



Multiple biomarker responses in *Prochilodus lineatus* subjected to short-term in situ exposure to streams from agricultural areas in Southern Brazil

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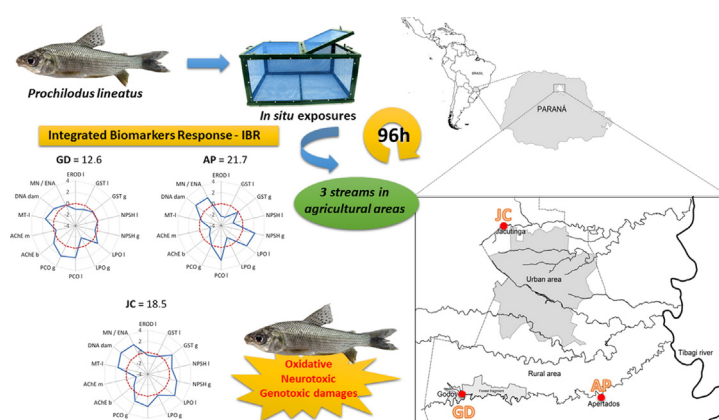
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HIGHLIGHTS

- We measured biochemical and genotoxic biomarkers in fish caged in three streams.
- AP and JC streams are more vulnerable to pesticides than GD stream.
- Pesticide concentrations were higher in AP, followed by JC and GD.
- The Integrated Biomarker Response Index (IBR) was AP 21.7 > JC 18.5 > GD 12.6.
- IBR correlated well with contaminants levels.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to assess the quality of streams susceptible to contamination by pesticides we apply biochemical and genotoxic biomarkers in the Neotropical fish *Prochilodus lineatus* submitted to in situ tests. Fish were caged, for 96 h, in two streams located in areas with intensive use of pesticides, the Apertados (AP) and the Jacutinga (JC), and in a small stream (Godoy stream – GD) found inside a forest fragment adjacent to a State Park. Biochemical parameters, such as biotransformation enzymes 7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST), non-protein thiols (NPSH), lipoperoxidation (LPO), protein carbonylation (PCO) and acetylcholinesterase (AChE) were evaluated in various fish organs, as well as genotoxic biomarkers (damage to DNA and occurrence of micronuclei and erythrocyte nuclear abnormalities). Samples of water and sediment were collected for analysis of metals (Cu, Cr, Pb, Ni, Mn, Cd and Zn), organochloride pesticides, and triazine and glyphosate herbicides. We observed an increase in liver GST activity in fish at AP and gill GST activity in fish at JC. An increase in liver LPO was also observed in fish exposed to AP and JC. The same animals also exhibited increased DNA damage and erythrocyte nuclear abnormalities (ENAs) compared to the fish kept in GD. A number of compounds

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showed concentrations higher than the permitted levels, in particular, dichlorodiphenyltrichloroethane (DDT), its metabolites dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), hexachlorocyclohexanes (HCH), heptachloride, diclofluanid and aldrins. These pesticides were detected at higher concentrations in water and sediment samples from AP, followed by JC and GD. The Integrated Biomarker Response Index (IBR) indicated that AP and JC (AP: 21.7 > JC: 18.5 > GD: 12.6) have the worst environmental quality. Integrated biomarker analysis revealed that the alterations observed related well with the levels of environmental contaminants, demonstrating the effectiveness of this biomonitoring approach.

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1. Introduction

Aquatic contamination became a serious environmental concern since the aquatic compartment was converted into a sink of several contaminants, such as pesticides (Guilherme et al., 2014). This is especially worrying in Brazil, which is one of the principal agricultural producers in the world and since 2008 the country became the world's largest consumer of pesticides and was responsible for 86% of Latin America consumption (Santana et al., 2013). From 2000 to 2012 the annual sales of pesticides in Brazil has enhanced by 194%, reaching 478 thousand tons of active ingredients sold on 2012 (IBAMA, 2013). The state of Paraná (Southern Brazil) came third in the national ranking for pesticide consumption, accounting for 11.5% of the total pesticide sales in the country in 2012 (IBAMA, 2013).

Pesticide contamination of surface waters has been well documented worldwide and constitutes a major issue that gives rise to concerns at local, regional, national and global scales (Cerejeira et al., 2003; Konstantinou et al., 2006). Although quantitative and qualitative analyses of pesticides in aquatic ecosystems are very important, these analyses alone cannot reveal the potential effects of complex mixtures of contaminants on the aquatic biota (Kerambrun et al., 2011). In this context, multidisciplinary studies combining chemical and biological measurements represent an added value to monitoring and management protocols in highly complex and heterogeneous environments (Bebianno et al., 2015).

Biomarkers are defined as biochemical, cellular, physiological or behavioral variations that can be measured in biological samples and provide evidence of exposure to and/or effects of, one or more contaminants (Depledge et al., 1995). The advantage of using biomarkers lies in their potential to anticipate damage at higher levels of biological organization, and for this reason they are used preventively, before ecological disruption occurs (Mouneyrac and Amiard-Triquet, 2013). However, there is no single biomarker that can fully diagnose the effects of environmental contaminants on organisms. For this reason, a battery of complementary biomarkers is recommended in order to obtain greater understanding of the way that organisms respond to the pollution in a given area (Lavado et al., 2006; Cazenave et al., 2009).

One of the most efficient methods for monitoring the effects of contaminant mixtures on the environment is in situ toxicity test using caged organisms, such as fish (Schlenk et al., 2008; Klobucar et al., 2010). In situ tests are more relevant in ecological terms than laboratory toxicity tests, since they take into account interactions among biotic variables (such as multi-species interactions), physical variables (such as light intensity and water flow rate) and chemical variables (mixtures of toxic substances) (Bonnineau et al., 2012). Furthermore, active biomonitoring using cages affords a number of advantages: precise knowledge of the location, the exact duration of exposure and the selection of species with specific biology and developmental stages (Oikari, 2006; Kerambrun et al., 2011). Besides, using transplanted animals from the same source also reduces inter-individual variability among exposed organisms and minimizes the influence of adaptive mechanisms (Klobucar et al., 2012). In this way, results from different sites can be validly compared.

In this study, the Neotropical fish *Prochilodus lineatus* (Valenciennes, 1836) was selected as the biomonitor organism, since it is a bottom feeder fish which is in contact with xenobiotics in water and in sediment

and rapidly responds to exposure to various pollutants, both in laboratory studies (Parma et al., 2007; Langiano and Martinez, 2008; Simonato et al., 2011; Bacchetta et al., 2011; Paulino et al., 2012) and in the field (Camargo and Martinez, 2006; Cazenave et al., 2009, 2014).

The northern region of Paraná, where activities related to agriculture are widespread, there is only 2 to 4% of the original ecosystem, represented by small remnants surrounded by areas of intensive farming (Reis et al., 2012). The intensive use of pesticides combined with the reduction in riparian forests mean that the streams in this region become increasingly susceptible to chemical contamination originating from agricultural runoff (Vieira et al., 2014). Thus, in this study we apply biochemical and genotoxic biomarkers in *P. lineatus* subjected to in situ tests in streams located in rural areas in Northern Paraná, in order to test the hypothesis that fish respond rapidly when exposed to various environmental contaminants and that the responses are an indication of the contamination levels in each environment. The biomarkers selected for this study together with the chemical analysis of the water and sediment were applied into the "Integrated Biomarker Response Index" (IBRv2), proposed by Sanchez et al. (2013), to provide a support tool to evaluate the quality of the environments investigated.

2. Material and methods

2.1. Study sites

The streams studied in this work are tributaries of the lower Tibagi basin (Fig. 1). They are located in rural areas of the municipality of Londrina (Northern Paraná), which are characterized by the deforestation caused by the large monocultures, with intensive use of pesticides throughout the year. For this study, we selected three experimental sites (briefly described below) due to their environmental conditions, which indicate distinct degrees of conservation.

Godoy stream (GD): a small stream found inside a forest fragment adjacent to "Mata dos Godoy State Park". The in situ tests were performed in a well preserved site (23°27'18.7" S–51°16'33.8" W) of the stream, which features native forest vegetation throughout its length. However, GD stream headwaters are located in a crop area. Apertados stream (AP): located in the southern municipality of Londrina. The site of in situ tests (23°27'44.6" S–51°07'38.6" W) presents banks surrounded by crops of corn, soybeans and wheat, depending on the season. This stream presents little or no riparian vegetation in some stretches.

Jacutinga stream (JC): located in the north limit between rural and urban area. The site of in situ tests (23°13'36.2" S–50°38'42.7" W) presents its banks with a predominance of grass and few trees, and corn and soybean crops arriving very close to the water.

2.2. In situ tests

Juveniles of *P. lineatus* with total length of 12.8 ± 0.1 cm and weighing 25.4 ± 0.8 g (mean \pm SD, $n = 96$; age: approximately six months) were supplied by the fish farming facility of the Londrina State University. Prior to in situ tests at each experimental site, 32 fish were acclimated for 7 days in 300 L-tanks, filled with dechlorinated

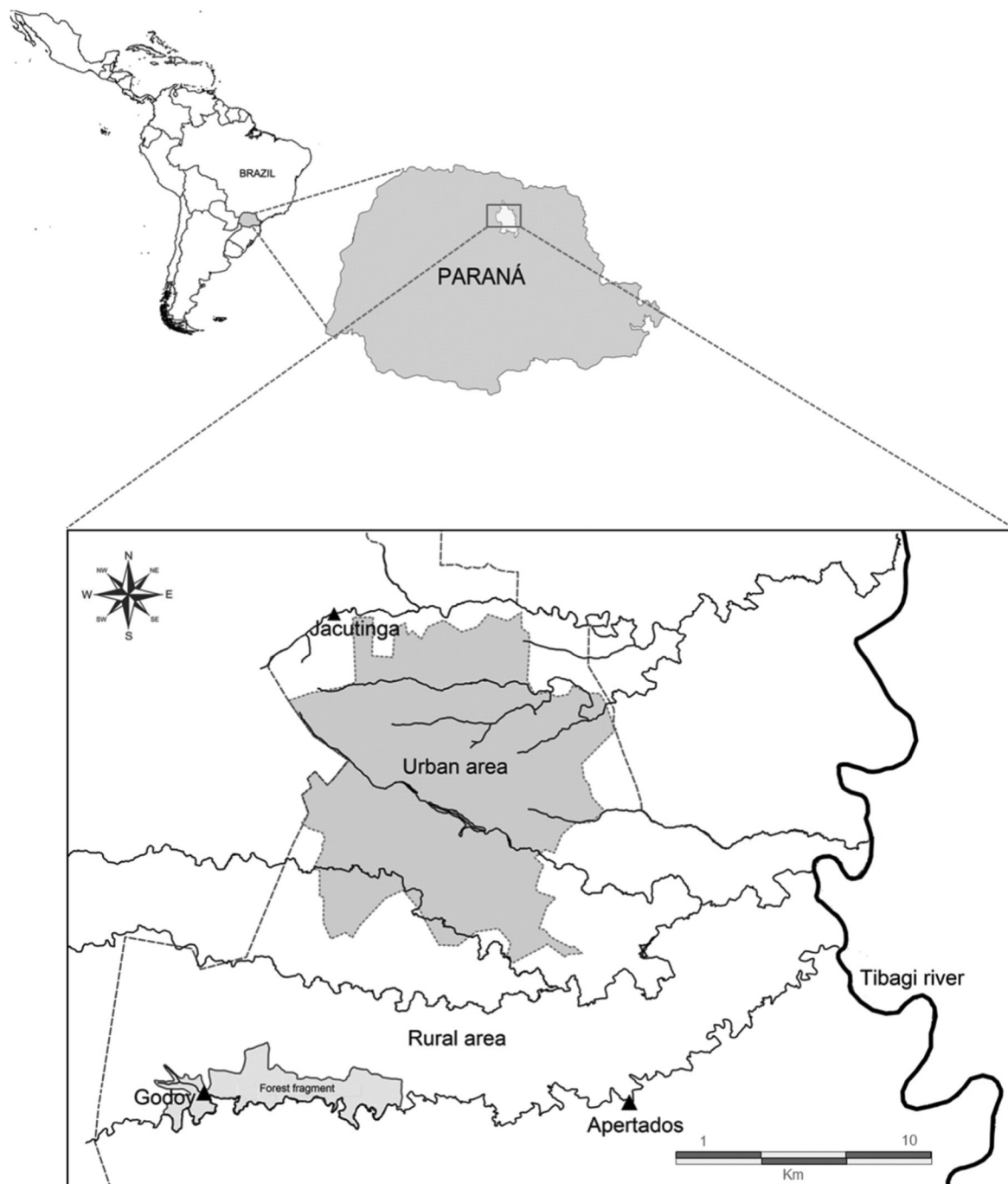


Fig. 1. Map of Brazil showing the Paraná State and the location of the municipality of Londrina (Northern Paraná). In detail the three sites (▲) where in situ tests were carried out: at Apertados (AP) and Jacutinga (JC) streams, sites in areas characterized by the intensive use of pesticides throughout the year, and at Godoy (GD) stream, a well preserved site inside a forest fragment.

water under constant aeration and a photoperiod of 12 h: 12 h. During this period, fish were fed every 2 days with commercial fish food (Guabi®, protein content of 36%). After acclimation, a group of fish ($n = 16$) were sampled for the verification of the baseline levels of biomarkers for this species and was named basal group (T_0). The other fish ($n = 16$) were transported to the selected experimental site in plastic bags containing water and oxygen. In the field, fish were transferred to cages ($60 \times 50 \times 40$ cm; 120 L) that had previously been placed under water, where they remained for 96 h. The cages, made of iron and covered with a 5-mm mesh screen that allowed water circulation, were completely submerged (depth ≤ 1.5 m) to permit fish access to the sediment for feeding.

The in situ tests were conducted during the summer (January–March, 2013), which corresponds to the rainy season for the region studied. The water parameters (pH, dissolved oxygen, temperature, conductivity and turbidity) were determined on the first and last day of fish caging, with a multiparameter probe (HORIBA-U52). Water and sediments samples were collected from each experimental site for posterior analysis.

2.3. Metal analysis in water and sediment samples

Samples of the superficial sediment and water were collected for metal quantification. In order to determine metals concentrations in

sediment samples, 1 g of sediment was previously dried at 60 °C and then subjected to horizontal shaking, for 2 h, with hydrochloric acid (HCl) 0.1 M. After this period, the suspension was filtered (8 µm pore size) and the filtrate was used for metals determination (Mozeto, 2004). Half of the water samples were filtered (0.45-µm pore size) and subsequently acidified (1% HNO₃) for the quantification of the dissolved metals concentrations; the remaining water samples were acidified without filtering for the quantification of total metals concentrations. The concentrations of Cr, Cu, Pb, Cd and Ni were determined by electro thermal ionization in a graphite furnace attached to an atomic absorption spectrophotometer (AAS) and Zn was determined by flame atomic absorption spectrometric (FAAS) (Perkin Elmer AAnalyst 700).

2.4. Pesticides analysis in water and sediment samples

Water samples were pre-concentrated and extracted by SPE tubes containing 500 mg of octadecylsilane (Chroma bond C18ec) with an average particle size of 45 µm. For the organochlorinated pesticides analyses a 250 mL volume of water samples was fortified by adding an established volume of stock solution of 100 µL of mixture of PCB-103 and PCB-198, resulting in concentration of 10 µg L⁻¹. Subsequently, the tubes were eluted with ethyl acetate and n-hexane. The final organic extracts together were down to exactly 1 mL using N₂ and transferred to a vial and added of internal standard (TCMX and PCB-209). An aliquot of 2 µL of each extract was subjected to GC-ECD analysis. For organochlorine analysis in sediment including extraction, clean-up, fractionation, and preparation to chromatographic injection was performed as described in Niencheski and Fillmann (2006).

Organochlorine compounds were quantified using a gas chromatograph (Perkin Elmer Clarus 500) equipped with a ⁶³Ni electron capture detector (ECD) and an ELITE 5MS capillary column. The analytes were quantified using the internal standard and individual calibration curves. Regular blank analyses were used to correct for eventual contamination. The identification of each analyte was confirmed by re-injecting the extracts under the same chromatographic conditions but using an Elite 17 column. Whenever required/possible the extracts were also re-injected in a Perkin Elmer Clarus 680 SQ-8 T gas chromatograph equipped with a mass spectrometer detector (GC/MS) fitted with an ELITE 5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness fused with silica).

Analyses of triazine, irgarol and clomazone herbicides were performed according to the methodology described by Demoliner et al. (2010). Before sample application, the SPE column was conditioned by passing consecutively methanol, purified water, and purified water acidified (pH 3.0) with phosphoric acid. The samples acidified were passed through the SPE tubes at 10 mL min⁻¹. After that, the tubes were eluted with methanol. The final organic extracts were directly analyzed by LC-ESI-MS-MS with injection volume of 20 µL. Herbicides analyses were performed on a Waters Alliance 2695 Separations Module HPLC, equipped with a quaternary pump, an automatic injector and a thermostatted column compartment (Waters, Milford, MA, USA). The chromatographic separation was performed with an XTerra® MS C18 column Waters (Milford, MA, Ireland. Analytical instrument control, data acquisition and treatment were performed by software MassLynx, version 4.1 (Micromass, Manchester, UK).

For glyphosate analysis in water and sediment samples, including extraction, clean-up, and preparation to chromatographic injection was performed as described in Harayashiki et al. (2013). The extracts was determined by ion chromatograph (IC Compact 881, Metrohm, Herisau, Switzerland) with conductometric detector, using an ion exchange column (Metrosep A Supp 5 150/4.0) and a chemical suppressor. The mobile phase was made with 9.6 mmol L⁻¹ of Na₂CO₃ and 3 mmol L⁻¹ of NaHCO₃ degassed for 30 min in an ultrasound bath. The solution for suppressor regeneration was prepared with ultra-pure water and 0.1 mol L⁻¹ of sulfuric acid. All injections were performed with a loop injection of 20 µL. The quantification and detection limit were 0.05 and 0.01 mg L⁻¹, respectively. Data collection and

treatment was performed using the Software MagicNet 2.3 (Metrohm, Herisau, Switzerland).

2.5. Fish sampling

After the exposure period (96 h), fish were removed from the cages, anesthetized with benzocaine (0.1 g L⁻¹) and the blood was taken from the caudal vein, using heparinized syringes. Blood samples (10 µL per fish) were preserved in microtubes containing fetal bovine serum (Gibco®) which were kept cool until the comet assay. After blood collection, the animals were killed by medullar section and samples of gills, liver, axial muscle and brain were taken. The samples were rapidly transported to the laboratory where they were stored in ultrafreezer (−80 °C) until biochemical analyses. These procedures were performed according to the protocol approved by Animal Ethics Committee of the State University of Londrina (Process 19559.2012.01).

2.6. Biochemical biomarkers

Samples of liver and gills were weighed, homogenized (1:10 w/v) in K phosphate buffer (0.1 M, pH 7.0) and centrifuged (16,000 × g, 20 min, 4 °C). The supernatant was separated for the analysis of the following biochemical parameters: activities of 7-ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferase (GST), concentrations of non-protein thiols (NPSH) and carbonyl proteins (PCO) and the occurrence of lipoperoxidation (TBARS). To determine the hepatic content of metallothionein-like proteins (MT-like) and the activity of acetylcholinesterase (AChE) in muscle and brain, tissues were homogenized in specific buffers.

EROD activity was estimated by the rate of conversion of 7-ethoxyresorufin to resorufin, according to the protocol of Eggens and Galgani (1992), with modifications. The reaction was initiated by adding the sample to the reactive mixture (0.1 M potassium phosphate buffer, pH 7.6; 2 mM NADPH and 0.1 mM 7-ethoxyresorufin). The progressive increase in fluorescence resulting from the formation of resorufin was measured at 1-min intervals for 10 min (ex/em: 530/590 nm). The EROD activity was expressed in pmol resorufin min⁻¹ mg of protein⁻¹.

GST activity was determined using the method described by Keen et al. (1976). This method is based on the GST catalyzed conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The increase in CDBN conjugate was monitored for 1 min in a spectrophotometer at 340 nm and the enzyme activity was expressed in nmol CDBN conjugate min⁻¹ mg of protein⁻¹.

NPSH concentration was determined according to the method of Beutler et al. (1963), by the reaction of glutathione with the color reagent 5,5-dithiobis-2-nitrobenzoic acid (DTNB), forming a thiolate anion (TNB), which was measured at 412 nm and the concentration was expressed in µg-SH·mg protein⁻¹.

Oxidative damage of proteins was analyzed by the quantification of PCO, according to Levine et al. (1994). A volume of the supernatant was transferred to a tube and mixed with DNPH solution (10 mM of 2,4-dinitrophenylhydrazine in 2.0 M of hydrochloric acid). For the blank, 2.0 M hydrochloric acid (without DNPH) was utilized. Samples were incubated at 35 °C during 90 min, proteins were precipitated (28% trichloroacetic acid), centrifuged (9000 × g, 10 min, 4 °C), and pelleted proteins were washed three times by suspension in ethanol/ethyl acetate (1:1) followed by centrifugation. Proteins were solubilized in 6.0 M of guanidine hydrochloride and centrifuged (9000 × g, 5 min, 4 °C) to remove any trace of insoluble material. The carbonyl content was determined spectrophotometrically at 360 nm, using the molar absorption coefficient of 2.1 × 10⁴ M⁻¹ cm⁻¹ for hydrazones, and normalized by total protein content quantified in an aliquot reserved from the first centrifugation procedure. The results were expressed in nmol carbonyl mg⁻¹ of protein.

Lipid peroxidation was determined by the TBARS (thiobarbituric acid reactive substances) assay, performed according to [Camejo et al. \(1998\)](#). Butylated hydroxytoluene (BHT 1 M), phosphate buffered saline (2 mM KCl, 1.4 mM NaH_2PO_4 , 357 mM NaCl, 10 mM Na_2HPO_4 , pH 7.4), trichloroacetic acid (TCA 50%) and thiobarbituric acid (TBA 1.3%) dissolved in 0.3% NaOH were added to the supernatant and the mixture was kept in an incubator at 60 °C for 1 h. A fluorescence reading was then made (ex/em: 535/590 nm) and the TBARS concentration was determined from a malondialdehyde (MDA) standard curve. The TBARS concentration was expressed in nmol TBARS mg of protein⁻¹.

The content of MT-like proteins was determined only in the livers, following the methodology described by [Viarengo et al. \(1997\)](#) with modifications. The livers were homogenized (1:5 m/v) in buffer (0.5 M sucrose, 26 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride, 1.3 mM β -mercaptoethanol) and centrifuged ($18,650 \times g$, 45 min, 4 °C), and the supernatant was subjected to ethanol/acid chloroform fractionation to obtain a partially purified metalloprotein fraction. In this fraction sulfhydryl groups (—SH) were quantified in a spectrophotometer at 412 nm, using Ellman's reagent (2 M NaCl, 0.43 mM DTNB buffered with 0.2 M Na-phosphate, pH 8). Glutathione (GSH) was used as standard and the metallothionein content was expressed in nmol MT-like mg of protein⁻¹.

Samples of brain and muscle tissue were homogenized (1:10 w/v) in K phosphate buffer (0.1 M, pH 7.5) and centrifuged ($16,000 \times g$, 20 min, 4 °C). The supernatant was used for the determination of AChE activity, according to the method described by [Ellman et al. \(1961\)](#), adapted to microplate by [Alves Costa et al. \(2007\)](#), using the substrate acetylcholine iodide (9 mM) and the color reagent DTNB (0.5 mM) at 415 nm. AChE activity was expressed in nmol min⁻¹·mg protein⁻¹.

The results of biochemical biomarkers were expressed per mg protein, determined by the method of [Bradford \(1976\)](#), based on the reaction of proteins with Coomassie Brilliant Blue G-250 dye. The calibration curve was prepared with bovine serum albumin (BSA) and absorbance was determined at 595 nm.

2.7. Genotoxic biomarkers

The alkaline comet assay with erythrocytes was performed according to [Singh et al. \(1988\)](#), with some modifications described by [Ramsdorf et al. \(2009\)](#). Only blood samples with cell viability above 80%, determined by the Trypan blue exclusion method, were used in the comet assay. After sampling, an aliquot of blood mixed with fetal bovine serum was added to the low melting point agarose. This mixture was placed on a glass slide previously covered with standard agarose, covered with coverslip, and remained in the refrigerator for 30 min. Then coverslips were removed and the slides were subjected to: a) lysis: 1 h at 4 °C, protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); b) DNA denaturation: 30 min in the dark in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); c) electrophoresis: 20 min, 300 mA, 25 V, 1 V cm⁻¹; and d) neutralization: three rinses for 5 min each with buffer (0.4 M Tris, pH 7.5). The slides were then fixed with absolute ethanol for 10 min and placed in the refrigerator until analysis. Subsequently, the slides were stained with gelRed (Uniscience®) and analyzed on a Leica microscope (DM 2500) adapted for fluorescence/epifluorescence, equipped with blue excitation filter (450–490 nm) and a 515 nm barrier filter, with a magnification of 1000 \times . All slides were blind-reviewed. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly select and non-overlapping cells per fish. DNA damage was classified into four classes according to [Kobayashi et al. \(1995\)](#): class 0 = no apparent damage; class 1 = tail shorter than the nucleoid diameter; class 2 = tail length corresponding to once or twice the diameter of the nucleoid; class 3 = tail length greater than twice the diameter of the nucleoid. The DNA damage score was obtained by multiplying the number of cells in each class by the class value. The results of DNA

damage was expressed by the mean of scores of damages for each group at each exposure site.

The micronucleus test (MN) was performed with fish erythrocytes according to the technique described by [Al-Sabti and Metcalfe \(1995\)](#) and the occurrence of erythrocytic nuclear abnormalities (ENAs) was analyzed according to [Carrasco et al. \(1990\)](#). The ENAs were classified in three categories: segmented nucleus, lobulated nucleus, and kidney-shaped nucleus, following [Monteiro et al. \(2011\)](#). Immediately after sampling, a small amount of blood withdrawn from each animal was smeared over two clean glass slides, dried at room temperature overnight, fixed with methanol for 10 min and stained with Giemsa (10%). A total of 3000 erythrocytes per fish were examined on an Olympus microscope (1000 \times magnification). The mean frequencies of micronuclei (MN) and erythrocytic nuclear abnormalities (ENAs) found in fish caged at each site was calculated and expressed per 1000 cells (%).

2.8. Integrated biomarker response index (IBR)

The biomarkers results were applied into the “Integrated Biomarker Response Index” (IBRv2), described by [Beliaeff and Burgeot \(2002\)](#) and modified by [Sanchez et al. \(2013\)](#). This novel version of IBR is based on the principle of reference deviation between a disturbed and undisturbed state ([Sanchez et al., 2013](#)). In the present work the deviation between biomarkers measured in fish caged for 96 h in rural streams (GD, AP e JC) were compared to those measured in fish recently collected from the fish farming and acclimated in clean water under controlled laboratory conditions (baseline levels). The baseline values of each biomarker (T_0) were used as a reference value. For each individual biomarker, the ratio between the mean value obtained at the each experimental site and the respective mean baseline value (T_0) was log-transformed (Y_i). In the next step, a general mean (μ) and standard deviation (s) was calculated, considering Y_i values of a given biomarker measured at each site. Then, Y_i values were standardized by the formula: $Z_i = (Y_i - \mu) / s$ and the difference between Z_i and Z_0 (T_0) were used to define the biomarker deviation index (A). To get an integrated multiple biomarkers response, the value of A of each biomarker was calculated for every caging site and IBRv2 was calculated for each site by the sum of the absolute values of A .

For each caging site (GD, AP and JC), A values calculated for each biomarker were reported in a star plot representing the reference deviation of each investigated biomarker. The area above 0 reflects biomarker induction, and the area below 0 indicates a biomarker inhibition.

2.9. Statistical analyses

The biomarker results were tested for normality and homogeneity of variance using the Shapiro-Wilks and Levene test, respectively. The biomarkers obtained for fish caged at the three experimental sites (GD \times AP \times JC) were compared using analysis of variance (ANOVA or Kruskal–Wallis) followed by Student–Newman–Keuls (SNK) or Dunn's test, when necessary, according to the distribution of the data. The significance level was set at $p < 0.05$.

3. Results

3.1. Physical and chemical analyses of water and sediment

The physical and chemical parameters of the water from the acclimation tanks, in the laboratory, and water collected from the caging sites where in situ tests were performed are given in [Table 1](#). The results revealed no major variations between the three sites assessed, with values within the normal range for this environment, and the values obtained in the first and last day of fish caging did not show large variations.

Table 1

Abiotic parameters of the water from the acclimation tanks, in the laboratory, and water collected from caging sites at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams in the first and last day of caging. The values for the water from acclimation are represented as mean \pm SD.

Parameters	Acclimation	Caging sites		
		AP	JC	GD
pH	6.1 \pm 1.5	6.8–7.2	5.9–6.5	6.5–6.8
Conductivity ($\mu\text{S cm}^{-1}$)	89 \pm 3.6	74–80	64–72	98–106
Turbidity (NTU)	2.6 \pm 0.7	70.4–56.1	116–102	101–38.3
Hardness (mg $\text{CaCO}_3 \text{ L}^{-1}$)	41.6 \pm 0	30.2–30.2	28.4–28.4	10.5–10.5
Temperature ($^{\circ}\text{C}$)	22.3 \pm 0.9	19.5–20.7	21.5–21.6	21.2–21.7

In regard to pesticide analysis, various organochloride insecticides were found, as well as some of the herbicides that are used for a wide variety of applications throughout the region (Table 2). A number of compounds were found at concentrations higher than the maximum permitted concentrations set by the Brazilian guidelines (BRASIL, 2005) for inland waters, including DDT and its metabolites (DDE and DDD), hexachlorocyclohexanes (HCH), heptachloride, dichlofluanid and aldrins. In general the Apertados stream (AP) exhibited the highest concentrations of the compounds analyzed, both in the water and sediment, followed by the Jacutinga (JC) and Godoy (GD) streams.

Table 2

Concentrations of selected pesticides in the water ($\mu\text{g} \cdot \text{L}^{-1}$) and sediment ($\mu\text{g} \cdot \text{kg}^{-1}$) collected from the caging sites at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams during in situ tests. The maximum permitted concentrations for some pesticides, set by the Brazilian guidelines (BRASIL, 2005) for inland waters, are indicated (MPC). Values that surpassed MPC are bolded in the Table.

Pesticides	Water				Sediment		
	AP	JC	GD	MPC	AP	JC	GD
Herbicides							
Atrazine	0.17	0.30	0.09	2	0.69	0.4527	0.3195
Glyphosate	nd	nd	nd	65	BDL	BDL	BDL
Irgarol	BDL	BDL	BDL	–	BDL	BDL	BDL
Simazine	0.0074	0.07	BDL	2	0.73	0.7731	0.4378
Ametrine	0.23	0.41	0.13	–	0.013	0.1875	0.091
Clomazone	BDL	BDL	BDL	–	BDL	BDL	BDL
Trifluralin	0.159	0.099	0.014	0.2	0.349	0.298	0.033
Organochloride insecticides							
α -Hexachlorocyclohexane	0.159	0.093	0.023	0.02	0.549	0.25	0.025
β -Hexachlorocyclohexane	0.127	0.061	0.012	0.02	0.439	0.126	0.032
γ -Hexachlorocyclohexane	0.05	0.032	0.008	0.02	0.166	0.077	0.022
δ -Hexachlorocyclohexane	0.001	BDL	BDL	0.02	0.004	0.001	BDL
Hexachlorobenzene	BDL	0.001	0.001	0.0065	BDL	0.004	0.005
Chlorothalonil	0.002	0.001	BDL	–	0.004	0.002	0.002
Oxychlorane	0.008	0.003	0.001	–	0.023	0.008	0.001
Dichlofluanid	0.038	0.027	0.002	–	0.212	0.01	0.006
Aldrin	0.027	0.021	0.006	0.005	0.078	0.056	0.032
Heptachlor epoxide	nd	BDL	0.001	0.01	nd	BDL	0.003
Heptachlor	0.147	0.08	0.016	0.01	0.356	0.012	0.043
α -Chlordane	nd	nd	nd	0.04	nd	nd	nd
γ -Chlordane	0.012	0.006	nd	0.04	0.024	0.012	nd
Transnonachlor	nd	nd	nd	–	nd	nd	nd
Endosulfan I	nd	nd	nd	0.056	nd	nd	nd
Endosulfan II	0.045	0.003	0.002	0.056	0.214	0.008	0.003
Endosulfan sulfate	nd	nd	nd	0.056	nd	nd	nd
Endrin aldehyde	nd	nd	nd	0.004	nd	nd	nd
Endrin	nd	nd	nd	0.004	nd	nd	nd
Endrin ketone	nd	0.001	0.002	0.004	nd	0.002	0.004
Dieldrin	nd	nd	nd	0.005	nd	nd	nd
op'-DDT	nd	nd	nd	0.002	nd	nd	nd
pp'-DDT	0.003	0.196	0.044	0.002	0.011	0.559	0.068
op'-DDE	0.006	0.005	0.002	0.002	0.028	0.011	0.003
pp'-DDE	0.006	0.004	0.002	0.002	0.022	0.021	0.003
op'-DDD	nd	BDL	BDL	0.002	BDL	BDL	BDL
pp'-DDD	0.025	0.016	0.006	0.002	0.058	0.035	0.009
Mirex	nd	nd	nd	–	nd	nd	nd

BDL – below detection limit of the method; nd = not detected.

The results for the presence of some metals in the water and sediment generally showed low concentrations, below the levels permitted by Brazilian guidelines for inland waters (BRASIL, 2005) (Table 3).

3.2. Biomarkers

3.2.1. Acetylcholinesterase (AChE)

A significant decrease in cerebral AChE activity ($p < 0.001$) was observed in fish confined at both AP and JC, compared to those kept in GD (Fig. 2A). There was also a significant reduction in muscle AChE activity in the fish confined at AP ($p = 0.006$) compared to those kept in GD, but fish caged at JC did not show any significant difference in muscle AChE in comparison to fish caged at the other sites (Fig. 2B).

3.2.2. Biotransformation and antioxidant defense enzymes (EROD)

In regard to liver EROD activity, no significant differences were observed among fish confined at the experimental sites (Fig. 3A). EROD activity in the gills was very low and could not be detected by the method used. However, hepatic GST activity showed an increase in the fish confined at AP ($p < 0.001$) compared to that of fish confined at GD (Fig. 3B). In regard to GST activity in the gills (Fig. 3C), there was a significant increase in enzymatic activity only in the fish confined at JC, compared to the other experimental sites ($p < 0.001$). There was no difference between the experimental groups in the concentration of non-protein thiols in both the liver ($p = 0.091$) and gills ($p = 0.079$) (Fig. 3D and E).

Table 3

Concentrations of selected metals in the water (total (T) and dissolved (D) concentration) and in sediment samples collected from the caging sites at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams during in situ tests. The maximum permitted concentrations (MPC) for each metal, set by the Brazilian guidelines (BRASIL, 2005) for inland waters, are indicated.

Metals	Water ($\mu\text{g}\cdot\text{L}^{-1}$)				Sediment ($\text{mg}\cdot\text{kg}^{-1}$)		
	AP	JC	GD	MPC	AP	JC	GD
Cu T	7.2 \pm 1.39	4.89 \pm 0.40	12.01 \pm 0.6		93	47.54	23.5
Cu D	2.95 \pm 0.30	3.46 \pm 0.67	5.78 \pm 0.71	9			
Cr T	7.08 \pm 3.16	1.22 \pm 0.11	8.41 \pm 0.18	50	4.08	5.45	1.89
Cr D	0.85 \pm 0.05	0.49 \pm 0.01	4.83 \pm 0.34				
Pb T	9.18 \pm 0.57	5.78 \pm 0.19	2.17 \pm 0.21	10	3.21	2.68	11.52
Pb D	5.47 \pm 0.14	4.29 \pm 0.16	1.93 \pm 0.01				
Cd T	0.92 \pm 0.05	nd	nd	1	0.07	0.015	0.023
Cd D	0.12 \pm 0.06	nd	nd				
Zn T	nd	nd	nd	180	9.90	17.75	1.55
Zn D	nd	nd	nd				
Ni T	1.8 \pm 0.04	nd	0.55 \pm 0.08	25	2.46	1.86	1.05
Ni D	0.74 \pm 0.02	nd	0.06 \pm 0.01				

Results for metal concentrations in the water are given as mean \pm SD ($n = 6$), obtained from the water samples collected in the first and last days of fish caging. Sediment samples were collected only in the last day of fish caging. nd = not detected.

3.2.3. Oxidative damage

There was an increase in MDA levels in the liver of fish confined at both AP and JC, compared to the fish confined at GD ($p = 0.026$) (Fig. 4A). In the gills, there was no significant increase in LPO ($p = 0.097$) in the fish confined at the three sites (Fig. 4B).

No significant change in PCO levels was observed in the liver of fish caged at any of the sites ($p = 0.083$) (Fig. 4C). However, concerning PCO levels in the gills ($p = 0.035$), fish exposed to AP showed significant lower values than those caged at GD, while fish caged at JC did not show any significant difference in PCO in comparison to fish caged at the other sites (Fig. 4D).

3.2.4. Genetic damage

The comet assay revealed a significant increase in DNA damage in the erythrocytes of fish confined at AP and JC compared to those kept in GD ($p < 0.001$) (Fig. 5A). However, there was no significant increase ($p = 0.125$) in the frequency of micronucleated cells (MN) in the fish confined at any of the experimental sites (Fig. 5B). Nevertheless, the frequency of erythrocyte nuclear abnormalities (ENAs) was significantly higher ($p = 0.002$) in the fish kept in AP and JC compared to the fish

kept in GD (Fig. 5C). The types of nuclear abnormality most commonly detected were kidney-shaped nucleus, segmented nucleus and lobulated nucleus.

3.2.5. MT-like protein

There was no significant difference ($p = 0.087$) in the content of MT-like protein in the liver of fish caged at the three experimental sites (mean \pm SE): GD: 6.36 \pm 0.23, AP: 5.32 \pm 0.42; and JC: 5.88 \pm 1.04 μg MT-like protein $^{-1}$.

3.3. Integrated biomarker response index

The baseline levels for the biomarkers analyzed in the various organs of *P. lineatus* are given in Table 4. As no significant difference was detected among baseline levels (T_0) determined for the three groups of fish, the results were grouped and are presented as a single value (mean \pm SE) for each biomarker. These baseline values were then used for IBR determination.

The IBR values calculated for each caging site are shown in Fig. 6 and indicate differences among the streams. AP showed the highest IBR value (21.7), followed by JC (18.5) and GD (12.6). The star plots indicate that the induction of both genotoxic biomarkers (DNA damage and MN/ENAs) and liver LPO, together with the reduction in muscular and brain AChE activities, were the most representative biomarker to differentiate AP and JC from GD, as they showed greater variations in comparison to the basal group and significant variations in relation to GD group. These biomarker results clearly indicate that the level of contamination at AP and JC streams is higher than that at GD stream, corroborating the pesticides and metals concentrations found at these sites.

4. Discussion

In this study fish exhibited responses in the biomarkers related to exposure to environmental contaminants, showing that all the three streams analyzed were affected by the ingress of chemical substances from agricultural activities, in varying degrees of intensity. The results also show how useful it can be to integrate a number of biomarkers to define exposure to and the effects of pesticides under actual field conditions. However, it is worth noting that the interpretation of field results is always a very complex operation, since many factors can influence the variables analyzed in uncontrollable ways (Zanette et al., 2006).

Chemical analyses of pesticides in the streams revealed that some compounds were present at levels much higher than those established by the current Brazilian water quality criteria (BRASIL, 2005). These

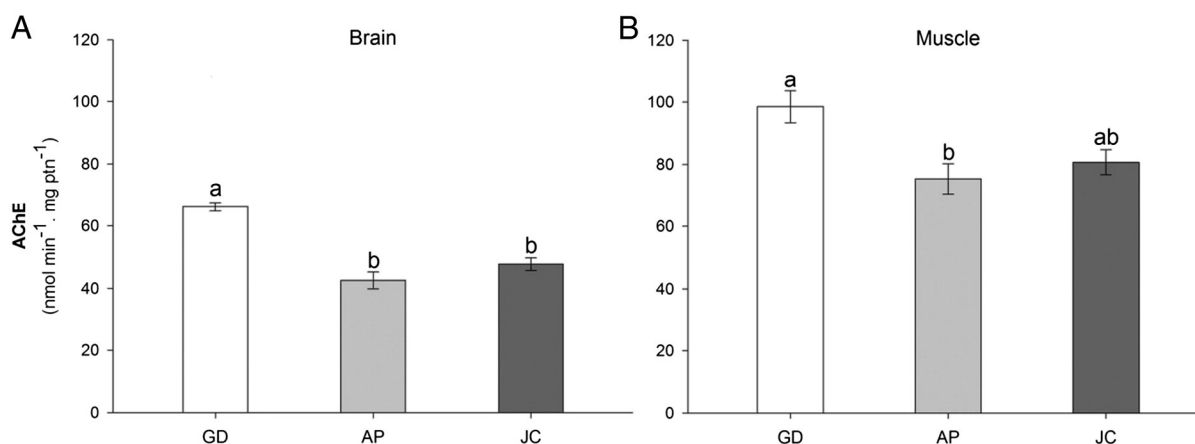


Fig. 2. Activity of acetylcholinesterase (AChE) in brain (A) and muscle (B) of *P. lineatus* caged at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams. Results are mean \pm SEM ($n = 8$). Different letters indicate significant differences between sites ($p < 0.05$).

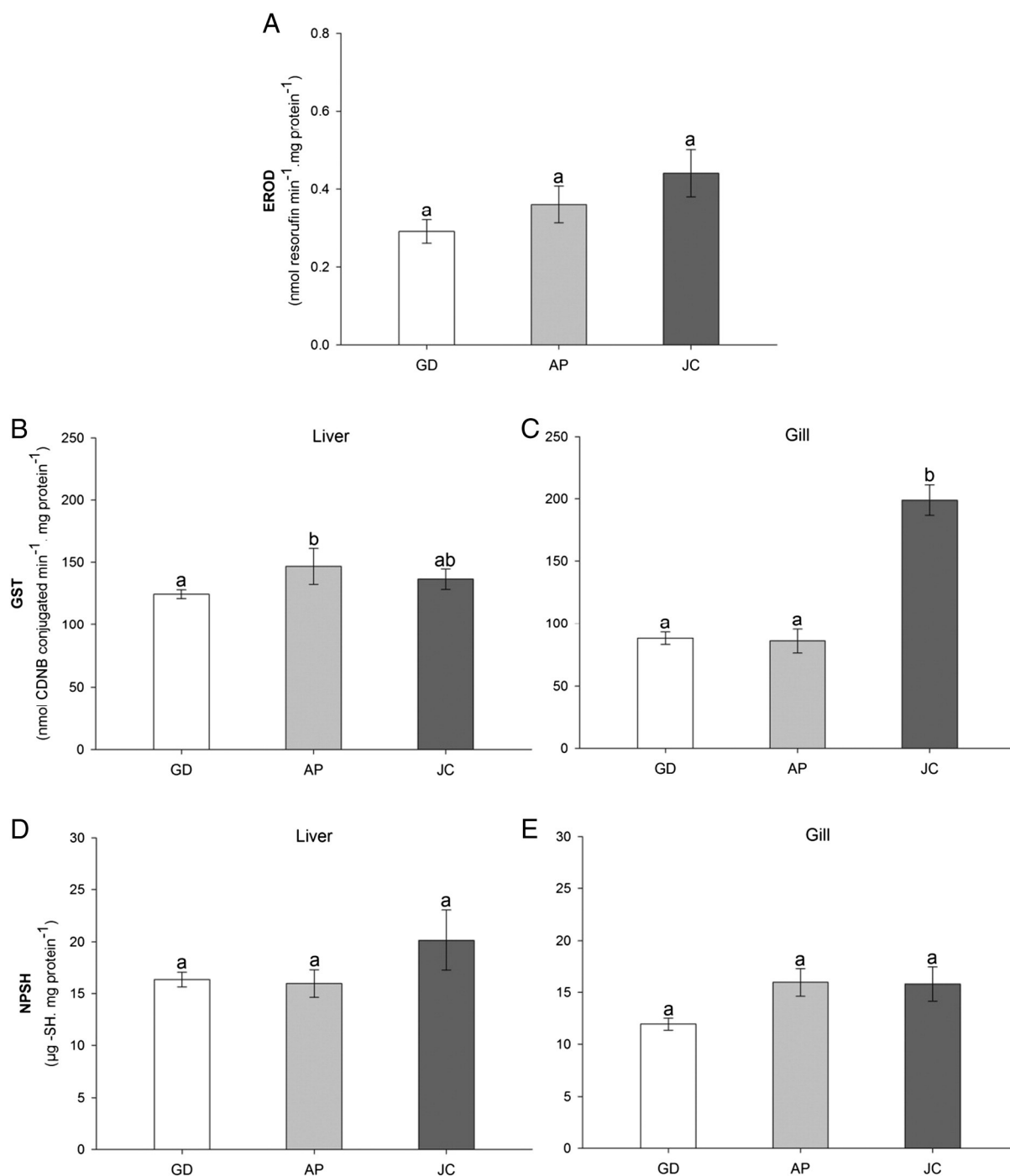


Fig. 3. Activity of 7-ethoxiresorufina-O-deethylase (EROD) in liver (A) and glutathione-S-transferase (GST) in liver (B) and gill (C), and concentrations of non-protein thiols (NPSH) in liver (D) and gill (E) of *P. lineatus* caged at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams. Results are mean \pm SEM (n = 8). Different letters indicate significant differences between sites (p < 0.05).

compounds included organochlorides such as hexachlorocyclohexanes (α , β and γ -HCH), with concentrations exceeding the recommended limits in AP and JC, and heptachlor, which exceeded these limits in all the streams studied. Moreover, all three streams showed high concentrations of pp-DDD and pp-DDT. The pp-DDT concentration found in JC ($0.196 \mu\text{g L}^{-1}$) was particularly high, indeed almost 100 times higher than the permitted level. The metabolites pp-DDE and pp-DDD also exceeded the permitted levels in JC and AP. In general, AP showed the highest concentrations of most of the pesticides analyzed, both in the water and the sediment, followed by JC and GD.

Analyses of metals in the water and sediment of the streams showed the presence of various metals, possibly introduced by human activity. Copper (Cu), chromium (Cr) and lead (Pb) were the most abundant, and nickel (Ni), zinc (Zn) and cadmium (Cd) were found in lower concentrations. The concentrations of these metals found in water were below the limits established by the current legislation (BRASIL, 2005). Because of these relatively low concentrations, metals were not considered a major problem induced by agricultural contamination in the region. Their presence could be related to agricultural practices, since the application of essential elements for stimulating plant growth,

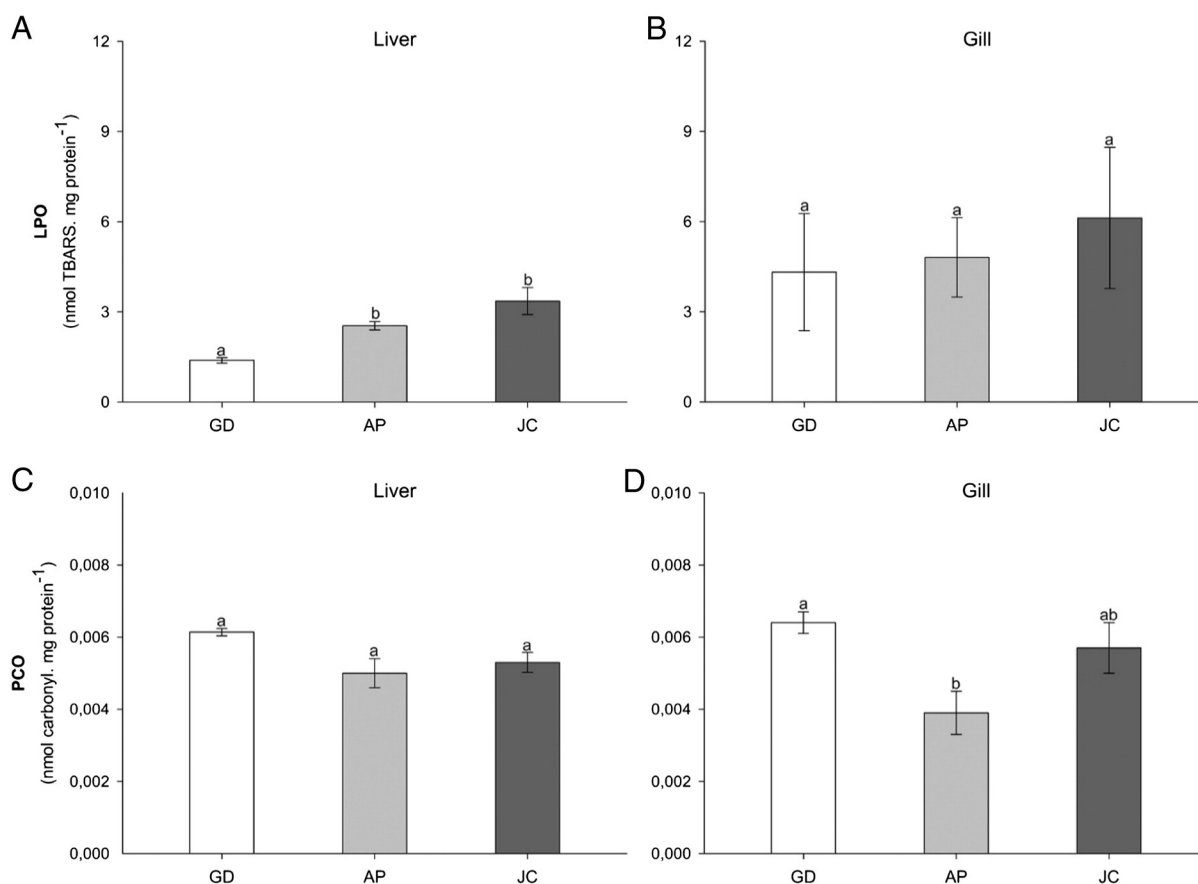


Fig. 4. Lipid peroxidation (LPO) in liver (A) and gill (B) and carbonyl proteins (PCO) in liver (C) and gill (D) of *P. lineatus* caged at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams. Results are mean \pm SEM (n = 8). Different letters indicate significant differences between sites (p < 0.05).

such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and boron (B), is common to remedy deficiencies in the soil. Furthermore, the formulation of many pesticides (fungicides, insecticides and herbicides) contains metals, and the application of fertilizers is one of the most consistent sources of trace elements, increasing metal concentrations in the water and sediment (Jiao et al., 2012).

The activity of EROD represents the activity of the phase I biotransformation enzyme, CYP1A. In many fish species, the activity of the CYP1A subfamily can be altered in the presence of pesticides and other pollutants in the aquatic environment (Van der Oost et al., 2003). The decrease in EROD activity induced by organochlorides is well-documented in the literature. In field studies, Couillard et al. (2005) found a strong correlation between the reduction in the hepatic activity of EROD in fish and exposure to organochlorides. Similarly, Kolankaya (2006) also observed a decline in EROD activity in fish exposed to surface waters containing organochlorides, such as BHC and HCHs. Some organophosphorus compounds can also cause the inhibition of P450 (Fabrizi et al., 1999). However, in our study we did not find any significant differences in the activity of this enzyme in the liver of the fish caged at the three experimental sites.

On the other hand, an increase in the activity of the Phase II biotransformation enzyme GST was observed in the liver of fish caged at AP and in the gills of fish caged at JC. These increases could be linked to exposure to organic compounds that are metabolized by conjugation with GSH. Various authors have already documented the activity of GST in different fish species exposed to pesticides, some of which are used in the region where this study was conducted (Oruc et al., 2004; Gluszczak et al., 2006; Modesto and Martinez, 2010a; Rossi et al., 2011; Cattaneo et al., 2011; Dong et al., 2013).

Glutathione (GSH), a tripeptide that plays a fundamental role in oxidation/reduction reactions, transportation of amino acids and

detoxification of many toxic agents, is the first line of defense against cell lesions mediated by oxidants (Van der Oost et al., 2003). In this study the levels of NPSH were used to estimate GSH concentrations and the results showed no significant variations of NPSH both in liver and gill of fish caged at the different sites. The lack of an increase in GSH might have impaired the antioxidant capacity of the organisms, leading to oxidative damage. In fact, the liver of fish exposed to AP and JC exhibited lipoperoxidation (LPO), characterizing oxidative stress. There was however no evidence of LPO in the gills. In general, pesticides induce oxidative changes that are evidenced by the build-up of reactive oxygen species (ROS), LPO and protein and DNA oxidation (Bagchi et al., 1995; Franco et al., 2009).

A number of studies have shown the oxidative effects of various organochlorides, such as endosulfan, DDT and its metabolites, and HCH, on different species of fish, including *P. lineatus* (Song et al., 2006; Bacchetta et al., 2011, 2014), *Odontesthes bonariensis* (Barni et al., 2014) and *Carassius carassius* (Dar et al., 2015). Therefore, the occurrence of lipoperoxidation in the liver of fish caged at AP and JC is a potential biomarker for oxidative stress as a result of exposure to different organochlorides present in these streams.

LPO can result in loss of membrane integrity and, with increased permeability, a change in the flow of ions across the membrane, dysfunction in the transportation of Na^+/K^+ , excessive inflow of calcium and activation of enzymes, such as proteases, phospholipases and nucleases (Meagher and Fitzgerald, 2000; Barreiros et al., 2006; Valavanidis et al., 2006). Based on the occurrence of genetic damage in the erythrocytes of fish caged at AP and JC, it is reasonable to suppose additional effects of the products of LPO on the oxidation of other cell components, such as nucleic acids.

Despite the occurrence of LPO in the liver of fish confined at the most contaminated sites, increased PCO was not observed. However, the gills

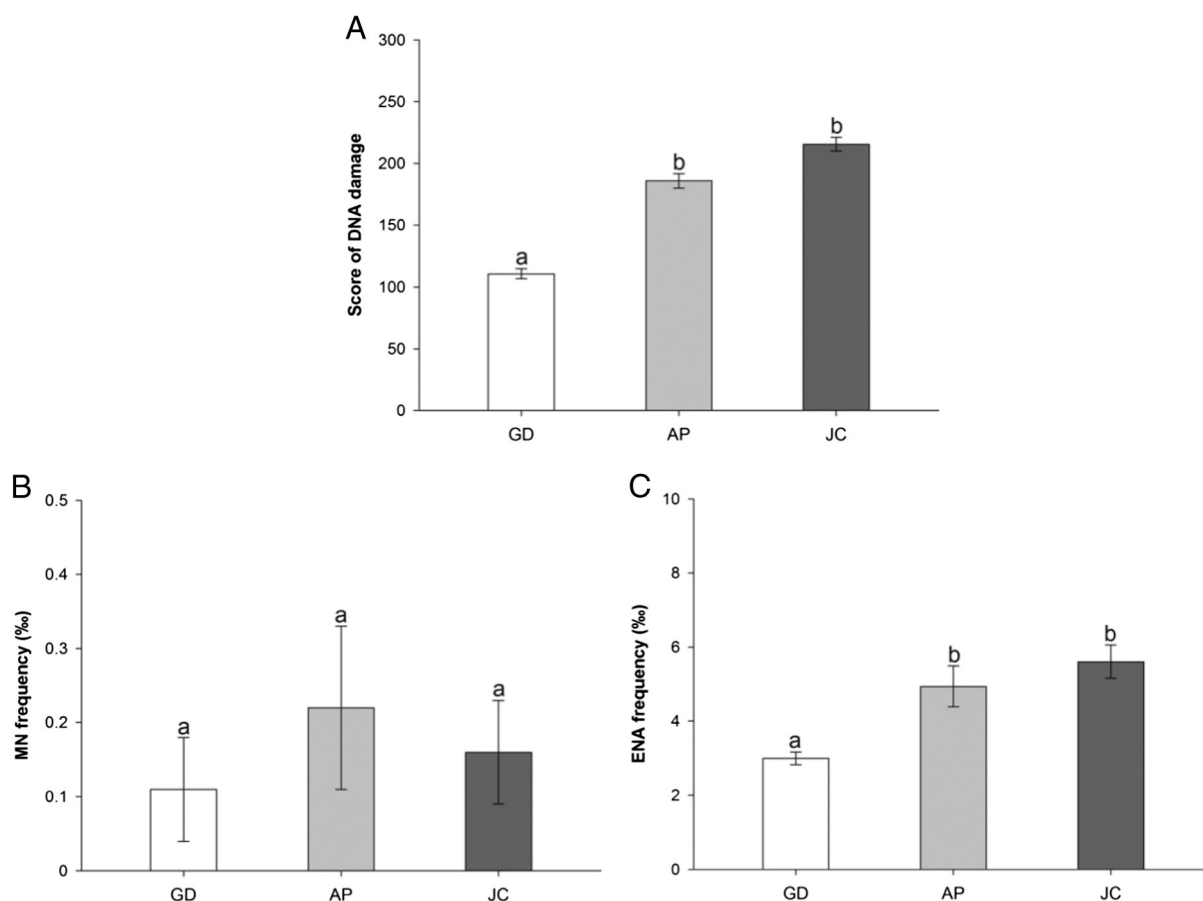


Fig. 5. Score of DNA damage (A) and frequency of micronuclei (MN) (B) and nuclear abnormalities (ENA) (C) in erythrocytes of *P. lineatus* caged at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams. Results are mean \pm SEM (n = 8). Different letters indicate significant differences between sites ($p < 0.05$).

of animals caged at AP showed a lower concentration of PCO compared to fish kept in GD. Grune et al. (2003) have shown that, at moderate oxidant concentrations, the degradation of damaged proteins increases, whereas higher oxidant concentrations can inhibit proteolytic degradation. Mild oxidation of soluble proteins enhances their proteolytic susceptibility while it appears that severely oxidized proteins may be stabilized due to aggregation, cross-linking and/or decreased solubility.

Table 4

Activity of 7-ethoxoresorufina-O-deethylase (EROD), glutathione-S-transferase (GST), acetylcholinesterase (AChE), concentrations of non-protein thiols (NPSH) and MT-like proteins (MT-like), lipid peroxidation (LPO), carbonyl proteins (PCO), and DNA damage (DNA dam) and erythrocytic nuclear abnormalities (ENA) in different tissues and cells of *P. lineatus* collected from the fish farming and acclimated in clean water under controlled laboratory conditions (baseline levels).

Biomarkers	Tissue-cell	Mean \pm SD (n = 48)
EROD (nmol resorufin min ⁻¹ ·mg protein ⁻¹)	Liver	0.37 \pm 0.03
GST (nmol CDNB conjugated min ⁻¹ ·mg protein ⁻¹)	Liver	155.62 \pm 5.47
	Gill	92.02 \pm 4.68
AChE (nmol min ⁻¹ ·mg protein ⁻¹)	Brain	55.87 \pm 1.94
	Muscle	105.2 \pm 4.08
NPSH (μg-SH·mg protein ⁻¹)	Liver	15.94 \pm 1.15
	Gill	11.63 \pm 0.93
MT-like (μg MT-like·mg protein ⁻¹)	Liver	4.78 \pm 0.36
LPO (nmol TBARS·mg protein ⁻¹)	Liver	0.59 \pm 0.04
	Gill	5.39 \pm 0.57
		0.0039 \pm 0.0004
PCO (nmol carbonyl·mg protein ⁻¹)	Liver	0.0031 \pm 0.0002
	Gill	0.0039 \pm 0.0004
Score of DNA damage	Erythrocytes	85.46 \pm 3.21
ENA frequency (%)	Erythrocytes	2.88 \pm 0.18

Thus, the decreased PCO levels in the gills of fish caged at AP may indicate an increase in proteolytic degradation due to mild oxidation, resulting in lower concentrations of PCO.

Inhibition of AChE activity was observed in both brain and muscle of fish exposed to AP and only in the brain of those confined at JC, in contrast to fish caged at GD. The inhibition of AChE is usually associated with the toxic action of organophosphate and carbamate insecticides (Aguar et al., 2004). However, AChE in fish can also be inhibited by other categories of pesticides, including organochlorides (Dutta and Arends, 2003) and herbicides such as glyphosate (Gluszczak et al., 2006, 2007; Cattaneo et al., 2011). It was already shown that glyphosate-based herbicides, such as Roundup® and Roundup Transorb®, inhibit AChE activity in brain and muscle of *P. lineatus* (Modesto and Martinez, 2010a, 2010b). The disruption in AChE activity can affect locomotion and balance in fish, compromising feeding, escape and reproductive behaviors (Bretaud et al., 2000; Pessoa et al., 2011).

Metallothioneins (MT) are important proteins for regulating and detoxifying essential and non-essential metals. They also play an important role in protecting cells against oxidative stress (Viarengo et al., 2000). In our study, the concentrations of MT-like proteins found in the liver of *P. lineatus* did not vary significantly. These results are probably due to the low concentrations of metals present in the three streams investigated, not sufficient to promote the synthesis of MT in the liver of the fish.

In regard to DNA damage, an increase in DNA damage scores in the erythrocytes of fish confined at AP and JC was observed. There was also an increase in the occurrence of ENAs in fish caged at AP and JC. The genotoxic effects of a number of pesticides, including organophosphates, organochlorides and pyrethroids, have already

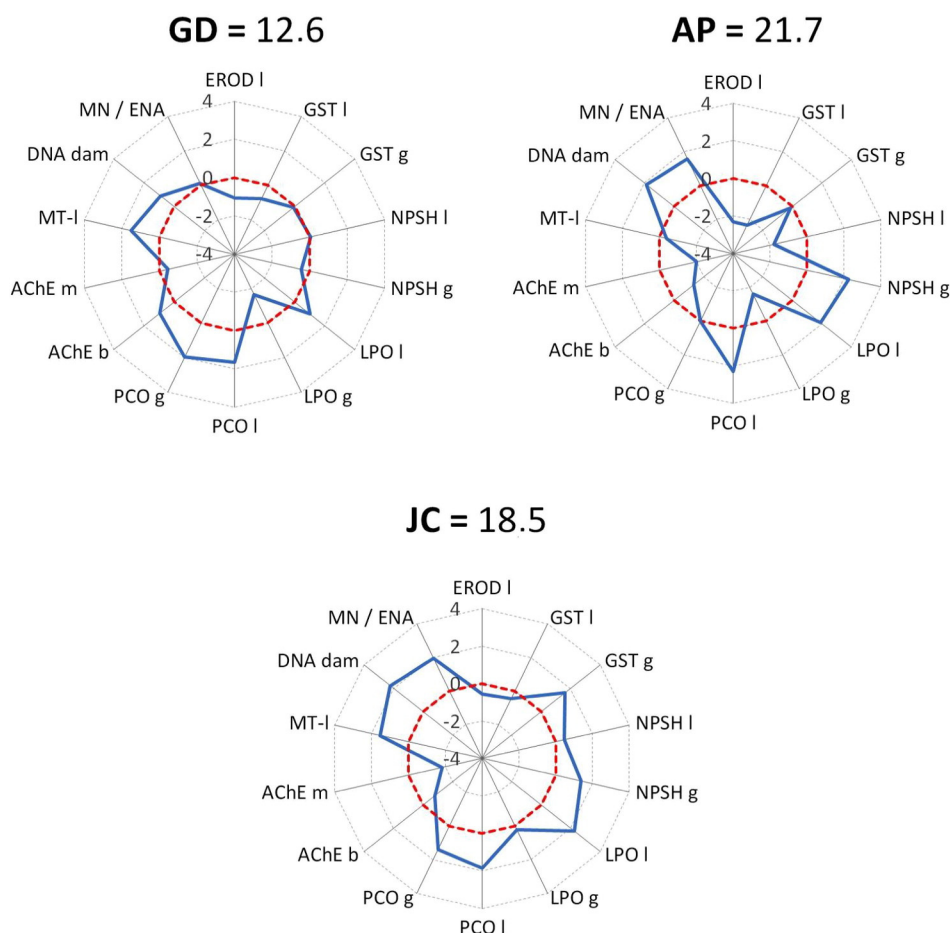


Fig. 6. Integrated biomarker response index (IBRv2) based on the following biomarkers: 7-ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), non-proteins thiols (NPSH), lipoperoxidation (LPO), carbonyl proteins (PCO), acetylcholinesterase (AChE), MT-like proteins (MT-like) in different tissues (l = liver, g = gill, b = brain, m = muscle), and DNA damage (DNA dam) and nuclear abnormalities (ENA) in erythrocytes of *P. lineatus* caged at Apertados (AP), Jacutinga (JC) and Godoy (GD) streams. Biomarkers results are represented in relation to the baseline group (dashed line). The area above 0 reflects induction of the biomarker and below 0 indicates reduction of the biomarker.

been documented in tests in vivo and in vitro (Bolognesi, 2003; Abdollahi et al., 2004; Kaushik and Kaushik, 2007). Monitoring an area potentially contaminated with pesticides, Ramsdorf et al. (2012) observed an increase in the frequency of ENAs and MNs, as well as DNA damage, in the fish *Astyanax* sp. Vieira et al. (2014) also found an increase in DNA breaks and occurrence of nuclear alterations in erythrocytes of a Neotropical fish caged at sites located in a region of intense agricultural activity.

Some pesticides detected in the streams in this study can also lead to genotoxic effects, even when tested alone. For example, the genotoxic effects of atrazine to fish were observed in several species, such as *P. lineatus* (Santos and Martinez, 2012), *Carassius auratus* (Çavas, 2011), *Oreochromis niloticus* (Ventura et al., 2008) and *Channa punctatus* (Nwani et al., 2011). In addition to this herbicide, an increase in the frequency of ENAs, MNs and DNA breaks has also been reported in fish exposed to endosulfan (Neuparth et al., 2006; Pandey et al., 2006).

Although the frequency of MNs did not increase significantly in any of the experiments, ENAs were augmented in fish confined at AP and JC. These results are in line with the DNA damage detected by using the comet assay. There are few explanations regarding the cause of these ENAs, but one of the most accepted was suggested by Shimizu et al. (1998), who stated that, when a damaged region in the DNA is detected, a repair process is initiated. The altered region of the DNA is then moved to the periphery of the nucleus and eliminated by exocytosis and before the process is completed, the nuclear membrane exhibits some imperfections, characterizing ENAs.

The occurrence of LPO in the liver together with the increased frequency of ENAs could indicate that oxidative stress is responsible for the formation of these nuclear alterations. LPO would lead to an increased permeability of the nuclear membrane, rendering the nucleus more susceptible to the xenobiotic-induced alterations (Seriani et al., 2011). The state of oxidative stress established in the fish could also explain, at least in part, the occurrence of DNA breaks. Among ROS, the most reactive with the DNA is the hydroxyl radical ($\text{HO}\cdot$), which can react with the deoxyribose component, resulting in DNA single-strand break, or can react with DNA bases, yielding oxidative base damage (Wang, 2008).

The Integrated Biomarker Response Index (IBRv2) has been found capable of discriminating scores based on the level of contamination of each site and the responses of a number of biomarkers. The IBR values were consistent with the differences found in the contamination levels at each site and the degree of anthropogenic interference in these environments, since the lowest IBR value was found in GD, inside the forest unit, confirming its better state of conservation compared to the other sites assessed. This is an evidence of the protective effect of the forest vegetation, which acts mainly as a barrier to surface runoff and the leaching of contaminants from the surrounding monocultures. In addition, the plant cover is capable of intercepting drifting pesticides sprayed from the air. In summary, the integrated analysis of various biomarkers reveals that the biochemical and genetic alterations observed correlate well with the levels of environmental contamination, demonstrating the efficiency of this biomonitoring approach. Other works comparing different levels of environmental contamination by using

biomarkers in aquatic organisms have already showed the efficiency of biomarkers integration for the environmental assessment (Damiens et al., 2007; Sanchez et al., 2008; Vieira et al., 2014).

In this study, we did not attempt to identify which pollutant at the experimental site was responsible for the effects on the fish, but rather to verify that biological effects are caused by a mixture of contaminants that can have environmental consequences. Despite the difficulty of interpreting the cause-and-effect relations of a given alteration in the organisms under field conditions, biomonitoring does allow a wider assessment of the natural conditions to which the organisms are subjected, providing promising instruments for identifying environmental impacts. Therefore, the results of this study are relevant for the development of new and ongoing monitoring programs in the region and warn about a serious environmental problem, which requires more detailed investigation in order to better understand the dynamics of the streams, resulting in greater or lesser toxicity for organisms. Our results could also contribute to the development of management and conservation actions for these streams.

Acknowledgments

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