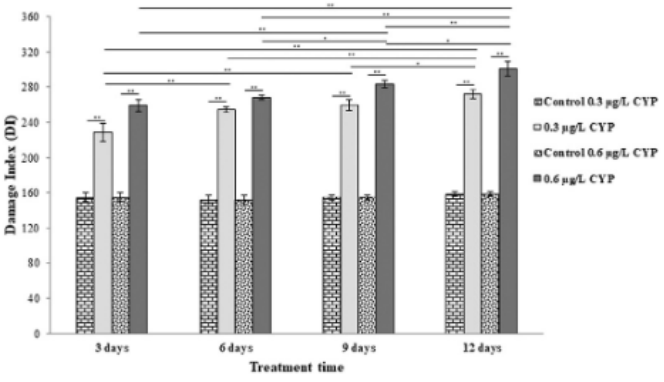

Fig. 1. DNA DI values in zebrafish gill cells exposed to CYP at 0.3 µg/L and 0.6 µg/L. Statistically significant differences are indicated by asterisks (* $p < 0.05$ and ** $p < 0.01$).

3.3. Effects of CYP on antioxidant enzyme activities

The enzymatic activity of SOD, in adult zebrafish exposed to 0.3 µg/L CYP and 0.6 µg/L CYP (Fig. 2A) showed a similar behavior. At 3 days of exposure, no significant differences were observed regarding to the control, while from 6 days of exposure and onward, the differences were very significant regarding the controls at all times studied. In relation to the different treatment times, all showed highly significant differences among each other. In terms of percentage, gill cells from fish exposed to 0.3 µg/L CYP showed an increase range from 3.5% at 3 days to 72.3% at 12 days of treatment respect to the controls, while in those exposed to 0.6 µg/L CYP, there was a remarkable increase range from 4.7% at 3 days to 177.8% at 12 days.

The enzymatic activity of CAT in gill cells of fish exposed to 0.3 µg/L of CYP at 3 days did not show significant differences respect to the control. From 6 days of treatment, the differences became very significant in all times studied. For the comparison between the different treatment times, all showed highly significant differences except those recorded between 9 and 12 days (Fig. 2B). In terms of percentage, gill cells showed moderate increases in enzymatic activity with respect to their controls after 6 days of exposure (25.2%), reaching values of 37.9% at 12 days. The enzymatic activity of CAT in the gill cells of fish exposed to 0.6 µg/L of CYP showed very significant increases over the respective untreated controls. From the analysis of different times of exposure, there were significant differences among all groups (Fig. 2B). In terms of percentage, gill cells showed that the increases in CAT activity ranged from 30.5% at 3 days, to 154.5% at 12 days of treatment, compared against the controls.

3.4. Effects of CYP on Mn-sod and cat gene expression

Based on the variations observed in the enzymatic activities of SOD and CAT, the gene expression analysis of both enzymes was performed in the gill cells of zebrafish exposed to 0.6 µg/L CYP, at 3 and 12 days. The gene expression levels showed that mRNA-*cat* increased significantly after 3 days of exposure, but no effect was observed on the mRNA-*Mn-sod* levels against the corresponding controls. In relation to *cat* and *Mn-sod* gene expression levels in fish exposed to 0.6 µg/L CYP at 12 days, very significant increases were observed, being this increment higher for *Mn-sod* than for *cat* (Fig. 3).

4. Discussion

Pesticides can be counted among the most important xenobiotics when the contamination processes of fresh water bodies are evaluated. The use of different fish species to map the fraction of bioavailable pollution in an aquatic ecosystem is due to the fact that these vertebrates participate in different levels of the trophic chain, react with low concentrations of toxic substances, and can accumulate harmful compounds directly from contaminated water and, indirectly, by feeding on contaminated aquatic organisms (Cavaş and Ergene-Gözükara, 2005).

It is widely accepted that CYP is extremely toxic to fish and aquatic arthropods under laboratory conditions. Its toxicity in fish varies depending on the species (Bradbury and Coats, 1989; Das and Mukherjee, 2003; Yılmaz et al., 2004; DeMicco et al., 2010). The high toxicity of pyrethroids in fish has been attributed to a combination of three factors: a sensitive central nervous system, a rather slow hydrolytic detoxification, and the route of internalization after exposure (i.e. direct absorption via the gills into the blood stream). Further, pyrethroids may also secondarily induce osmotic imbalance that contributes to its toxicity (Saha and Kaviraj, 2008).

In a recent work carried out on zebrafish, our group demonstrated very significant changes in retina structure layers, alteration in the expression of two proteins involved in DNA repair pathways and cellular apoptosis (γ-H2AX and caspase-3), DNA damage, as well as alteration in the activity, and gene expression of antioxidant enzymes. These results allowed us to show the genotoxic effect and the induction of oxidative stress on retinal cells of adult zebrafish exposed to environmental concentrations of CYP (Paravani et al., 2018).

Due to their lipophilic character, pyrethroids insecticides have a high rate of gill absorption even at low concentrations, which leads to fish high sensitivity at aqueous pyrethroid exposures. This occurs, because they are unable to efficiently metabolize the pyrethroids a fact that affects the transport rates and enzyme activities (Viran et al., 2003).

In this work, prior to in vivo evaluation of possible damage to DNA in gill cells subjected to CYP exposure, a series of controls were

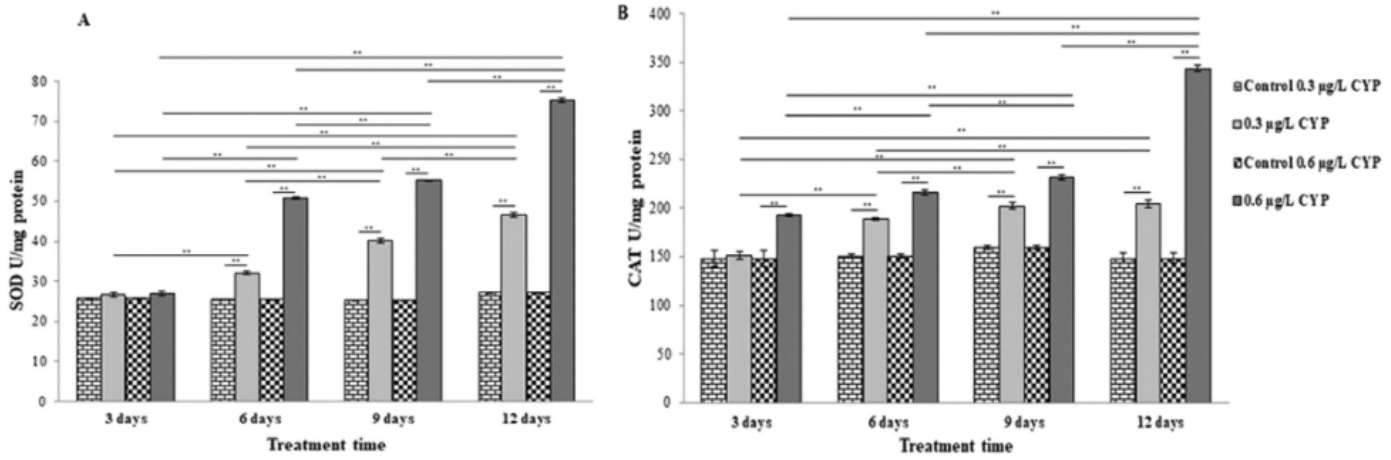

Fig. 2. SOD (A) and CAT (B) enzymatic activities in the gill cells of adult zebrafish exposed to CYP. Statistically significant differences are indicated by asterisks (** $p < 0.01$).

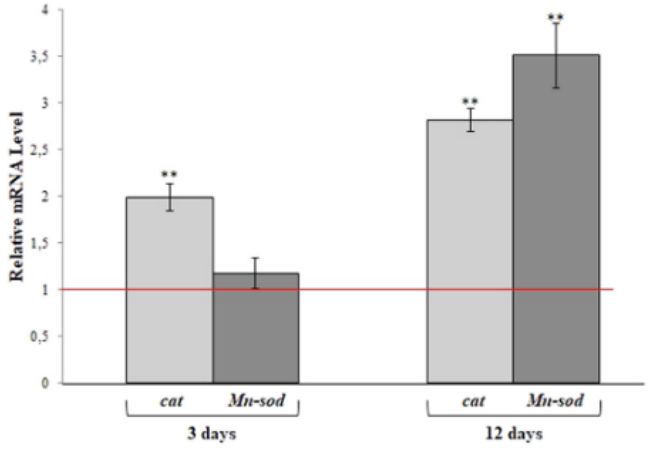

Fig. 3. Gene expression levels of *cat* and *Mn-sod* mRNAs in the gill cells of zebrafish exposed to 0.6 µg/L CYP. Values were normalized against *β-actin* (used as a house-keeping gene), and represent the mean mRNA expression value ± SE relative to those of the controls. *cat*: Catalase; *Mn-sod*: Superoxide Dismutase. The asterisk represents a statistically significant difference when compared with the control (** $p < 0.01$).

performed to standardize the CA technique and to obtain reference values for both the damage index and the sensitivity to oxidative stress. In relation to the sensitivity to oxidative damage caused by H_2O_2 (in vitro assay), incremental values of 22.2% and 58.6% were verified for gill cells exposed to 2.5 µM and 10.0 µM of H_2O_2 during 10 min, regarding to the control group. Comparing these data with the in vitro studies in retinal cells of adult zebrafish under similar experimental conditions (Paravani et al., 2018), gill cells are more sensitive than retinal cells throughout the study. Also, analyzing these data obtained in gill cells of *P. lineatus* (Poletta et al., 2013) under similar experimental conditions, but employing higher H_2O_2 concentrations (25 µM and 50 µM) and longer exposure times, it was observed that the DI are also dependent on the concentration of H_2O_2, resulting in increments of 36% and 108% in relation to the control group. Because the H_2O_2 concentrations and exposure time used here, are below those used in a previous study, adult zebrafish gill cells seem to be more sensitive than the *Prochilodus lineatu*. Similarly, de Miranda Cabral Gontijo et al. (2003) reported concentration dependency over the DI of *Nile tilapia* erythrocytes exposed in vitro to 1.0 – 100.0 µM H_2O_2 for 5 min. Since the DI observed in our study are greater than those found in *Nile tilapia* erythrocytes exposed to the same concentration and exposure time, zebrafish gill cells can be used to evaluate the oxidative damage caused by H_2O_2, being a suitable cell type for the application of the CA in biomonitoring of oxidative damage in aquatic environments.

According to Mitchelmore and Chipman (1998), DNA strand breaks, particularly measured by the comet assay, can be considered as a biomarker of genotoxicity in fish and other aquatic species, but this approach should be combined with the use of complementary studies. In this sense, in our zebrafish CYP bioassay, the gill cells showed very significant values of DNA damage index following dose-dependent and time-dependent patterns. Our results are like those found for *P. lineatus* (Poletta et al., 2013). One possible factor accounting for the genotoxic effects of CYP is that, because of its small size and hydrophobicity, it can easily cross cell membranes, reaching and interacting with DNA through its acid moieties (Saxena et al., 2005). Thus, this binding to DNA could lead to structure destabilization and duplex unwinding, inducing chromosomal damage.

Oxidative stress occurs when there is an imbalance in the biological oxidant-to-antioxidant radio, and can trigger oxidative damage to lipid, proteins, carbohydrates, and nucleic acids. In general, the abnormal ROS generation, which can significantly damage the cell structure, is considered an important signal of oxidative damage (Barzilai and Yamamoto, 2004). Organisms possess self-defense systems for protecting against the damaging effects of ROS. Therefore, examining the activity changes of antioxidant enzymes such as SOD and CAT, can become a method for assessing oxidative stress. However, up to now there are only few studies demonstrating that CYP can induce oxidative stress in different organisms. Between these, Giray et al. (2001) have reported that either single or repeated oral administration of CYP produced significant oxidative stress in rat brain and liver. In the same way, Jin and coworkers, have reported that CYP induces oxidative stress affecting several zebrafish liver processes (Jin et al., 2011).

In this study, the activity of SOD and CAT enzymes were induced significantly with both CYP concentrations after 6 days of exposure, following a dose- and time-dependent behavior. Comparing our previous results obtained with the retinal cells of adult zebrafish (Paravani et al., 2018), it can be observed that the activities of both enzymes turn out to be more sensitive in the gill cells than in the retinal cells, exposed to the same concentrations and exposure times. In addition, in the gill cells, the activity of SOD exhibits significant differences with a lower concentration of CYP, compared to that observed in retinal cells. As previously indicated, these results are consistent with the fact that gill cells are directly and constantly exposed to environmental contaminants in the water, therefore gill cells are very suitable for the evaluation of genotoxic effects of water contaminants.

Furthermore, the analysis of the gene expression of SOD and CAT has been also used to detect toxicity and to monitor the effects of chemical pollutants (Sheader et al., 2006; Woo et al., 2009). In this

study we have demonstrated that the mRNA levels of these genes were significantly altered in the gill cells of CYP treated adult zebrafish, suggesting that the SOD and CAT pathways were directly involved as a response to oxidative stress caused by CYP exposure. Thus, these results are consistent with those found in hepatocytes (Jin et al., 2011), and in retinal cells (Paravani et al., 2018) in adult zebrafish, altering mRNA levels of the antioxidant enzymes SOD and CAT, after exposure of CYP.

To our knowledge, this is the first study that combines biochemical and molecular techniques to elucidate the effect of CYP on gill cells, under environmentally relevant concentrations. The results obtained show that contamination with CYP can become dangerous for aquatic ecosystems and this fact must be taken into consideration when this insecticide is used in agriculture. The behavior of the enzymes responsible for the elimination of ROS, as well as the changes in their gene expression levels, make it possible to postulate that CYP triggers processes of oxidative stress in gill cells. However, as it was previously discussed, comet assay does not rule out that CYP can interact directly with DNA molecules, causing their rupture and in some cases, apoptotic cell death.

5. Conclusion

This study shows that gill cells of zebrafish are an extremely sensitive organ for the comet assay method, demonstrating DNA damage in response to oxidative agents, such as H_2O_2 and to the insecticide CYP. We have demonstrated that enzymatic activity and the mRNA levels of *Mn-sod* and *cat* genes were significantly altered in the gill cells of CYP-treated zebrafish, which allows us to postulate that the combination of biochemist and molecular techniques is very appropriate for the determination of oxidative disruption effects on zebrafish gill cells. In addition to previous studies of our group, this work allows us to confirm new mechanisms of CYP induced toxicity in fish and other aquatic vertebrates.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.02.004.

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