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Integrated biomarker response index using a Neotropical fish to assess the water quality in agricultural areas

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Aquatic ecosystems in areas with intense agricultural activity are subject to pesticide contamination, which may compromise the health of the fish. In order to verify the quality of the water and the possible effects of pesticides on fish, a method that combines different biomarker responses into an index named "integrated biomarker response" (IBR) was applied using the biological alterations in the Neotropical fish *Astyanax altiparanae*. Fish were maintained *in situ* at five sites along a stream that runs in an agricultural area and in a stream within a forest fragment, considered a reference site. After seven days of exposure the following alterations were observed in fish confined at experimental sites: increased activity of glutathione-S-transferase (GST) and catalase (CAT) and increase in the content of reduced glutathione (GSH) in liver and gills, reduction of acetylcholinesterase (AChE) activity in the brain and muscle, increase in the occurrence of DNA strand breaks and in the frequency of micronuclei (MN) and nuclear abnormalities (ENA) in erythrocytes. The IBR highlighted three sites as the most affected, as the animals confined at these sites showed greater variations in biological responses. The biomarkers most important for the IBR results were GST, AChE, DNA breaks and ENA.

Ecossistemas aquáticos inseridos em áreas com intensa atividade agrícola estão susceptíveis à contaminação por pesticidas, os quais podem comprometer a saúde dos peixes. A fim de verificar a qualidade da água e os possíveis efeitos de pesticidas sobre peixes, um método integrando o uso de diferentes biomarcadores, denominado índice integrado de respostas de biomarcadores (IBR), foi aplicado utilizando-se as alterações biológicas avaliadas em vários órgãos do peixe neotropical *Astyanax altiparanae*. Os peixes foram confinados *in situ* em cinco pontos ao longo de um ribeirão localizado em área de produção agrícola e em um córrego dentro de um fragmento florestal, considerado um local de referência. Após sete dias foram observadas as seguintes alterações nos peixes confinados nos pontos experimentais: aumento da atividade da glutationa-S-transferase (GST) e catalase (CAT) e aumento do conteúdo de glutationa-reduzida (GSH) em fígado e brânquias, redução da atividade da acetilcolinesterase (AChE) no músculo e cérebro, aumento de quebras no DNA e na frequência de micronúcleos (MN) e alterações nucleares (ENA) em eritrócitos. O IBR destacou três dos pontos estudados como os mais afetados pela contaminação agrícola, uma vez que foram observados nos animais confinados nestes locais maiores variações nas respostas biológicas. Os biomarcadores mais significativos para os resultados de IBR foram a GST, AChE, quebras no DNA e ENA.

Key words: *Astyanax altiparanae*, Biomonitoring, Biotransformation, Genotoxicity, Pesticides.

Introduction

The agricultural expansion in Brazil was characterized by lack of planning and consequent destruction of natural resources, particularly forests. Throughout the history of the country the original forest cover has been giving more space for agricultural and livestock activities (Martins, 2001). As a result of this expansion, only in the last decade there has been an increase of 190% in the internal market of pesticides, making Brazil the world record in the consumption of these products (SINDAG, 2009). Between 2010 and 2011, about 936 tons of pesticides were used throughout the national territory

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(ANVISA & UFPR, 2012). In turn the Paraná State stands out in the Brazilian agricultural scenario, being in the third place in the national ranking of pesticide consumption, equivalent to 13% of the total sold in Brazil (ANVISA & UFPR, 2012).

Environmental pollution caused by these pesticides, especially in aquatic ecosystems has been documented worldwide and is a major problem both at local and global scales (Cerejeira *et al*., 2003; Spalding *et al*., 2003). However field studies that demonstrate the effect of these contaminants on aquatic fauna are lacking as well as the effects of the reduction of riparian vegetation and the use of the adjacent landscape, the quality of water resources and the health of aquatic biota, have been little investigated.

In order to monitor the effects of pesticides in the environment, *in situ* tests have proven useful, especially because they combine ecological relevance to toxicity testing, by incorporating field fluctuations under semi-controlled conditions. Fish have been extensively employed in *in situ* tests, because containment can be a useful strategy for monitoring of toxic agents in the aquatic environment (Barbee *et al*., 2008; Schlenk *et al*., 2008; Klobucar *et al*., 2010) through the analysis of different parameters.

Among these parameters, biomarkers are defined as variations induced by toxic agents in molecular or cellular components, processes, structures and functions, measurable in biological systems or samples (Depledge *et al*., 1995). These biomarkers may be able to provide an early warning signal well before severe environmental degradation has already occurred (Mouneyrac & Amiard-Triquet, 2013).

The application of biomarkers in environmental assessment is limited without an integrated system to overcome difficulties in relating information and in categorizing sites according to pollutant-induced changes in the health status of the organisms (Cravo *et al.*, 2012). The use of a methodology that integrates the responses of different biomarkers into a single value or graph has proven advantageous, as it allows a better understanding of the results and large-scale deployment of these tools in environmental monitoring (Sanchez *et al*., 2011). Among these indices, the Integrated Biomaker Response (IBR), described by Beliaeff & Burgeot (2002), is one of the most used in field and laboratory studies (Arzate-Cardenas & Martinez-Jeronimo, 2011; Serafim *et al*., 2012). Recently, Sanchez *et al.* (2013) proposed a second version for the index (IBRv2) to eliminate some drawbacks of the first version, and achieved satisfactory results.

Species of *Astyanax* have been pointed out as useful in environmental monitoring in several field studies (Schultz & Martins-Junior, 2000; Winkaler *et al*., 2001; Silva & Martinez, 2007; Lemos *et al*., 2008) and in laboratory (Akaishi *et al*., 2004; Rossi *et al*., 2011). The fish *Astyanax altiparanae* Garutti & Britski, 2000, is important in fishing and as food and is found in the microbasin of the Água das Araras Stream. The region where this microbasin is inserted, in northern Paraná State, consists of a complex of water springs that form important water bodies, placed into a mosaic of agricultural areas and forest fragments. The intensive use of pesticides, along with the reduction of riparian vegetation makes these water bodies even more susceptible to chemical contamination from agricultural activities. In this context this study aimed at evaluating the water quality of the Água das Araras Stream through biochemical and genotoxic biomarkers on the fish species *A. altiparanae* subjected to *in situ* tests at different sites along the stream.

Material and Methods

Study area

The Água das Araras stream is located in Paraná State, Southern Brazil, and runs about 18 km from its source to its mouth, on the left bank of the Laranjinha River. The region of the stream is characterized by intensive agricultural activity, with predominance of crops of corn, wheat and soybean. Along its course, besides crossing agricultural areas, the stream passes through a conservation unit, the Parque Estadual Mata São Francisco (PEMSF). For this study, we selected five experimental sites along this stream (Fig. 1).

Site 1 (23°09’39.2”S 50°35’52.4”W): artificial impoundment used for aquaculture, near the main source of the stream, with banks devoid of riparian vegetation, surrounded by corn monoculture.

Site 2 (23°10’05.2”S 50°33’18.3”W): at the southern limit of the PEMSF, close to wheat and corn fields. Although this stretch is still protected by the vegetation of the park, proximity to these crops may be interfering with the water quality.

Site 3 (23°09’38.4”S 50°31’27”W): in the middle portion of the stream, with banks lacking riparian vegetation, predominance of grass, surrounded by cornfields. At this location, there is a station for water abstraction that supplies the municipality of Santa Mariana, Paraná State.

Site 4 (23°09’48.5”S 50°30’08.9”W): in the upper-middle portion of the stream, with banks also devoid of riparian vegetation, and proximity to wheat and corn crops.

Site 5 (23°09’59.0”S 50°28’57.0”W): in the upper portion of the stream, with total absence of riparian vegetation. Among all the sites evaluated, this is the one that may be receiving the largest load of agricultural contaminants, since monocultures in the surroundings reach a few meters from the bed of the stream.

Due to the lowest level of human interference it was taken as reference (Ref) a site (23°09’23.6”S 50°34’13.8”W) in a small stream located inside the PEMSF, which has its bed protected by forest vegetation.

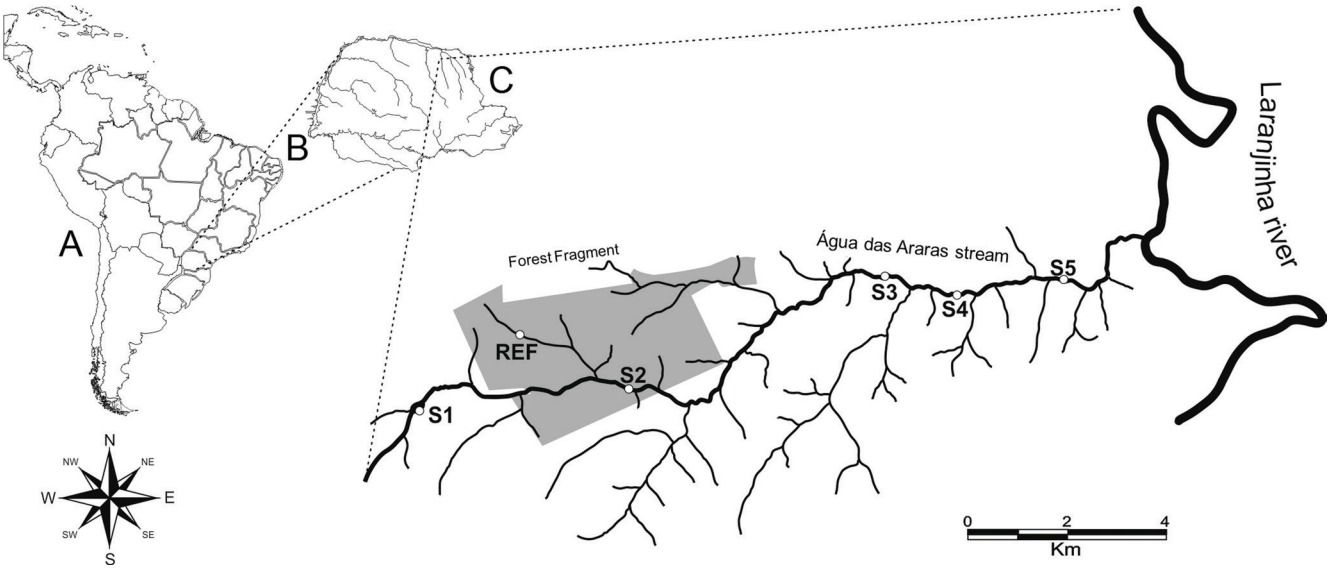

Fig. 1. Maps are showing South America (A), Paraná State (B) and Água das Araras stream (C) indicating the five experimental sites along of the stream (S1, S2, S3, S4, and S5) and the reference site (Ref), which is a small stream within a forest fragment named "Parque Estadual Mata São Francisco".

***In situ* tests**

Specimens of *A. altiparanae* (N = 48) weighing 8.09 ± 0.35 g and total length of 8.43 ± 0.09 cm (mean ± SE), obtained from a fish farm, were kept in tanks containing dechlorinated water with constant aeration for at least seven days before the start of the *in situ* tests, which were conducted during the winter (July and August 2011). Fish were transported to the field in plastic bags containing water and oxygen and confined (N = 8 per site) in cages (50 x 50 x 50 cm) in the different experimental sites, where they remained for seven days. Voucher specimen of *A. altiparanae* (MZUEL 6465) was deposited in the fish coleção do Museu de Zoologia da Universidade Estadual de Londrina (MZUEL), Brazil.

Water parameters (pH, dissolved oxygen, temperature, and conductivity) were determined on the first and last day of exposure, with a multiparameter probe (HANNA- HI 9828) in all the sites. It was also conducted a survey with farmers in the study area in order to check the most used pesticides.

Sampling

After the exposure period, fish were removed from the cages, anaesthetized with benzocaine (0.1g 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, muscle and brain were taken and maintained in dry ice. In the laboratory, samples were stored in ultrafreezer (-80°C) until biochemical analyses. These procedures were performed according to the protocol approved by the Animal Experiments Committee of Londrina State University.

Biochemical analysis

Samples of liver, gill, muscle and brain were weighed, homogenized (1:10 w/v) in potassium phosphate buffer (0.1M, pH 7.0) and centrifuged (10000 g, 20 min, 4°C). The supernatant was used for biochemical analyses.

The activity of glutathione S-transferase (GST) was determined by monitoring the complexation of reduced glutathione (GSH) with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) in a spectrophotometer at 340 nm (Keen *et al*., 1976). The enzyme activity was expressed as nmol CDNB conjugates. min^{-1}. mg $protein^{-1}$.

The activity of catalase (CAT) was determined from the rate of decomposition of H_2O_2 by the enzyme, based on the decrease in absorbance at 240 nm (Beutler, 1975). The enzyme activity was expressed as µmol $H_2O_2.min^{-1}$.mg $protein^{-1}$.

The concentration of reduced glutathione (GSH) 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. The GSH concentration was expressed in µg GSH.mg $protein^{-1}$.

The activity of acetylcholinesterase (AChE) was determined according to the method described by Ellman *et al.* (1961) adapted to microplate by Alves Costa *et al.* (2007),

using the substrate acetylcholine iodide and the color regent 5,5-dithiobis-2-nitrobenzoic acid (DTNB) at 415 nm. AChE activity was expressed in nmol min^{-1}.mg $protein^{-1}$.

The total protein concentration was determined in a spectrophotometer at 700 nm according to the method of Lowry *et al.* (1951) using a standard curve of bovine serum albumin (BSA).

Genotoxic analysis

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: 1h 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 1000X. All slides were analyzed in blind test, being evaluated 100 nucleoids per fish. The extent of DNA damage was quantified by the length of the tail formed by the migration of DNA fragments and were 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 Heddle (1973) and Schmid (1975) and the occurrence of erythrocytic nuclear abnormalities (ENA) was analyzed according to Carrasco *et al.* (1990). The ENA were classified according to Monteiro *et al.* (2011) in three categories: segmented nucleus, lobulated nucleus, and kidney-shaped nucleus. 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 1000 erythrocytes per fish were examined on an Olympus microscope (1000x magnification). The mean frequency of micronuclei (MN) and erythrocytic nuclear abnormalities (ENA) of each site was calculated and expressed per 1000 cells (‰).

Integrated Biomarker Response (IBR)

The biomarkers results were applied into the "integrated biomaker response" (IBR) index, described by Beliaeff & Burgeot (2002) and modified by Sanchez *et al.* (2013). For IBR calculation, the ratio between the experimental sites individually and the reference site for each biomarker was log-transformed (Yi) and then the overall mean (μ) and standard deviation (s) was calculated. Then, Yi values were standardized by the formula: $Z_i = (Y_i - \mu)/s$ and the difference between Z_i and Z_0 (reference) determined A values. IBR value was calculated for each exposure site by the sum of A values.

For each site data were represented in a radar chart indicating the deviation of biomarker investigated in relation to the reference site (0). The area above 0 reflects induction of the biomarker, and below 0 indicates reduction of the biomarker.

Table 1. Physical and chemical parameters of the water from the reference site (Ref) and from the sites along Água das Araras stream (S1, S2, S3, S4, and S5) collected in the first and last day of fish exposure.

Site	pH	Dissolved oxygen (mg.L^{-1})	Temperature (**°C**)	Conductivity (**μS**.cm^{-1})
Ref	7.28 - 6.89	8.50 - 8.10	17.90 - 19.11	92 - 96
S1	7.53 - 7.37	5.41 - 5.38	20.42 - 19.37	92 - 91
S2	8.00 - 8.50	5.63 - 5.68	16.32 - 17.32	79 - 76
S3	7.46 - 7.21	8.10 - 8.36	18.10 - 19.50	90 - 91
S4	7.20 - 7.43	7.71 - 7.56	19.50 - 19.00	121 - 119
S5	7.56 - 7.58	8.58 - 8.24	17.90 - 20.20	120 - 126

Table 2. Most used pesticides in the watershed area of the Água das Araras stream. Class: H = herbicide; I = inseticide; IA = inseticide/acaricide; F = fungicide.

Trade name	Class	Chemical group	Active ingredient
Roundup®	H	derived glycine	glyphosate
Curyom 550 CE®	I	organofosforate	profenofos + lufenuron
Endosulfan Nortox 350 EC®	IA	organochlorine	endosulfan
Actara 250 WG®	I	neonicotinoid	thiamethoxam
Metamidofos Fersol 600®	IA	organofosforate	methamidophos
Engeo Pleno ®	I	neonicotinoid/pyrethroid	lambda cialotrine
Connect®	I	neonicotinoid/pyrethroid	imidacloprid + beta-ciflutrine
Karate Zeon 50®	I	pyrethroid	lambda cialotrine
Match®	I	acilurea	lufenuron
Rimon 100®	I	benzophenyl urea	novalurom
Mentox 600 ®	IA	organofosforate	methyl parathion
Atrazina Nortox 500®	H	triazine	atrazine
Sanson 40®	H	sulfonylurea	nicosulfuron
Tamaron BR ®	IA	organofosforate	methamidophos
Ally®	H	sulfonylurea	metsulfuron- methyl
Ampligo®	I	pyrethroid	lambda cialotrine
Premio®	I	antranilamide	clorantraniliprole
Talstar 100 CE®	IA	pyrethroid	bifentrine
Priori Xtra®	F	estrobilurin/ triazole	azoxistrobine + ciproconazole
U-46 BR®	H	ariloxialcanoic acid	2,4 D

Statistical analysis

The mean values obtained from each biological variable analyzed in fish from different exposure sites were compared with each other by parametric analysis of variance (ANOVA), after checking for normality and homogeneity of variance. When necessary, differences were identified by Student-Newman-Keuls *post hoc* test. The significance level was set at $P < 0.05$.

Results

The results of chemical and physical parameters of the water collected from the different sites at the beginning and at the end of the exposure period of the animals are shown in Table 1. There were no marked differences in evaluated parameters between the first and last day of the experiment. Sites 1 and 2 showed the lowest concentration of dissolved oxygen. This can be explained because these stretches have slower water flow, compared with the other sites. Likewise, the site 1 also presented the highest temperature because it is an impoundment, which receives a higher incidence of solar radiation. The survey with local farmers identified the 20 agrochemical contaminants that could be present in the study area (Table 2). Among the pesticides used in the region, 45% correspond only to insecticides, 25% are herbicides, standing out glyphosate and atrazine as the most widely used in the region, and the rest are insecticides/ acaricide and fungicides.

The data obtained from analyses with biomarkers showed a significant increase in GST activity in the liver of fish exposed in all experimental sites with respect to the reference site (Fig. 2A). In the site 1, it was observed a significant increase in enzyme activity with respect to all other sites. In the gills, there was a significant increase in GST activity in animals kept in sites 3, 4, and 5 compared to the reference site (Fig. 2B).

The activity of liver CAT was significantly increased in sites 3 and 5 in relation to the others (Fig. 3A), and in the gills was also observed a significant increase in enzyme activity in sites 1, 3, 4, and 5 (Fig. 3B). It should be emphasized that the CAT activity determined in the gills was very low and in fish from the reference site the enzyme activity was about 50 times lower than in the liver.

The concentration of GSH increased significantly in the liver of fish confined in the sites 1, 4, and 5 (Fig. 4A) and in the gills of fish from sites 1, 3, 4, and 5 (Fig. 4B), with respect to the reference site.

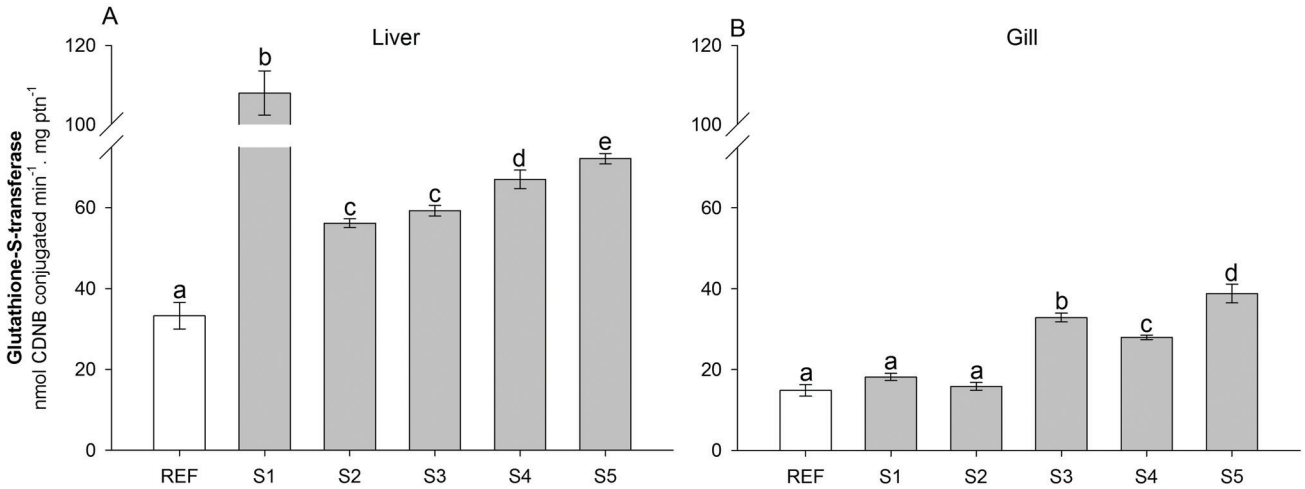

Fig. 2. Activity (mean ± SEM, n = 8) of glutathione S-transferase in liver (A) and gills (B) of *A. altiparanae* exposed *in situ* for seven days in five sites along Água das Araras stream (S1, S2, S3, S4, and S5) and in a reference site (Ref). Different letters indicate significant differences between sites ($P < 0.05$).

Results showed a significant reduction in AChE activity both in muscle (Fig. 5A) and brain (Fig. 5B) of fish confined in sites 1, 2 and 4 compared to the other sites.

The results of the comet assay showed a significant increase in the occurrence of DNA damage in fish erythrocytes in all sites of the Água das Araras stream, with respect to fish kept in the reference site (Fig. 6). Fish from site 5 showed a higher DNA damage score than all other locations. The frequency of MN (Table 3) was significant higher in fish from sites 1, 3, and 5 in relation to animals from the reference site, while ENA frequency was significantly higher only in fish from site 5 in relation to the reference.

IBR values for each location of the *in situ* tests are shown in Fig.7. The results demonstrated a possible discrimination between the sites in terms of the level of contamination along the stream. The site 5 had the highest IBR value (24.42) being considered the location possibly most affected by agricultural contaminants. Increased GST, CAT, GSH content, DNA strand breaks and frequency of MN and ENA were the most discriminant factors for this site. Sites 1 and 4 showed almost the same value of IBR (20.11 and 20.34, respectively). In both locations, variations in the activity of GST, liver GSH concentration, AChE activity and mutagenic and genotoxic damages, were the most relevant responses that explain the IBR value. The site 2 presented the lowest IBR value (11.98) once it was observed minor variations in analyzed biomarkers.

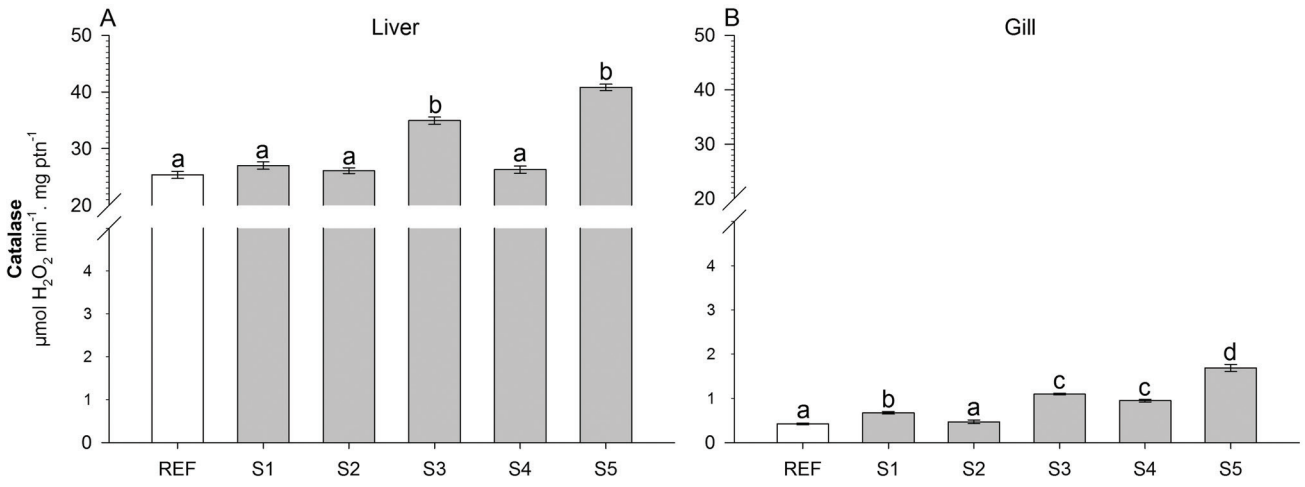

Fig. 3. Activity (mean ± SEM, n = 8) of catalase in liver (A) and gills (B) of *A. altiparanae* exposed *in situ* for seven days in five sites along Água das Araras stream (S1, S2, S3, S4, and S5) and in a reference site (Ref). Different letters indicate significant differences between sites ($P < 0.05$).

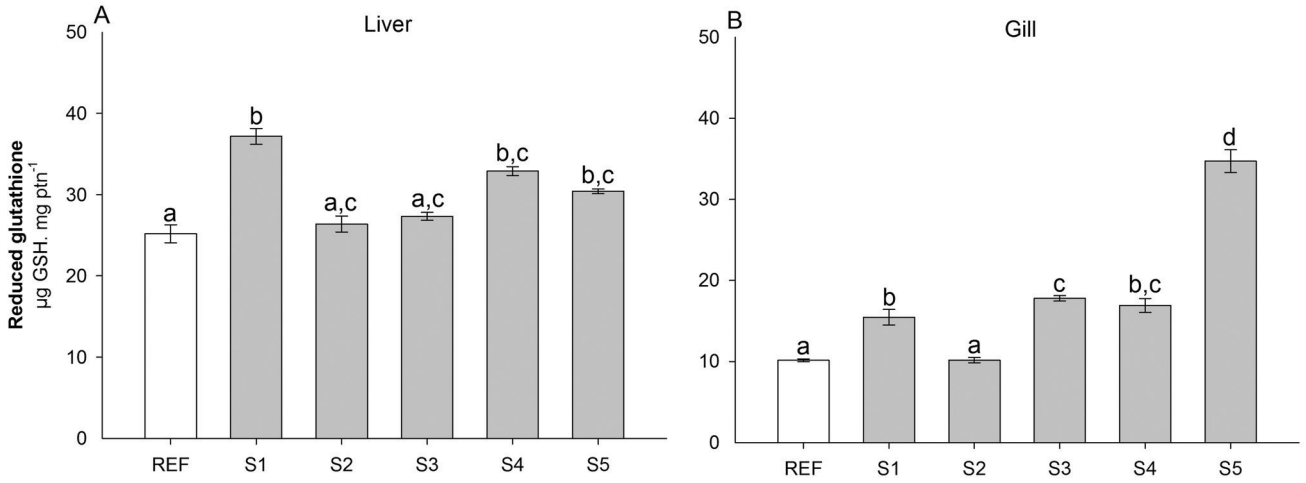

Fig. 4. Content (mean ± SEM, n = 8) of glutathione in liver (A) and gills (B) of *A. altiparanae* exposed *in situ* for seven days in five sites along Água das Araras stream (S1, S2, S3, S4, and S5) and in a reference site (Ref). Different letters indicate significant differences between sites (P<0.05).

Discussion

One of the most investigated biochemical biomarkers in fish are the enzymes involved in the detoxification of toxic agents and their metabolites, such as biotransformation and antioxidant defense enzymes. The family of glutathione-S-transferase (GST) enzymes is essential in the protection against damage from potentially reactive compounds, combining them with endogenous molecules such as reduced glutathione (GSH), to be later eliminated by the body. The activity of this enzyme is considered a good biomarker of exposure to environmental pollutants (Van der Oost *et al*., 2003).

The increased GST activity in the liver and gills of fish caged at various sites along the Água das Araras stream can indicate the presence of xenobiotics that are metabolized by conjugation with GSH, to be eliminated from the body. Several authors have reported increased GST activity in different fish species exposed to pesticides, some of which are used in the study region. For example, Dong *et al.* (2013) reported increased GST activity in *Danio rerio* larvae exposed to the organochlorine endosulfan and Oruc *et al.* (2004) observed increased activity of this enzyme in two species of fish exposed to 2,4 D. Paulino *et al.* (2012) observed an increase in GST activity in gills of the Neotropical fish *Prochilodus lineatus* after sub-chronic exposure to atrazine, a herbicide widely used to control undesirable organisms in corn crops (Mudiam *et al*., 2012). The present study also observed an increase in GST activity in gills of fish exposed to sites 3, 4, and 5, which have close proximity to corn fields.

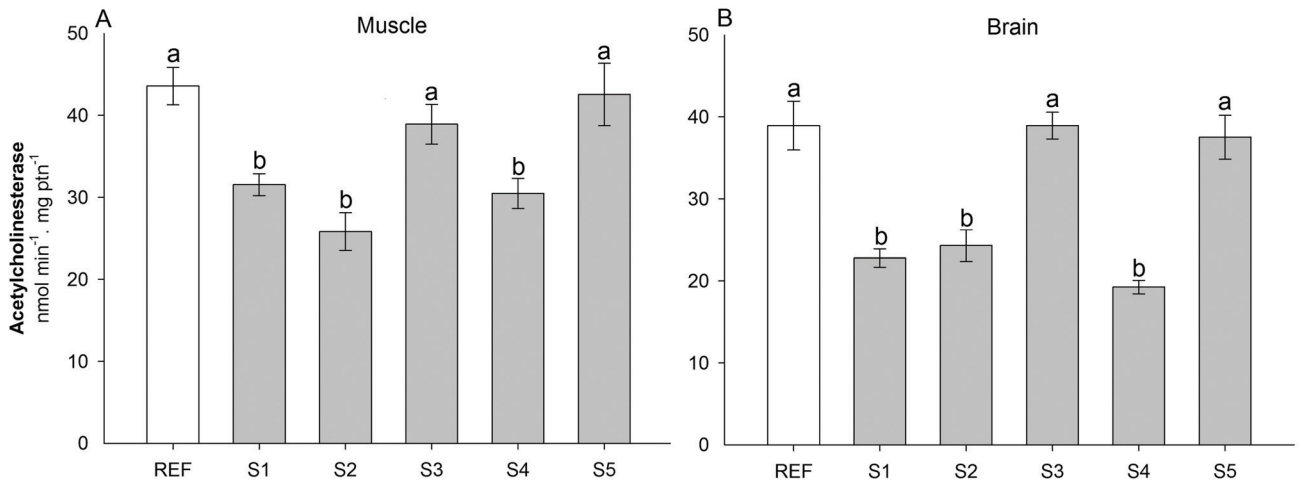

Fig. 5. Activity (mean ± SEM, n = 8) of acetylcholinesterase in muscle (A) and brain (B) of *A. altiparanae* exposed *in situ* for seven days in five sites along Água das Araras stream (S1, S2, S3, S4, and S5) and in a reference site (Ref). Different letters indicate significant differences between sites (P < 0.05).

Table 3. Frequency of micronuclei (MN) and other nuclear abnormalities (ENA) in erythrocytes of *A. altiparanae*, taking into account the total number of fish (N) analyzed from the reference site (Ref) and from each site located along Água das Araras stream (S1, S2, S3, S4, and S5). Different letters indicate significant differences between sites ($P < 0.05$).

Site	N	MN frequency (‰)	ENA frequency (‰)
Ref	8	0.50 ± 0.18 [a]	1.57 ± 0.29 [a]
S1	8	2.85 ± 0.69 [b]	3.71 ± 0.83 [a,b]
S2	8	1.25 ± 0.25 [a,b]	2.87 ± 0.89 [a,b]
S3	8	2.37 ± 0.32 [b]	4.50 ± 0.56 [a,b]
S4	8	1.66 ± 0.28 [a,b]	3.57 ± 0.84 [a,b]
S5	8	3.62 ± 0.37 [b]	6.14 ± 0.85 [b]

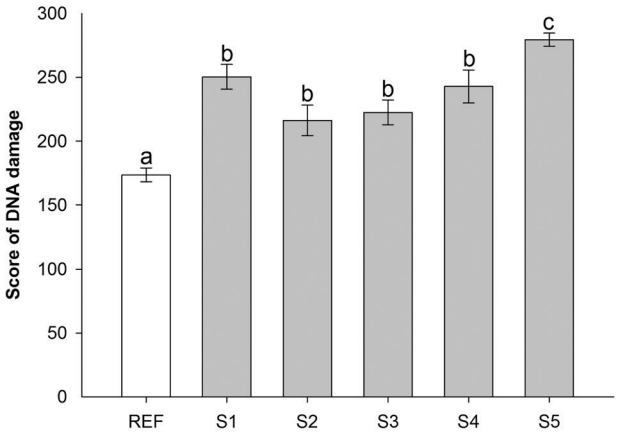

Fig. 6. DNA damage scores (mean ± SEM, n = 8) in erythrocytes of *A. altiparanae* exposed *in situ* for seven days in five sites along Água das Araras stream (S1, S2, S3, S4, and S5) and in a reference site (Ref). Different letters indicate significant differences between sites ($P < 0.05$).

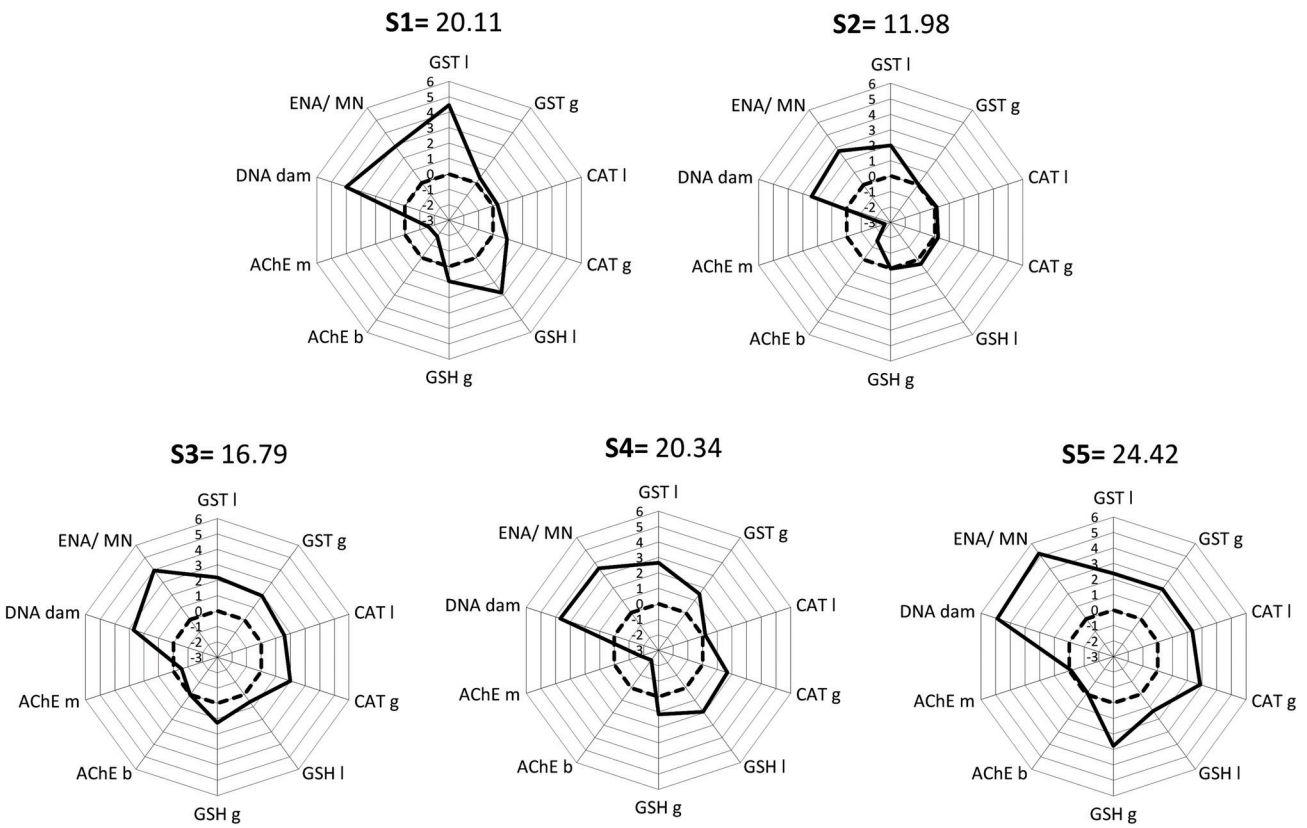

Fig. 7. Integrated biomarkers response index (IBR) values for each experimental site along Água das Araras stream (S1, S2, S3, S4, and S5) where *in situ* tests were performed. Biomarkers results are represented in relation to the reference site (0). The area above 0 reflects induction of the biomarker, and below 0 indicates reduction of the biomarker.

Another widely used herbicide in the study area is Roundup®, a glyphosate based product. The effects of this herbicide on aquatic organisms have been addressed, especially in fish (Glusczak *et al*., 2006; Cattaneo, 2011; Rossi *et al*., 2011). Modesto & Martinez (2010a) have shown that this herbicide increases the activity of liver GST and other enzymes involved in antioxidant defense of *P. lineatus*.

The antioxidant defense system has been increasingly studied given the ability of oxiradicals to promote responses that are used as biomarkers (Di Giulio *et al*., 1989; Winston & Di Giulio, 1991). The main components of this defense system are enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). The catalase (CAT) works in eliminating H_2O_2, producing H_2O and O_2. In this study, an increase in the activity of this enzyme was observed in the liver of fish from sites 3 and 5 and in the gills of animals from sites 1, 3, 4 and 5, suggesting an increase in antioxidant defense to eliminate reactive oxygen species (ROS), mainly formed during the metabolism of chemical compounds. Although it was observed an increase in CAT activity in the gills, it should be borne in mind that this organ has very low activity of this enzyme, compared with the liver, as demonstrated for other fish species, such as *P. lineatus* (Simonato *et al*., 2011). Wilhelm Filho *et al.* (1994) suggest that gills may have alternative mechanisms to eliminate hydrogen peroxide.

Various pesticides can lead the organism to a state of oxidative stress, causing an increase in ROS generation and changes in antioxidant defense mechanisms. Several studies have shown that organophosphate pesticides promote oxidative changes in fish species, such as in *Cyprinus carpio*, *Ictalurus nebulosus* (Hai *et al*., 1997) and *Anguilla anguilla* (Peña-Llopis *et al*., 2003) exposed to dichlorvos, in *Brycon cephalus* exposed to Folisuper BR 600 (methyl parathion) (Monteiro *et al*., 2009) and in *Oreochromis niloticus* exposed to fenthion (Piner *et al*., 2007) and trichlorfon (Thomaz *et al*., 2009). Glyphosate-based herbicides are also reported to induce oxidative stress in different fish species (Lushchak *et al*., 2009), including *Leporinus obtusidens* (Glusczak *et al*., 2006), *Rhamdia quelen* (Glusczak *et al*., 2007) and *P. lineatus* (Modesto & Martinez, 2010a).

Glutathione (GSH), a tripeptide that plays a key role in reactions of oxidation/reduction, amino acid transport and detoxification of many toxic agents, is the first line of defense against cellular damage mediated by oxidants (Van der Oost *et al*., 2003). The increased concentration of liver GSH in fish confined in sites 1, 4, and 5 may be related to increased production of ROS, since GSH can be directly used in neutralizing these reactive oxygen species. This increase may also indicate a higher synthesis of GSH to sustain the increased GST activity, which uses this peptide in the conjugation with xenobiotics, considering that the activity of this biotransformation enzyme was also high in these same sites. In the gills, it was also observed an increased concentration of GSH in the sites 1, 3, 4, and 5, where it was found increased gill CAT, indicating a possible adaptation of antioxidant defenses.

In relation to AChE activity, there was an inhibition of this enzyme activity in both brain and muscle of fish exposed in the sites 1, 2, and 4. This enzyme, which occurs in cholinergic synapses and motor end-plates, is responsible for the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid. Inhibition of AChE is classically associated with the mechanism of toxic action of organophosphates and carbamates insecticides (Payne *et al*., 1996) and disturbances on its activity may affect locomotion and balance in fishes, impairing feeding, escape and reproductive behavior (Pessoa *et al*., 2011). AChE activity in fish can also be modified by other classes of pesticides, like organochlorines such as endosulfan (Dutta & Arends, 2003). Other authors have also demonstrated the anticholinesterase effect of glyphosate in different fish species (Glusczak *et al*., 2006; Glusczak *et al*., 2007; Cattaneo *et al*., 2011) as well as glyphosate based-products, Roundup® and Roundup Transorb® on *Prochilodus lineatus* (Modesto & Martinez, 2010a, 2010b).

In assessing DNA damages, it was observed an increase in damage scores in erythrocytes of fish confined in all experimental sites in relation to the control. There was also an increase in the occurrence of MN in erythrocytes of fish confined in the sites 1, 3, and 5 and increased frequency of ENA in fish of the site 5. Genotoxic effects of various groups of pesticides, such as organophosphates, organochlorines, and pyrethroids have been demonstrated *in vivo* and *in vitro* tests (Bolognesi, 2003; Abdollahi *et al.*, 2004; Kaushik & Kaushik, 2007).

The effects of atrazine on genetic material have also been described for fish. Santos & Martinez (2012) observed increased occurrence of DNA damage in blood, liver and gill cells in fish exposed to this herbicide. Cavas (2011) observed a significant increase in the occurrence of DNA damage and the frequency of micronuclei in erythrocytes of *Carassius auratus* after exposure to atrazine. Ventura *et al.* (2008) observed an increase in DNA strand breaks and in the frequency of MN and ENA in erythrocytes of *O. niloticus* exposed to different concentrations of atrazine after only 72 hours of exposure. Likewise, Nwani *et al.* (2011) observed an increase of MN for *Channa punctatus* after seven days of exposure to atrazine.

Genotoxic effects of glyphosate on fish have also been reported in the literature. Cavalcante *et al.* (2008) verified an increase in DNA strand breaks in blood cells of *P. lineatus* exposed to commercial formulation of the herbicide. Similarly, Rossi *et al.* (2011) reported an increased frequency of MN and ENA in *Astyanax* sp. exposed to this pesticide. Ramsdorf *et al.* (2012) also observed an increase in the frequency of

ENA and MN and DNA damage in species of *Astyanax* sp. collected in the area potentially contaminated with pesticides, including Roundup, compared with the amount of changes found in fish collected from a reference site. In the same way, Cavas & Könen (2007) registered an increase in the frequency of MN and ENA in *Carassius auratus* after four days of exposure to different concentrations of glyphosate. In addition to these pesticides, increased frequency of ENA and MN and DNA strand breaks have also been described in fish exposed to herbicide endosulfan (Neuparth *et al*., 2006; Pandey *et al*., 2006).

Formation of micronuclei is a short term response to a genotoxic substance, so that their expression depends on the intensity of exposure to contaminants and probably independent of the duration of such exposure (Heddle *et al*., 1991). The increased frequency of MN and ENA in some sites evaluated may indicate the presence of pesticides able to promote mutagenic damage in erythrocytes of *A. altiparanae*.

The integrated biomarker index (IBR) was able to discriminate the sites based on the biomarkers responses. The sites with higher IBR values were 5, 4, and 1, respectively. These results are consistent with the degree of local human interference, as they are the stretches of the stream with the lowest cover of riparian vegetation, which is non-existent in some stretches, and are located in areas with more intensive farming activities, which come very close to the bed of the stream. The site 2 also located within the forest unit, had the lowest IBR value among the evaluated sites. Thus, we can assume the protective effect of this vegetation as a barrier to the runoff and leaching of these contaminants coming from the surrounding monocultures. Nevertheless, important variations observed in some parameters evaluated in fish confined in the site 2 still indicate the presence of contaminants in these waters.

As proposed by Beliaeff & Burgeot (2002) the IBR can be associated with a star or radar chart that shows the specific responses of biomarkers in each site analyzed. In the present study, several biomarkers exhibited a response that was induced or inhibited according to the sampling site and the spatial arrangement of these biomarkers in the star plot allowed visualizing more clearly which biomarkers were the most sensitive in this kind of evaluation. Thus, the comet assay that assesses DNA damage, the enzymatic activity of GST in the liver and the frequency of MN and ENA, besides the activity of AChE were the biomarkers that proved to be more efficient in this study.

In summary, our results show that the quality of the Água das Araras stream is impaired from the headwaters to the mouth, with some attenuation in the site located within the conservation unit, and suggest that contamination by pesticides is the main responsible for the reduced water quality of this stream. This *in situ* approach using biomarkers in *Astyanax altiparanae* was effective to evaluate water quality. New and ongoing monitoring programs in these sites should be established, combining the use of biomarkers and *in situ* exposure with chemical analysis of water, aiming to identify the pesticides present in this mixture and relate them to the observed effects in animals.

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