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Comet assay in gill cells of *Prochilodus lineatus* exposed *in vivo* to cypermethrin

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ABSTRACT

Agricultural chemicals can induce genetic alterations on aquatic organisms that have been associated with effects on growth, reproduction and population dynamics. The evaluation of DNA damage in fish using the comet assay (CA) frequently involves the utilization of erythrocytes. However, epithelial gill cells (EGC) can be more sensitive, as they are constantly dividing and in direct contact with potentially stressing compounds from the aquatic environment. The aim of the present study was to evaluate (1) the sensitivity and suitability of epithelial gill cells of *Prochilodus lineatus* in response to different genotoxic agents through the application of the CA, (2) the induction of DNA damage in this cell population after *in vivo* exposure to cypermethrin. Baseline value of the CA damage index (DI) for EGC of juvenile *P. lineatus* was 144.68 ± 5.69. Damage increased in a dose-dependent manner after *in vitro* exposure of EGC to methyl methanesulfonate (MMS) and H_2O_2, two known genotoxic agents. *In vivo* exposure of fish to cypermethrin induced a significant increase in DNA DI of EGC at 0.150 μg/l (DI: 239.62 ± 6.21) and 0.300 μg/l (270.63 ± 2.09) compared to control (150.25 ± 4.38) but no effect was observed at 0.075 μg/l (168.50 ± 10.77). This study shows that EGC of this species are sensitive for the application of the CA, demonstrating DNA damage in response to alkylation (MMS), oxidative damage (H_2O_2), and to the insecticide cypermethryn. These data, together with our previous study on DNA damage induction on erythrocytes of this species, provides useful information for future work involving biomonitoring in regions where *P. lineatus* is naturally exposed to pesticides and other genotoxic agents.

1. Introduction

Agriculture practices produce an ever-increasing use of pesticides throughout the world and aquatic environments are receiving growing amount of pesticides by drift, run-off, and leaching down through the soil [1]. These agricultural chemicals can induce genetic alterations on aquatic organisms that have been associated with effects on growth, reproduction and population dynamics [2,3]. For this reason, there is increasing interest in using native species to study the effects of aquatic pollutants at the genomic level. In this regard, fish, particularly Teleost, are suitable organisms in that they are responsive to mutagens, even at low concentrations [4–6]. Cypermethrin (Cyp) is a very toxic synthetic pyrethroid insecticide used nowadays to control many pests in a variety of crops in Argentina. Particularly, it is the main insecticide used in soybean (followed by chlorpyrifos and endosulfan), representing more than half of the total insecticide consumption in this country [7]. Although considered to be relatively safe for mammals, it is highly toxic to fish and aquatic invertebrates [8]. The hypersensitivity of fish to pyrethroid intoxication is mainly due to the increased sensitivity of the piscine nervous system to these pesticides [9]. The intensive pesticide use that is taken place in the crops of the Paraná and Salado River basins make it necessary to study potential effects in native fish species, considering the particular features of the neotropical ecosystems [10,11]. Nevertheless, very few native fish species have been used in *in vivo* genotoxic evaluation in Argentina [12,13]. *Prochilodus lineatus* (Pisces, Curimatidae) is a widely distributed neotropical fish

Abbreviations: BV, basal values; CA, comet assay; CYP, cypermethrin; DI, damage index; EB, ethidium bromide; EGC, epithelial gill cells; LMPA, low melting point agarose; MN, micronucleus; MMS, methyl methanesulfonate; NC, negative control; NMPA, normal melting point agarose.

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that represents more than 50% of the total ichthyomass of the Paraná and Salado rivers, therefore, it could be an important indicator to characterize contaminated sites [14,13]. Besides, *P. lineatus* is a bottom feeder that comes in contact with sediments and the sediment–water interface, where pyrethroid insecticides are expected to accumulate [15].

Among the variety of methods developed for detecting DNA damage, the alkaline comet assay (CA) is recommended because it detects a broad spectrum of DNA lesions with a very high sensitivity [3]. Tissue specific variation in DNA lesions is expected because different cell types have distinct background levels of DNA single-strand breaks due to variation in excision repair activity, metabolic activity, antioxidant concentrations, or other factors [2]. In fish, the cell type mainly used for CA application are erythrocyte [4,6]. However, gills are directly and constantly exposed to environmental contaminants in water, being the main target organ for a variety of insults. Therefore, gill cells are supposed to be very suitable for the evaluation of genotoxic effects of water contaminants [16]. In a previous work, we successfully applied the CA to erythrocytes of *P. lineatus* and reported genotoxicity after *in vivo* exposure to sublethal concentrations of Cyp [13]. Cavalcante et al. [17] determine that the CA applied to EGC of *P. lineatus* is a complementary tool for detecting genotocixity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. The aim of the present study was to evaluate (1) the sensitivity and suitability of epithelial gill cells of *P. lineatus* in response to different genotoxic agents through the application of the CA; (2) the induction of DNA damage in this cell population after *in vivo* exposure to sublethal concentrations of the widely used insecticide Cyp.

2. Materials and methods

2.1. Chemicals

Cypermethrin (Cyp) commercial formulation SHERPA®: 25% of Cyp as active ingredient was used. Hydrogen peroxide (H_2O_2) was purchased from Cicarelli, and Dimethyl sulphoxide (DMSO) was purchased from Fluka. Low melting point agarose (LMPA), normal melting point agarose (NMPA), ethidium bromide, methyl methanesulfonate (MMS), the rest of the reagents for CA and general laboratory chemicals were provided by Sigma. RPMI-1640 medium was purchased from HyClone.

2.2. Animals

This study was evaluated and approved by the 'Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina)' (N° 13-11) and is in accordance with the 'EU Directive 2010/63/EU for animal experiments'.

A total of 80 juvenile *Prochilodus lineatus* with an average weight and length of 39.8 ± 11.8 g and 109.68 ± 14.77 mm respectively, were used for the study. They were collected from a pristine environment situated at 31° 39′ 36″S, 60° 35′ 26″W, in Santa Fe province (Argentina), previously used as a control area in different toxicological studies [13–15]. They were transported to the laboratory in oxygenated recipients, and were acclimated for 24 h at 25 ± 2 °C, pH 8.2–8.5, with constant aeration, and under a photoperiod of 12:12 h.

2.3. Epithelial gill cells extraction

Gills from both sides of each specimen were excised, filaments separated from arches, sliced, and washed two times with Ca^{2+}- and Mg^{2+}-free PBS to remove blood cells. Then they were placed in a tube containing 1 ml of PBS and 1 ml 0.05 trypsin, and softly dissociated for approximately 2–3 min. The supernatant containing the free cells was transferred to another tube with 3 ml of PBS and centrifuged at 45*g* for 5 min [18]. After removing most of the supernatant, 50 μl of the cell suspension were mixed with 950 μl of RPMI for *in vitro* exposure or directly used for CA application in the case of *in vivo* exposure.

2.4. Basal values determination and in vitro exposure to MMS and H_2O_2

As there was no previous data on CA applied to gill cells in *P. lineatus*, 20 specimens were used to determined basal values (BV) of DNA damage in gill cells and to standardize the assay conditions using MMS and H_2O_2 as known genotoxic agents.

Immediately after acclimation period, fish were sacrificed by a thermal shock in ice water, and epithelial gill cells obtained as it was explained before. Half of the samples were used for BV determinations and the rest for standardization of the technique as follows: cell suspension was made 1:19 (v/v) with RPMI and 10 aliquots from each specimen were used to conduct *in vitro* exposure for 1 h at 25 °C, per duplicate: negative control (NC), MMS (20 and 40 μM) and H_2O_2 (25 and 50 μM). Then, tubes were centrifuged at 400*g* for 10 min. and cells used for the application of the CA. The criteria used to select MMS and H_2O_2 concentrations were based on previous studies conducted in this and other species under similar experimental conditions [19,20,13]. We chose the temperature of 25 °C taking into account that we *P. lineatus* is a neotropical fish species.

2.5. In vivo exposure to cypermethrin

Exposure was conducted during 96 h in 25 l containers containing 3 specimens each, placed in an acclimated room with a constant temperature of 25 ± 2 °C, photoperiod of 12:12 h, pH 8.2–8.5 and oxygen concentration between 5.7 and 6.8 mg/l.

Sixty specimens were randomly distributed in four groups of 15 animals each: a NC group without chemical exposure, and three groups exposed to the following Cyp sublethal concentrations: 0.300, 0.150 and 0.075 μg/l [21,14]. Considering breakdown in water, under normal environmental temperatures and pH, cypermethrin is stable to hydrolysis with a half-life greater than 50 days and to photodegradation with a half-life greater than 100 days [22]. The static test method of acute toxicity was used. Temperature, pH, and dissolved O_2 were monitored continuously. Fish feeding was suspended 24 h before the beginning of the experiment and mortality, immobility and behavioral alterations were registered every 24 h. At the end of the exposure period, specimens were sacrificed by cervical section and epithelial gill cells obtained as described above.

2.6. Cypermethrin analytical determination

Cypermethrin determination in the water of eack tank was conducted by Gas chromatographic method (GC) following the AOAC Official Method 985.03 [23]. Sample was dissolved in CH_2CL_2 containing dicyclohexyl phthalate, and 1.0 ml is injected into capillary GC in split mode, with flame ionisation detection. Peak areas are measured for each cypermethin isomer and dicyclohexyl phthalate and compared with those from standard injection.

2.7. Comet assay

Cell viability was determined before the application of the CA by fluorescent DNA-binding dyes. A cell suspension was mixed with a dye-mix working solution of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide (EB), prepared in Ca^{2+}- and Mg^{2+}-free PBS and then examined under a fluorescent microscope (40×). A total of 100 cells were counted per sample and the percentage of viable cells was determined [24]. The CA was performed

as described for Singh et al. [25] with modifications required by *P. lineatus*: DNA unwinding during 10 min and electrophoresis at 300 mA, 0.7 V/cm, during 10 min too [13]. Epithelial cells obtained after *in vitro* and *in vivo* exposure were used for CA as follows: 50 µl cells were mixed with 200 µl 1% LMPA and slides prepared following standard procedure. Slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%; pH 10) for 24–72 h, incubated in freshly made alkaline solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13) during 10 min, electrophoresed 10 min. at 300 mA and 25 V (0.70 V/cm) and then neutralized (0.4 M trizma base, pH 7.5). Finally, they were dehydrated in ethanol and left to dry [13]. Lysis, unwinding and electrophoresis were conducted at low temperature (4–10 °C) and the preparations were kept in the dark to prevent additional DNA damage. All samples were coded for 'blind' analysis, stained with EB (2 µg/ml) and comet images of 100 randomly selected cells (50 cells from each of two replicated slides) were scored from each sample under a fluorescent microscope. Cells were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, 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 [13].

2.8. Statistical analysis

Statistical analysis was performed using the software SPSS 14.0 for Windows (2005). Results are informed as mean values ± standard error (SE) per group. Variables were tested for normality with Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by Levene test. We used the one-way ANOVA followed by Dunnett's test for the comparison of DI between each group and the NC in both *in vitro* and *in vivo* exposures. A linear regression was conducted for the *in vivo* exposure to determine the existence of a concentration–response relationship between DI and Cyp. A difference of $p < 0.05$ was considered statistically significant.

3. Results

Analytical cypermethrin determination done in the aqueous solutions of each tank gave the following results: 0.066, 0.127 and 0.260 µg/l, corresponding to the experimental concentrations of 0.075, 0.150 and 0.300 µg/l, respectively. All determinations indicate a 85–90% of the active ingredient respect to the value informed in the label.

Results of cell viability obtained by fluorescent DNA-binging dye method were in the range of 90–95% for all samples. Basal values of DI for epithelial gill cells of juvenile *P. lineatus* was 144.68 ± 5.69.

Results for *in vitro* exposure of epithelial gill cells to MMS and H_2O_2 demonstrated a significant dose-dependent increase in DI for both compounds, compared to control ($p < 0.001$; Fig. 1). Exposure to 25 µM H_2O_2 induced a DI = 170.6 ± 3.13 while 50 µM H_2O_2 a DI = 261 ± 9.83. This means an increase over DI of the NC (125.5 ± 3.51) of 35.9% and 107.97% respectively. Epithelial gill cells exposed to MMS showed higher damage, with an increase over NC of 175.69% at 20 µM (DI = 346 ± 2.79) and 206.85% at 40 µM (DI = 385.1 ± 1.18).

No fish mortality or behavioral alterations were observed during the *in vivo* exposure experiment. Cypermethrin induced a significant increase in DNA damage of epithelial gill cells at 0.150 µg/l (DI: 239.62 ± 6.21) and 0.300 µg/l (270.63 ± 2.09) compared to the NC (150.25 ± 4.38; $p < 0.001$) but no effect was observed at 0.075 µg/l (168.50 ± 10.77; $p > 0.05$; Fig. 2). In terms of

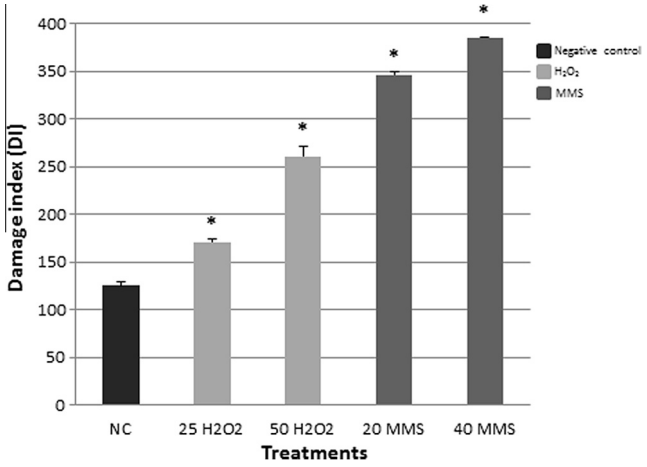

Fig. 1. Damage index (DI) in epithelial gill cells of *P. lineatus* after *in vitro* exposure to the known genotoxic agents MMS and H_2O_2. NC: negative control; 25 H_2O_2: 25 µM H_2O_2; 50 H_2O_2: 50 µM H_2O_2; 20 MMS: 20 µM MMS; 40 MMS: 40 µM MMS. *Significantly different compared to the NC ($p < 0.05$).

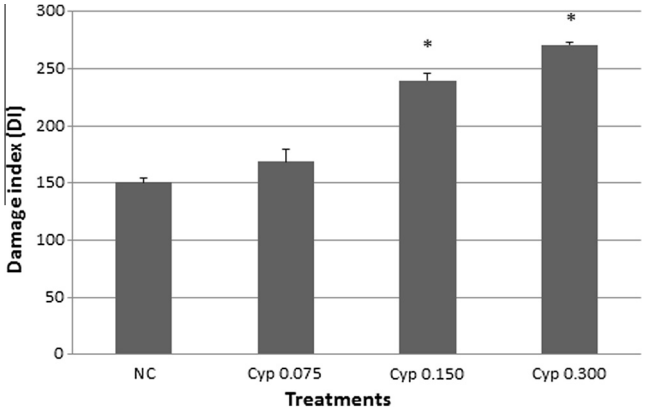

Fig. 2. Damage index in epithelial gill cells of *P. lineatus* after *in vivo* exposure to cypermethrin (96 h). NC: negative control; Cyp: groups exposed to 0.075, 0.150 and 0.300 µg/l Cypermethrin. *Statistically significant compared to the NC ($p < 0.05$).

percentage, the group exposed to 0.075 µg/l showed an increase of 12.15% over control while at Cyp 0.150 µg/l and 0.300 µg/l, DI increased 59.48% and 80.11%, respectively. We found a moderate concentration-dependent effect of Cyp ($R^2 = 0.576$, $p < 0.001$), showing an increment in DNA damage as concentration increases.

4. Discussion

Growing awareness about aquatic pollutants generated an increasing interest in fish as bioindicators for the monitoring of environmental mutagens, carcinogens and teratogens.

Fish are often used as sentinel organism because they play a number of roles in the trophic web, respond to low concentrations of mutagens and accumulate toxic substances directly from contaminated water, and indirectly, by feeding on contaminated aquatic organisms [26,27]. Therefore, the use of fish biomarkers as signs of contamination effects permits early detection of aquatic environmental problems. Pesticides are one of the most important groups of compounds concerning water contamination. Fish are generally good indicators of pesticide exposure due to the fact that they frequently change metabolic state in response to the presence of pesticide [28,29]. Various fishes (freshwater and marine) have been used as models for genotoxicity monitoring in aquatic

environments. The comet assay has found wide application as a simple and sensitive method for evaluating *in vivo*, *in vitro* and *in situ* DNA damage in different fish tissues (gills, liver, blood) after exposure to various xenobiotics in the aquatic environment [30].

The evaluation of DNA damage in fish using the comet assay frequently involves the utilization of erythrocytes because of their ready availability and ease of collection, as 97% of total fish blood cells are erythrocytes. Solid tissues such as gill require cell dissociation and isolation prior to the CA procedure, using techniques that themselves do not cause DNA damage. However, when comparing cells types it is usually reported that circulating blood cells are usually less sensitive than gill cells [18,31,32]. This is likely due to the fact that gills represent an important tissue for the uptake of contaminants, they are directly exposed to a potentially stressful environment and cells are constantly dividing, so they can demonstrate more frequent DNA damage than erythrocytes [27,17].

In this work, we used the comet assay for gill cells of *P. lineatus*, one of the most common species native to the Litoral region of Argentina, in order to evaluate sensitivity of this cell type for the evaluation of genotoxicity induced by cypermethrin and compared it with a previous work done with erythrocytes [13]. Results found in the present work show a higher DI for gill cells in all exposed groups compared to the previous results found in erythrocytes, with the same concentrations and exposure conditions (Table 1) [13], indicating that gill cells are more sensitive than blood cells. In both studies carried out, animals were capture from the same pristine area and in the same period of the year to avoid the influence of natural fluctuation, such as seasonal temperature change [20]. This higher sensitivity of gill cells to the action of cypermethrin agrees with the results reported by several authors for different compounds. Cavalcante et al. [17] found that fish exposed to Roundup® (glyphosate) exhibited significantly higher DNA damage than controls both in erythrocytes and gill cells after 6 h of exposure, but while DNA damage in erythrocytes diminished returning to the control values at 24 h, it remained increased in gill cells. According to the authors, a possible explanation for this difference would be that the repair system in gill cells is slower than in erythrocytes, resulting in an increased comet score after 24 h.

Ali et al. [33,34] indicated that gill cells of *Channa punctatus* exhibited comparatively higher DNA damage than lymphocyte cells at most of the concentrations of chlorpyrifos analyzed after short- and long-term exposure. They stated that the higher DNA damage in gill cells could be explained as gill is an organ directly and constantly exposed to the DNA damaging chemicals dissolved in the water, whereas lymphocytes and erythrocytes receive chemicals after circulation. In other work, the same species exposed to acute concentrations of the organochlorine pesticide endosulfan showed that the gill cells were more sensitive to the pesticide exposure than the kidney cells, but both cell types responded in a dose-dependent manner [32]. The suitability of gill tissue for genotoxicity studies has also been demonstrated earlier using shellfish [26]. Different responses between gill cells and blood cells may be due to tissue specific physiochemical activities, concerning either activation/detoxification mechanisms or the repair of different types of strand breaks [33]. Besides, the number of alkali-labile sites in DNA could vary from different tissues, and different cell types can have very different levels of DNA single strand breaks due to variation in excision repair activity, metabolic activity, antioxidant concentrations and other factors [2]. This is also truth for other biomarkers of genotoxicity, such as the micronucleus (MN) test. Cavas and Ergene- Gözükara [35,27] reported that gill cells are more sensitive than erythrocytes to MN induction by different compounds. Similarly, Hayashi et al. [18] reported that MN frequencies in five different fish species captured from the field were significantly higher in gill cells than in erythrocytes. However, Sharma et al. [16] reported no tissue-specific variation in DNA damage comparing gill, liver and blood cells.

Table 1
Damage index (DI) in erythrocytes of juvenile *Prochilodus lineatus* exposed *in vivo* to different concentration of Cypermethrin (from Simoniello et al., 2009).

Treatment (μg/l Cyp)	DI[a]
0.075	115.33 ± 15.94
0.150	119.00 ± 10.74
0.300	202.87 ± 35.84

[a] DI, damage index (mean ± SE).

Frenzilli et al. [6] recommended the calibration of the CA for its application to aquatic species. Therefore, effort is required to establish standardized protocols for the main species and cell type commonly used in environmental studies, and the utilization of negative and positive control groups is part of the recommended guidelines. In this context, we exposed gill cells *in vitro* to two known genotoxins: MMS and H_2O_2. MMS was used as a model for alkylation damage as alkylating agents are expected to be the most potent and abundant chemical DNA-damaging agents found in aquatic environment [36]. On the other hand, H_2O_2 was applied as a model for oxidative damage, which can happen endogenously or exogenously [19].

De Andrade et al. [20] reported that MMS at the same concentrations (20 and 40 μM), time of exposure (1 h) and temperature (25 °C) tested here induced a similar increase in DI compared to controls in erythrocytes of *Mugil* sp. The same was evaluated for sea catfish *Netuma* sp., showing a DI much lower than for *Mugil* sp. as well as for *P. lineatus* observed in our study, but anyway, with a significant increase at all concentration tested compared to the controls, and a clear dose–response. These results show that gill cells, like erythrocytes, are sensitive to alkylation damage caused here by MMS, so they are suitable as a cell type for the application of the CA in biomonitoring of alkilating agents in aquatic environments.

Gill cells of *P. lineatus* also demonstrated to be sensitive to oxidative DNA damage produced by 25 and 50 μM hydrogen peroxide, with increasing damage as the concentration increases. Similarly, de Miranda Cabral Gontijo et al. [19] reported clear concentration-related effects in the DI of Nile tilapia erythrocytes exposed *in vitro* to 1–100 μM H_2O_2 for 5 min. DI observed in our study at 25 μM H_2O_2 (170.6 ± 3.13) is a little higher than that found previously in erythrocytes exposed to the same concentration (161.71 ± 44.65) [13], but this different is not statistically significant.

Although pyrethroids are known to be extremely toxic to fish, little is known about their possible effects to native fish species. Acute effects are considered of special relevance in the assessment of effects induced by pyrethroids due to their relatively low environmental persistence [7]. Relatively low-molecular-weight organic compounds, as cypermethrin, are readily absorbed across the gill [37,38]. In our study, we found a significant increase in DNA damage of epithelial gill cells after *in vivo* acute exposure (96 h) of *P. lineatus* to 0.15 and 0.30 μg/l cypermethrin compared to the controls. Ansari et al. [39] reported increased frequencies of chromosome aberration and MN in *C. punctatus* exposed for 48 and 72 h to concentrations of cypermethrin a little higher than those tested in our study (0.4, 0.8 and 1.2 μg/l).

Carriquiriborde et al. [7] found cypermethrin was highly toxic in terms of 96 h-LC_{50} to *Cnesterodon decemmaculatus* exposed under laboratory conditions. However, authors indicated that in the environment, the actual effect of pesticide on resident biota might be rather different due to pyrethroid interaction with suspended matter, bottom sediments, and aquatic plants. Besides, effects induced by pyrethroids also depend on field-use conditions. Conventional practices involve a ground spraying using track-mounted

blowers with no attenuation strips near watercourses, so that cypermethrin residues were found in water at levels of 0.46 and 0.29 mg/l after field spraying, levels comparable to the estimated 96 h LC_{50} for that species [40]. However, no mortality or behavioral changes attributable to the insecticide were detected in resident populations of *C. decemmaculatus* exposed under field-use conditions. Authors indicated that the lack of effects can be explained by the buffering capacity of natural waters to reduce up to one order of magnitude cypermethrin toxicity to fish. Protective capacity was mainly associated with the organic matter content in the dissolved and particulate fractions [7]. These results are extremely important in terms of mortality, but sub-lethal effect, such as DNA damage or enzymatic alterations induced by long term exposure to pesticides in natural environment could have deep negative consequences for fish populations and other aquatic organisms [13,41]. It is important to highlight that sediment-feeders fish, like *P. lineatus*, are particularly vulnerable to those compounds associated to sediment particles and therefore, especially suitable and recommended for biomonitoring studies concerning these compounds [20].

In conclusion, this study shows that epithelial gill cells of *P. lineatus* are sensitive for the application of the CA, demonstrating DNA damage in response to alkylation (MMS), oxidative damage (H_2O_2), and the insecticide cypermethryn. These data, together with our previous study on cypermethrin DNA damage induction on erythrocytes of *P. lineatus* [13], provides useful information for future work involving the biomonitoring of regions where this species is naturally exposed to different pesticides, including cypermethrin, and other genotoxic agents.

Conflict of interest

The authors report no declarations of interest.

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