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Evaluation of metal contamination effects in piranhas through biomonitoring and multi biomarkers approach

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ABSTRACT

The present field study aimed to assess the water quality of the Itacaiúnas River, located at the eastern part of the Brazilian Amazon, using water's physicochemical parameters, ecological risk assessment in sediments, biomarkers and metal bioaccumulation in piranhas at two points: upstream (P1) and downstream (P2), and the type of season (Dry and Rainy). We revealed a significant difference between the points and the seasons. Concerning, the concentration of metals (Cd, Cu, Zn, Cr, and Hg) in sediment and water, we reported significant concentrations of Cd and Cu especially on P2 at dry season. The fish gills collected in P2 showed various types of damages (moderate and severe), furthermore, the Degree of Tissue Change (DTC) reported a significant difference between points, highlighting the dreadful condition in animals' health originated from this point. In terms of the biotransformation enzyme, the GST activity was higher in fishes from P2 in both seasons. The obtained results showed clear signs of stress in fish from the downstream point. Linear correlation analysis exhibited that the biomarkers' response could be linked to the detected metals bioaccumulation. This field investigation provides baseline data on pollution status in this region and the results showed that although the overall potential ecological risks of the metals were considered low at our sampling sites including cadmium, however, Cd posed a noteworthy monomial potential ecological risk factor. Strong evidence of correlation was obtained between Cd in the environment with the gills' damage in fishes from P2. The results also indicated that *S. rhombeus* could be useful for biomonitoring species for assessing metal contamination.

1. Introduction

The Itacaiúnas River Basin (IRB) (05°10′ to 07°15′S latitude, 48°37′to 51°25′ longitude), a Tocantins tributary and part of the Amazon Biome, with approximately 41,500 km2 is located in the Brazilian Amazonia, Carajás region, Pará State, Northern Brazil (Damous et al., 2002; Pontes et al., 2019) The Carajás complex comprises one of the largest mineral reserves in the world. There, among other activities that include iron and gold mining, a metallurgic project is under implementation to extract and upgrade copper from sulfide ore. About 1.2 × 109 tons of copper is present in three deposits extending over an area of 25,000 ha (Moreto et al., 2015; Sahoo et al., 2019).

The population of the IRB is approx. 700,000 people in an area of approx. 41,500 km2; the basin is economically important and is intensely mined. Originally, tropical rainforest was the predominating land cover in the IRB with subordinate mountain savanna, but at the present, the tropical rainforest is mostly limited to environmental protected areas and indigenous lands, which cover 11,700 km2 or approximately a quarter of the total area pasture lands. IRB had 50% of its area deforested between the 1970s and 2010s, causing an increase of temperature (+1.7 °C) and the reduction of air relative humidity (−10%) in the basin (Souza-Filho et al., 2016). The remaining forested areas are concentrated in a block of continuous protected areas and it is monitored and protected by a partnership between the Chico Mendes Biodiversity Conservation Institute (ICMBIO) and Vale S.A. (Pontes et al., 2019).

The studied region presents intense mining activity and has large economic, environmental and social relevance. For this reason, studies to assess the impact of mining activity were developed. They include research on the conservation of biodiversity (Lanes et al., 2018) and water quality, sediment geochemistry and geomorphologic aspects

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(Guimarães et al., 2019; Sahoo et al., 2019) and metals biomonitoring (Lacerda et al., 1994). (Borges et al., 2018) also at Itacaiúnas River, have reported concentrations of Hg among other parameters above the limits established by the Brazilian legislation. Additionally, the authors showed tissues damages in *Serrasalmus rombeus.*

Since the major causes of metal emission are mining operations, due to the emitted metals that continue to persist in the environment, causing serious damages even long after the activities have ceased (Ahmad et al., 2012; Krishna et al., 2013). Thus, it is extremely necessary to evaluate this environment through biomonitoring studies to have an overview of the health of this region. The assessment of environmental monitoring through multi biomarkers has been widely used in many studies (Ballesteros et al., 2017; de Oliveira et al., 2019; Regoli and Giuliani, 2014). Despite the well-known literature with this type of investigation, it is essential to evaluate these effects in different species of animals and in specific environments of very peculiar characteristics where these can directly influence the bioavailability of the contaminants. Therefore, the consideration of the Amazonian ecosystem becomes extremely unique.

Biomarkers that show the effects caused by the action of pollutants are determined and evaluated to have an insight into the environmental situation and aquatic organisms, such as fish, have been often used in this study over the years (Colin et al., 2016). Histological-ultrastructural analysis, and biochemical changes in different fish tissues, such as gill, is widely used in biomonitoring and aquatic toxicology (da Silva Montes et al., 2015; Tabassum et al., 2016). Thus, the morphological and physiological changes in the gills of fish can provide an assessment method to evaluate how environmental stressors can affect fish populations (Van der Oost et al., 2003).

The successful biomonitoring program begins with the choice of appropriate species. In this case, the target organisms must have a well-defined characteristic to be a very good bio monitor, such as: well described physiology, non-migratory organisms and abundant in the area. Fish play a major ecological role due to their function as an energy carrier from lower to higher trophic levels (Stankovic et al., 2014; Zhou et al., 2008). According to their different hydrological setting, different species of fishes may represent different biomonitoring sources. *Serrasalmus rhombeus*, popularly known as piranha, is widely distributed in rivers of Amazon and is the most abundant predators. It is a pelagic specie, inhabiting the lakes and backwaters of white, clear and black water rivers with low flow, and easy to capture (Naccari et al., 2015). Belongs to the genus Serrasalmus and it's distributed throughout the tropical regions of South America eastward from the Andean Mountains. The rhombeus group comprises of six to nine species that are recognized by a deep and compressed body, eye pierced by a vertical black band. They consume other smaller fish, larvae of aquatic insects, and crustaceans (Nakayama et al., 2001). Since it is a carnivorous, non-migratory, and abundant in the region of study, this animal becomes a potentially bioindicator species of aquatic pollution.

In this context, the present work aimed to assess the water quality of two points (upstream and downstream) of the Itacaiúnas River using water physicochemical parameters, ecological risk assessment in sediments, biomarkers and metal bioaccumulation in piranhas.

2. Materials and methods

2.1. Study area

The Itacaiúnas River is a tributary of the Tocantins River located at eastern part of the Brazilian Amazon. This river drains an area of approximately 41,300 km2 and is located approximately 600 km southward of the Equator line (Pontes et al., 2019). This river plays a very important role in the economic formation of the state of Pará, Brazil, however, it has been undergoing great anthropogenic pressures due to deforestation and mineral prospecting nearby the headwaters of the river's tributaries (Souza-Filho et al., 2016). The region experiences a tropical conditions, with a mean annual precipitation of 1899 mm and 29 °C throughout year, but with only 17% of precipitation occurring during the dry season (June to October). Two sampling sites were selected in the Itacaiúnas River: Point 1 (P1) is located at the upstream of the river (5°59′68″ - 50°43′36″), whereas Point 2 (P2) is located at downstream of the river (5°51′40″ - 50°27′25″), 50 km from point 1 and also exists an important geographical barriers between the sampling sites (Figure 1). Point 1 is a pristine area, since it a zone protected by Brazilian agency, Chico Mendes Biodiversity Conservation Institute (ICMBIO). On the other hand, point 2 is affected by several exploration mines (gold, manganese and copper). The present study is also considering the seasonal influence, therefore the samples were collected on rainy (march) and dry (august) season.

2.2. Fish collection and sampling design

The collection of animals in this study was authorized both by the federal government agency (ICMBIO) and by the Ethics Committee on Research with Experimental Animals (CEPAE/UFPA). All the samples and water data (Sediment, surface water and fish) were collected simultaneously. Three replicate 500-mL water samples were collected from each sampling site using acid-washed polyethylene bottles, than the water parameters such as: pH, temperature (°C), Electrical conductivity (μS.cm-1), total dissolved solids (mg/L) and dissolved oxygen (mg.l^{-1}) were measured three times using a previously calibrated HI 9828 HANNA$^{®}$ multi probe (model HI9828) using YSI Incorporated as reference (Table 1). 20 fish were caught from each point (P1 and P2) and seasons, then a total of 80 fishes were evaluated during the study. The animals were immediately anesthetized and euthanized, according to the guidelines of the National Council of Animal Experimentation Control (CONCEA). The piranhas were measured (cm) and weighed (g). Gills and muscle fragments were removed from the fish for analysis purposes. The tissues were conditioned according to the respective types of biomarker analysis, for histological biomarkers the tissues were stored in glass pots with Bouin's solution; for ultrastructure biomarkers in glass pots with karnosvsky solution; for biochemical biomarkers they were first cooled for 2 h and then stored in liquid nitrogen and finally being stored in– 80°. Ten replicates of bottom sediment were collected using a Van-Veen stainless steel drag in areas of deposition and low water flow. The sediment samples were collected in low-depth sediment deposition zones, which presented low hydrodynamic flow speed. After the bottom sediment collection, sediment samples were placed in plastic bags, which were previously identified and conditioned in thermal boxes until the beginning of the analytical procedure. Simultaneously, 15 replicates of surface water samples were collected at both sampling points and stored in 500 ml polyethylene bottles, previously washed with HCl, and then cooled on ice and stored at -20 °C until the analytical procedures.

2.3. Trace metals analysis (Zn, Cd, Cr, Cu)

At the laboratory the sediment samples were dried at 60 °C, sieved using a 9mnm sieve and then macerated to obtain a fine sediment extract, next sieved again (1mm sieve size) and the resulting sediment was dried at 80 °C. The water samples were filtered under vacuum using cellulose acetate filter of 0.45 uM pore size and 22 mm diameter to obtain the dissolved and particulate fractions. The samples of sediment, water and fish tissue were previously acidified at (HNO3 and $HClO_4$ (1:1), 5 ml H_2SO_4). The acid digestion was followed by the microwaves Mars, CEM procedure. Next, the contents were transferred to falcon tubes and milli-Q water was added to a final volume of 25 ml. The analyses were performed on an Optical Emission Spectrometer with Induced Plasma (ICP-OES). All analyses were run in duplicate and followed by quality control using certified reference material CRM DOLT-3 with analytical recovery 99.1% (3.340 ± 0.343).

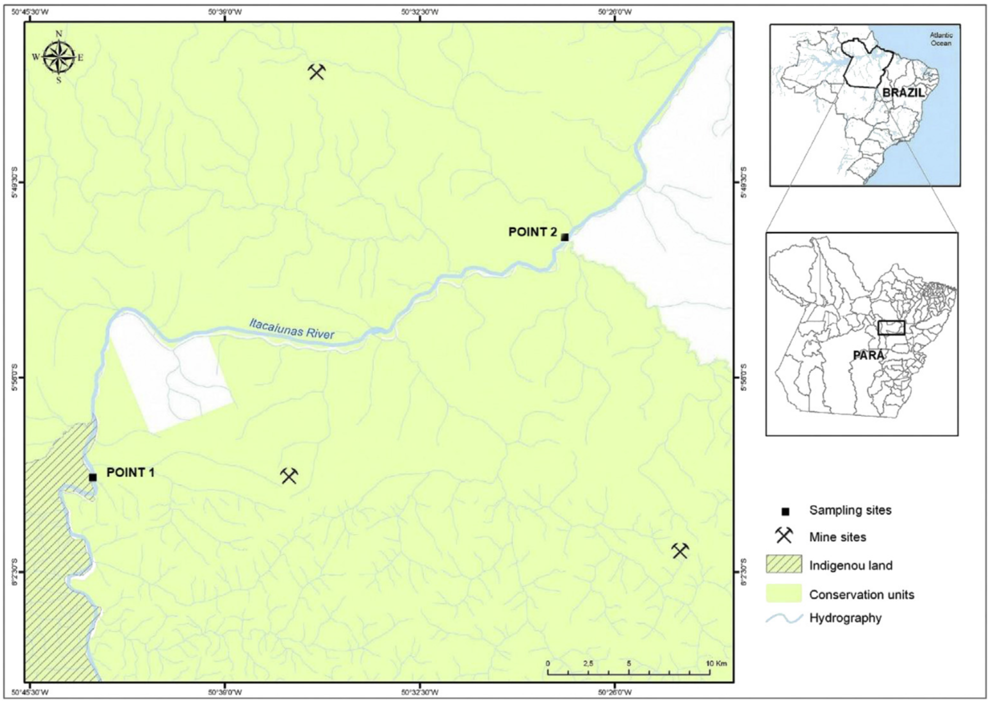

Figure 1. Map showing Itacaiunas River located at north of Brazil in Para State springs and irrigation channels; POINT 1(Upstream), POINT 2 (Downstream).

Table 1. Mean metal concentrations (mg L^{-1}) of water samples compared to WHO (2006) permissible limits and Mean ± SE of water Variables from two points in Itacaiunas River (Para - Brazil).

Metals	Dry season		Rainy season		WHO (2006)
	POINT 1	POINT 2	POINT 1	POINT 2	
Cd	0.002	**0.0054***	0.0019	0.0026	0.003
Cr	0.002	0.003	0.005	0.047	0.05
Cu	1.5	**2.6***	1.3	**3.4***	2
Zn	2.78	3.12	4.14	**5.6***	5
Hg	0.003	0.005	0.0022	0.0032	0.006
Variables					ISI Incorporated
Temperature (C°)	29 ± 1	31 ± 1.5	25.5 ± 2	26 ± 1	
pH	6.9 ± 0.2	6.8 ± 0.3	7.1 ± 0.1	6.8 ± 0.0	
Electrical conductivity (μS.cm-1)	45 ± 6	49 ± 3	33 ± 4	53 ± 9	
total dissolved solids (mg/l)	300 ± 90	420 ± 72	260 ± 67a	450 ± 107b	
dissolved oxygen (DO) (mg.l-1)	6 ± 1.5	5.2 ± 1	7 ± 1.5	6 ± 1	

Note; *: Values are higher than the recommended WHO (2006) drinking water quality guidelines. a: significantly different between seasons and b: between points.

2.4. *Total mercury analysis*

For THg analysis, the samples were prepared and digested in a concentrated acid mixture of 2 ml HNO3 and HClO4 (1:1), 5 ml of H_2SO_4 and 1 ml of H_2O, in a heating plate at 230–250 °C for 20 min, cooled to room temperature, measured in 50 ml volumetric flasks and homogenized. The available mercury in ionic solution was analyzed by atomic absorption spectrophotometry by cold steam generation (EAA-VF/G) with a Mercury Analyzer HG-3500, according to the method proposed by (Akagi et al., 1995).

2.5. *Assessment of metal contamination status*

The potential ecological risk index (RI) was determined to assess the ecological risks of the toxic metals in the sediments using Hakanson (1980) as reference in this study. This method gives a comprehensive evaluation of ecological risks and is calculated as follows a specific equation based on the findings values of metals in sediment at study area and the references values. After the calculations, the result shows the toxicity of each metal (within a rank of 5 categories) and the IR of each area (within a rank of 4 categories) (S1).

$$C_f^i = \frac{C_n^i}{C_0^i} \qquad E_r^i = T_r^i \times C_f^i$$

$$RI = \sum_{i=1}^{n} E_r^i$$

where C^i_f—the single metal pollution index; C^i_n—concentration of metal i in the sediment; C^i_0—the reference value for metal i; E^i_r—the monomial potential ecological risk fator; T^i_r—the metal toxic response factor; RI— the potential ecological risk caused by the overall contamination (for *n* metals). (Hakanson, 1980).

2.6. Histopathological analysis

Gill tissues were fixed in Bouin's. After, the samples were dehydrated in increasing concentrations of alcohol, diaphanized in xylol, embedded and included in paraffin blocks to obtain 5 mm thick cuts using a rotary microtome (Leica RM 2245). The sections were stained with hematoxylin and eosin (HE) and examined under a light microscope (Nikon Eclipse ci). The presence of histological alterations for each organ was evaluated semi-quantitatively by the degree of tissue change (DTC). For DTC calculation, the alterations in gills were classified in progressive stages of damage to the tissue: stage I alterations, which do not alter the normal functioning of the tissue; stage II, which are more severe and impair the normal functioning of the tissue; and stage III, which are very severe and cause irreparable damage. A value of DTC was calculated for each animal by the formula: $DTC = (1 \times SI) + (10 \times SII) + (100 \times SIII)$ where I, II and III correspond to the number of alterations of stages I, II and III, respectively. The DTC value obtained for each fish was used to calculate the average index for each sampling site. DTC values between 0 and 10 indicate normal functioning of the organ; values between 11 and 20 indicate slight damage to the organ; values between 21 and 50 indicate moderate changes in the organ; values between 51 and 100 indicate severe lesions and values above 100 indicate irreversible damage to the organ (Poleksić and Mitrović-Tutundžić, 1994).

2.7. Transmission electron microscopy (TEM)

The samples were fixed in Karnovsky. After fixation, the tissues were washed in 0.1M sodium cacodylate buffer pH 7.4 and post-fixed in 2% osmium tetroxide. They were then dehydrated in series of acetone, embedded in Epon 812 and included in blocks. Next, they were cut in ultramicrotome and thin sections were stained with 1% toluidine blue to define the area to be used for ultrathin sections. The ultrathin sections were contrasted with uranyl acetate and lead citrate, analyzed and photographed in a transmission electron microscope (LEO 906 E).

2.8. Oxidative stress biomarkers analyses

The gill samples were homogenized (1:4; p/v) in buffer with pH set to 7.6, according to (Bainy et al., 1996) methodology. The homogenates were centrifuged at 20,000 x g per 20 min at −4 °C. The supernatant was removed, aliquoted and packed at −80 °C until the moment of the dosages. The analyses of total proteins was performed with commercial kit (Doles LTDA, Brazil), based on the biuret test (citratetrisodium 114 $mmol.L^{-1}$, sodium carbonate 210 $mmol.L^{-1}$ and copper sulphate 10 $mmol. L^{-1}$) for proteins, the readings were performed in multimodal microplate reader (Victor X3, Perkin Elmer) at 550 nm. The results were expressed in milligrams of proteins/mL. The activity of Glutathione-S-transferase was analyzed based on works of (Habig et al., 1974) and (Habig and Jakoby, 1981). The readings were expressed in UGST/mg of protein that represents the amount needed of the enzyme to conjugate 1μMol of CDNB/min/mg of protein, at 25 °C and pH 7.0. The lipoperoxidation was determined according to Hermes-Lima et al. (1995) adapted for microplates according to Monserrat et al. (2003). The samples were homogenized (1:6m/v) in methanol 100% in the cold (4 °C). The homogenates were centrifuged at 1000×g, during10 min at 4 °C. The readings were performed in spectrophotometer in the length of 550nm. The content of lipid peroxides was expressed as equivalent of nM CHP/g of wet.

2.9. Estimation of catalase (CAT) (EC 1.11.1.6)

The catalase (CAT) activities were determined according to the method described by (Beutler, 1984), measuring by spectrophotometer enzymatic decomposition rate of H2O2 at 240 nm. The absorbance decrease by the enzyme activity was expressed in CAT unit/mg of protein, where one unit is the amount of enzyme required to hydrolyze 1μm H_2O_2/min/mg protein at 30 °C and pH 8.

2.10. Superoxide dismutase (SOD) (EC 1.15.1.1)

Activities were measured by the ferricytochrome c method using xantine/xantine oxidase as a source of superoxide radicals. Enzyme activity was reported in units of SOD per milligram of Hb or protein. One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate (McCord and Fridovich, 1969).

2.11. Statistical analysis

Firstly, the assumptions of normality were performed by Kolmogorov-Smirnov test. Then, datas of biomarkers were submitted to the Mann-Whitney test and Kruskal-Wallis test followed by the Dunn multiple comparisons test. Data of metal concentrations in muscle were submitted to Student's t test and one-way analysis of variance (ANOVA). The Spearman correlation analysis (r) was used to verify the influence of some environmental parameters, such as metal concentrations in water and sediment, in the biochemical biomarkers responses and metal bioaccumulation in fish muscle. All analyses were performed using the Statistical Package GraphPad Prism 7. Differences were considered significant when $p < 0.05$. Data are expressed in mean ± standard deviation.

3. Results

3.1. Water analysis

Water physicochemical parameters are presented in Table 1. The values of temperature, pH, electrical conductivity did not vary among the sampling points and seasons, nevertheless, the value of oxygen (DO) at P2 – dry season and Total solid Dissolved at P1 – Rainy season were lower than compared to the others.

Table 2. Mean ± SE of metal concentrations in sediments (ug/g dry weight) of the two points in the Itacaiúnas River. Reference values according to (Hakanson, 1980) and the potential ecological risk index (RI) for sediment at each site and season from Itacaiunas River; the monomial potential risk index (Ei r) for each of the metals calculated from metal toxic response factor (Ti r).

Metals/toxic response factor (Ti r)	Dry		Rainy		Reference
	POINT 1	POINT 2	POINT 1	POINT 2	Ci 0 (μg/g dw)
Cd (30)	0.162 ± 0.01 **(24.3)**	0.41 ± 0.006* **(61.5)**	0.018 ± 0.07 **(2.7)**	0.053 ± 0.02 **(8)**	0.2
Cr (2)	35 ± 12 **(0.8)**	46 ± 8 **(1)**	37 ± 9 **(0.8)**	56 ± 11 **(1.24)**	90
Cu (5)	17 ± 1 **(2.4)**	32 ± 2 **(4.5)**	28 ± 3 **(4)**	46 ± 4* **(5)**	35
Zn (1)	78 ± 2 **(0.8)**	22 ± 8 **(0.22)**	98 ± 21 **(1)**	125 ± 17* **(1.25)**	100
Hg (40)	0.08 ± 0.01 **(12.8)**	0.1 ± 0.02 **(16)**	0.075 ± 0.01 **(12)**	0.13 ± 0.01 **(20)**	0.25
RI	**41**	**83**	**20.5**	**35.5**	

[*] Values are higher than the recommended.

Table 3. Terminology used to describe the indices Ei r and potential ecological risk factor RI.

E_r^i	The monomial potential ecological risk factor	RI	Potential ecological risk for all factors
$E_r^i < 40$	Low	$RI < 95$	Low
$40 \leq E_r^i \leq 80$	Moderate	$95 \leq RI \leq 190$	Moderate
$80 \leq E_r^i \leq 160$	Considerable	$190 \leq RI \leq 380$	Considerable
$160 \leq E_r^i \leq 320$	High	$RI \geq 380$	Very high
$E_r^i \geq 320$	Very high		

Suggested by (Hakanson, 1980).

Of the five metals (Cd, Cu, Zn, Hg, and Cr) appraised in the water from the studied points along the Itacaiúnas river (Table 1), three (Cd, Cu, and Zn) were above limits. The following findings showed a distinctive metallic behavior for Cd, Zn and Cu, with regards season. Cd presented high values at P2 in the dry season, whereas Zn was higher at P2 in the rainy season. Moreover, Cu reported high levels at both seasons at P2, but significantly higher in the rainy season.

3.2. Sediment analysis

Table 2 shows the values of metal concentration from the sediments collected in the study areas as well as the respective risk assessment. It can be seen that the metals Cd, Cu, and Zn are well above the reference limits, according to Hakanson (1980). Another important finding is that these metals are in high concentrations only at point 2, however, cadmium has higher values in the dry period while the others had higher values in the rainy period. The potential ecological risk index (RI) calculated based on the sediment data and the metal toxic response factor (Ti r) showed that the Point 2 on the dry season has the greatest RI value and also pointed out that cadmium values were the most expressive in the final ecological risk index calculation because of the monomial potential risk index (Ei r) (Tables 2 and 3).

3.3. Bioaccumulation of trace elements (Cd, Zn, Cr, Cu, Hg)

Metal detection data in fish muscle and the limits for safe human consumption are shown in Table 4. Significant differences between sites were only observed for concentrations of Cd and Hg. Higher concentrations of Cd were observed at Point 2 on the dry season ($p = 0.004$). Likewise, higher concentrations of Hg were observed at Point 2 on the dry season ($p = 0.03$). Additionally, significant temporal variation was also observed. Concentrations of Cr and Cu were higher on point 2 the dry season ($p = 0.047$, $p = 0.002$). Among the metals analyzed, values of Cd and Cr in all the samples exceeded the limits recommended for safe human consumption by the Brazilian National Health Surveillance Agency. The adult fishes captured at the study period weighed 400 ± 87 g and the total body length corresponded to 25 ± 1.8 cm average, there was no significant difference between the biometry of the specimens captured.

3.4. Morphological biomarkers

The architecture of the gills of the collected fish in P1 showed primary filaments and secondary lamellae, slender appearance, and well-defined cell types, such as: pillars, epithelial cells without stratification, and mucus cells (Figure 2A and B). The fish gills collected in P2 in the Itacaiunas river showed various types of damages (moderate and severe) in the gill tissues such as: Lamellar aneurysm, rupture of the lamellar epithelium, necrosis, aneurysm and lifting of the epithelial layer (Table 5) causing swelling of the lamellas and reducing the superficial respiration area. The necrotic lamellae fused together and with mucous depositions along the surface (Figure 2C and D). The Degree of Tissue Change (DTC) reported a clear difference between points and seasons evaluated at this study. The point 2 – the dry season presented a DTC of 155, highlighting the dreadful condition of the animals' health originated from this point (Table 5). Transmission electron microscopy confirmed the results obtained by light microscopy. The ultra-thin section of the control fish gills exhibited normal pavement cells varied in form from squamous to polygonal with an elongated nucleus, electro-dense cytoplasmic matrix with well-developed Golgi apparatus, rough endoplasmatic reticulum, ribosomes, and a small number of mitochondria in comparison with the chloride cells (Figure 3A and B). While the piranhas in P2 showed a completely different lamellar structure, with thicker lamella and irregular shape, the tissue in the filament region showed cell proliferation, where the intense proliferation of mitochondria in mucus cell can characterize tissue damage (Figure 3C and D).

3.5. Biochemical biomarkers

The Antioxidant enzyme activities from piranhas collected at Itacaiunas river at different points and seasons are presented in Figure 4. The results exhibit a significant difference between the enzyme activities of fishes from POINT 1 compared to POINT 2. In terms of biotransformation enzyme, GST activity was higher in fishes from POINT 2 in both seasons. Concerning antioxidants, the SOD activity presented a similar pattern to GST and reveled high values at POINT 2 on both seasons ($P < 0.05$). In contrast, differential change in CAT activity was pointed at P2 – the rainy season, the activity was below than other points and season ($P < 0.05$). Lipid peroxidation was evidenced by the increased TBARS in the

Table 4. Bioaccumulation (mg/Kg $^{-1}$) in the muscles of *S. rhombeus* from Itacaiúnas River.

Metals	Dry season		Rainy season		Reference
	POINT 1	POINT 2	POINT 1	POINT 2	
Cd	0.014 ± 0.006a	**0.052 ± 0.017b**	0.037 ± 0.02	**0.0509 ± 0.02**	0.05
Cr	0.14 ± 0.05	**0.2 ± 0.09A**	0.094 ± 0.01	0.05 ± 0.03B	0.2
Cu	1.05 ± 0.005	**2.090 ± 0.008A**	0.7 ± 0.02	0.6 ± 0.01B	2
Zn	13.34 ± 4.2	16.1 ± 6	11 ± 3.8	14 ± 3	50
Hg	0.26 ± 0.12a	0.417 ± 0.05b	0.175 ± 0.04a	0.322 ± 0.012b	0.5

Means with different lower case letters indicate significant difference between sampling sites in the same period. Means with different upper case letters indicate significant difference between sampling periods in the same sampling site. Reference values for safe human consumption according to the Brazilian National Health Surveillance Agency.

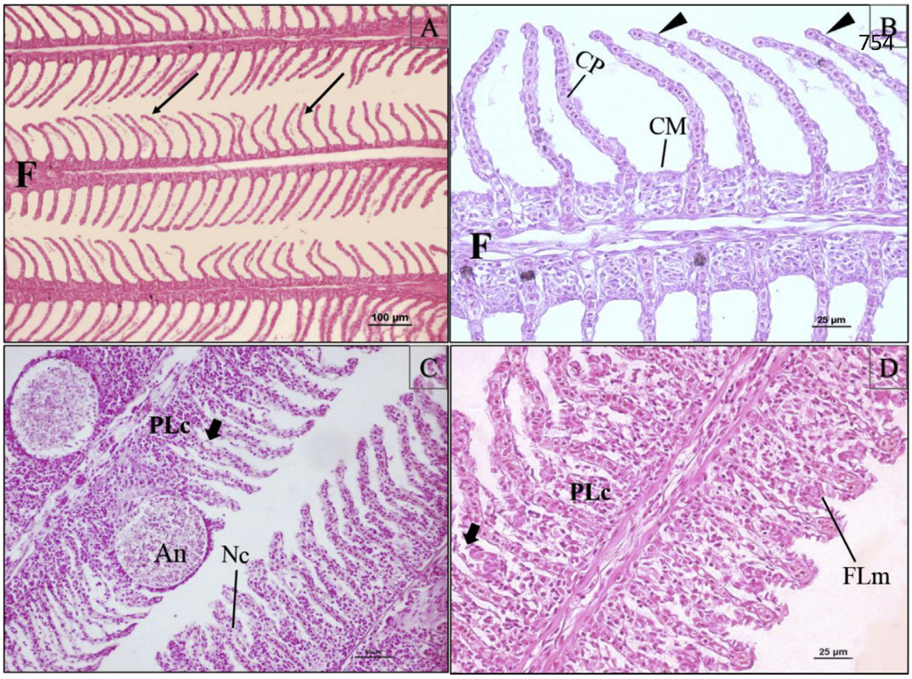

Figure 2. Longitudinal sections of gill filaments of *S. rhombeus*. (A) Fishes captured in P1 with normal structure for teleost Branchial: filament (F) and second lamella (*large arrow*); (B) Detail of the second lamella with a slender appearance and without tissue damages: CP – Pillar cell, CM – Mucous cell; Epithelial cell (*head arrow*) (C) Gills tissues from *S. rhombeus* captured in P2 presenting lesions: Aneurism (An), Necrosis (Nc), Proliferation of cells (PLc), lifting of epithelium (*short arrow*); (D) Filament with severe alterations caused by cell proliferation generating completely Lamellar fusion (FLm).

gills' tissue and also showed higher levels of lipid peroxidation in animals captured at POINT 2 in both seasons. These results demonstrate the effects of season and especially the location in the Itacaiunas River on the piranha's enzyme activities.

The correlation analysis carried out between season and other variables analyzed in the present study, revealed a significant strong negative correlation with concentrations of Cr in the water ($r = -0.63$, $p = 0.004$), a strong positive correlation with concentrations of Cd and Hg in the sediment ($r = 0.61$, $p = 0.05$; $r = 0.68$, $p = 0.03$, respectively), and with LPO ($r = 0.68$, $p = 0.03$).

4. Discussion

The morphological changes and biochemical enzymes in fishes have been used as biomarkers against aquatic pollution to detect the biological effects of chemical contaminates even at low concentrations. The complex mixture of pollutants increased the study of relationships between exposure to chemical contaminants and alterations in several molecular and cellular processes in the organisms to use the biomarkers (Lushchak, 2016). However, in the present study, we have described the role of cellular and oxidative stress against metal pollution in Amazonian important river, the Itacaiúnas River. We have studied five metals (Cadmium, Chromium, Copper, Zinc, and Mercury) in water, sediment, and muscle of piranhas from two sampling points along the river and also at two different seasons (dry and rainy). Bioaccumulation of metallic compound is usually employed combined with water physicochemical analysis or/and with other biomarkers in order to gather more information that can more accurately point out the environment scenario at the moment. In the present study, the results showed clearly a difference among the points.

Differences in the water physicochemical parameter could be associated with the marked anthropogenic changes observed in the Carajás

Table 5. Histological damages in gills of *S.rhombeus* collected from the two points in Itacaiunas river. Respective stages of tissue damage, frequency of occurrence and degree of tissue change (**DTC**).

Lesions	STAGE	Dry season		Rainy season	
		P1	P2	P1	P2
Hyperplasia of the gill epithelium	I	+	+++	+	++
Hypertrophy of the gill epithelium	I	++	+++	+	+++
Blood congestion	I	+	0+	+	++
Dilation of the marginal channel	I	0+	++	0	++
Epithelial lifting of the lamellae	I	0+	+++	0	++
Lamellar fusion	I	0	++	0	++
Lamellar aneurysm	II	0	++	0	++
Rupture of epithelial cells with hemorrhage	II	0	+	0	+
Complete fusion of all the lamellae	II	0	++	0	+
Rupture of pillar cells	II	0	++	0	++
Rupture of the lamellar epithelium	II	0+	+++	0+	++
Necrosis	III	0	+	0	0+
Degree of tissue change (DTC)		3.6 ± 1	$155 \pm 30^{a***}$	3.5 ± 1	$66 \pm 12^{b***}$

Note: 0, absent; 0+, rare; +, low frequency; ++, frequent; +++, very frequent.
Different letters denote difference between points, * denote difference between seasons ($p < 0,05$).

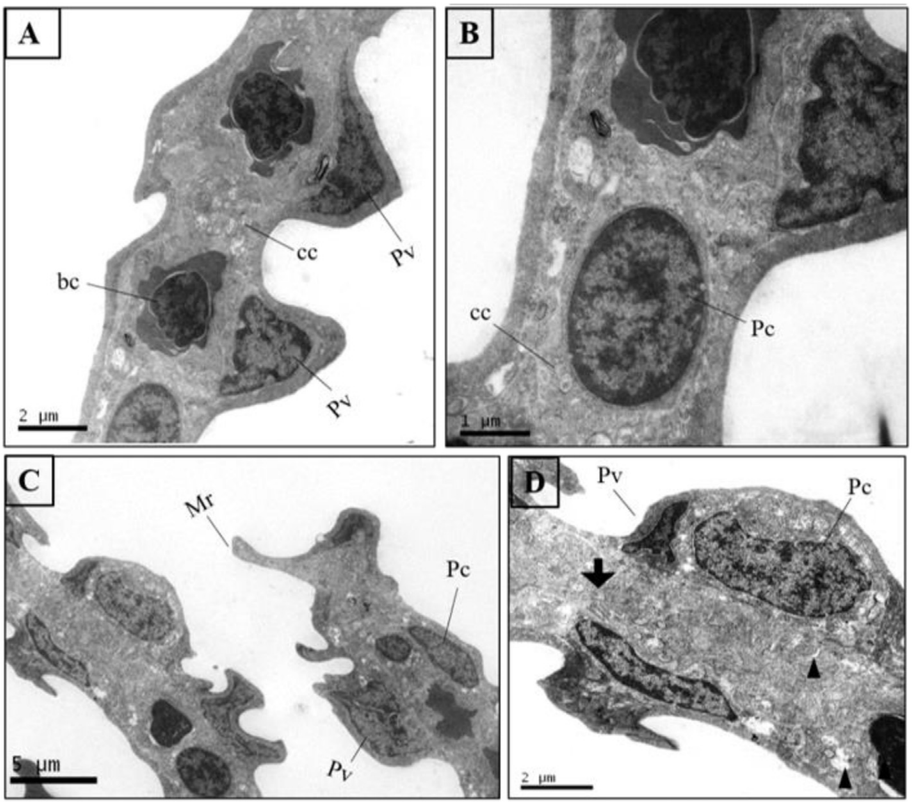

Figure 3. Gill epithelial of *S. rhombeus* cell under transmission electron microscopy (TEM) (A) Normal appearance of the secondary lamellae; distinct paviment cell (Pv), Chloride cell (CC) and Blood Cell (bc). (B) Detail of a normal secondary lamella presenting a slender and regular cells such as: Pillar Cells (Pc) and Chloride cell (CC). (C) Detail of altered lamellae, such as prominent microridges (Mr), hypertrophied and disarrangement in the architecture of the tissue; paviment cell (Pv) and Pilar cell (Pc). (D) Detail of the secondary lamellae reporting the tubular system of the chloride cells altered (*black arrow*), hypertrophied Paviment Cell (Pv) and dilated mitochondria (*head arrow*).

region. In this context, it is important to note that point 1 is approximately 50 km distant to point 2, thus showing abundant vegetation and higher production of vegetal biomass. In turn, point 2 is located in the old mining area, thus being characterized by low vegetation and limited production of vegetal biomass (Pontes et al., 2019). This could explain the higher concentration total dissolved solids found at point 2, especially at the rainy season.

Contamination by metallic compounds occurs most often as a mixture, however most environmental risk assessment frameworks to date have been based on metal-by-metal analysis (Nys et al., 2017). When

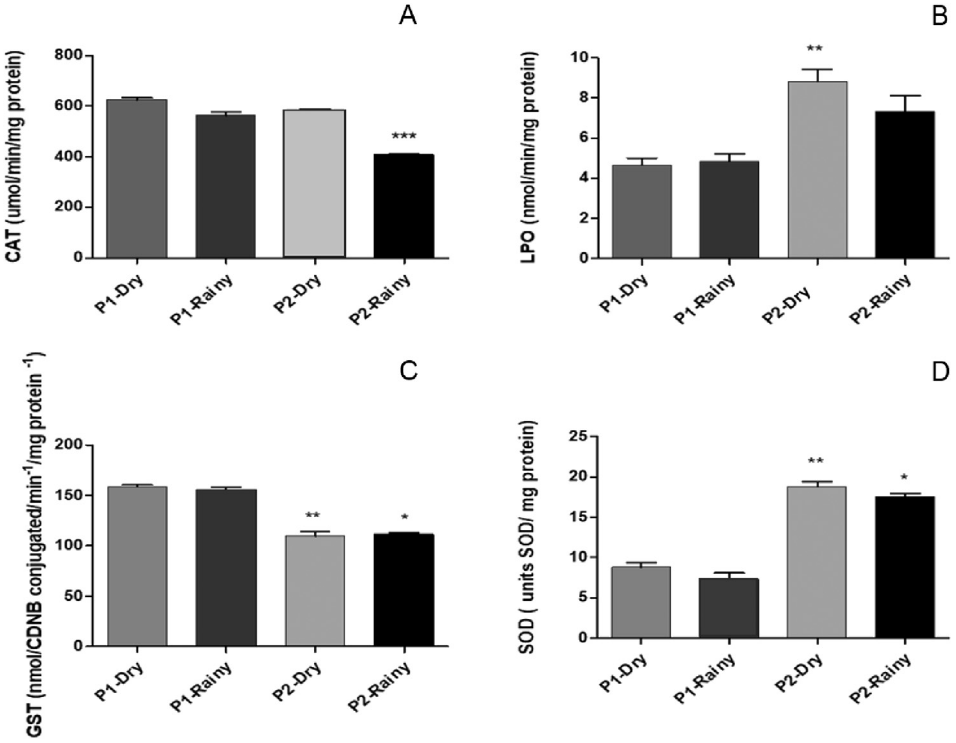

Figure 4. Biochemical biomarkers in the gills of *S. rhombeus* (catalase (A), GST (B), LPO (C) and SOD (D)). One-way ANOVA test followed by tukey test, $p < 0.05$.

looking at the overall potential ecological risks in sediment posed by the metals at each site, RI values ranged from 20 (POINT 1 rainy season) to 83 (POINT 2 rainy season), since the average RI for the studied sampling sites was below 150, it appears to be a low potential ecological risk due to contamination by these specific metals. However, cadmium was the metal with more contribution for the RI, the monomial potential risk index for this metal was very high, following mercury and copper. This finding corroborates the results of bioaccumulation in piranhas and water analysis, which pointed to the high levels of cadmium in fishes and water, above the permit limits. Regarding the concentrations of metals in water, we can observe a different behavior between metals as well as water's physical-chemical variables. Cadmium showed the highest values in point 2 only in the dry period, while the copper showed the highest values in point 2 in both periods, while the Zn highest value in point 2 only in the dry period. pH might also affect the concentration of soluble zinc, where high concentrations of this metal are found in well-oxidized conditions (pH 5 to 6.5), whereas low concentrations might be observed in redox conditions (pH 8) (Adams et al., 2020).

Cadmium is classified as a human carcinogen, chrome has toxic and carcinogenic effects and the chronic Cu toxicity can result in liver disease and severe neurological defects (Mirsha et al., 2018) this study revealed higher concentrations of Cd, Cu, and Cr in fish muscle were observed at point 2 on the dry period. Interestingly, chromium was not detected in the water samples of both sites, evidencing the presence of this contaminant in the environment as well as its bioaccumulation in the fish muscle. In the case of Cr, trivalent chromium is an essential element, but at high concentrations or in different forms, such as hexavalent Cr, it is toxic even at low concentrations (Beiras, 2018). Zinc can bind to metallothioneins, but since copper has a greater affinity for proteins, zinc concentration might be low due to the presence of copper (Langston, 2018). The elevated values of Cadmium in the tissues of piranhas from Itacaiunas River at point 2 are probably related to a high influx of metals as a result of pollution from mining combined with the rainfall period thereby increased bioavailability to the fish.

The presence of a metal in sediment, water, and also the biota evaluated in this research denotes the extent of this metal in the environment. The presence of such elements can induce changes in gill functions and structural and biochemical alterations have been suggested as useful biomarkers of environmental contamination (Costa et al., 2009; Souza et al., 2013). Since this tissue is fundamental in the uptake of dissolved substances from water, it represents the prime target for toxic action of waterborne metals (Raftopoulou and Dimitriadis, 2011). Therefore, it is the most important organ for environmental investigation using biomarkers. Studies about the toxic effects of cadmium in freshwater fish demonstrated the ability of Cd to inhibit active Ca^{2+} uptake in transporting epithelia. Along this process, the presence of Cd interferes with the normal transport or handling, leading to hypocalcemia, and potentially death during more extreme exposures of Cd (Galvez et al., 2007). A full understanding of heavy metal kinetics in fish is important for natural resource management and the human consumption of fish (Sabullah et al., 2015).

Fish gills have a basic structural design with a single epithelium surrounded by different cell types but with a complex mechanism to perform important functions, and in the presence of toxin accumulation, it may cause deleterious effects (Evans et al., 2005). The study of the prevalence of lesions demonstrated that most fishes had some degree of gill injury even in the reference point. This information indicates that in natural environments complex mixtures affect the organisms and many factors can interfere in the health status of the fishes, this result can be interpreted as an initial adaptation of certain pollutants. Once mucous cells can be efficient in seizing the toxic agents and thereby assist in preventing the entrance of these agents into the gills, the detachment can reduce the entrance of toxicants into the circulatory system (Laurent and Perry, 1991). However, it's important to accentuate that only POINT 2 presents permanent injuries in the gills architecture, such as necrosis, elucidating the fact that these animals present a history of exposure and damage in this point of the river, as they exhibited high cadmium and copper concentrations in the tissues analyzed and severe damages in the gill tissues, but no mortality was reported.

The ultrastructural analysis presented chloride cells with an abundance of mitochondria in their cytoplasm. The profile of these mitochondria varied from ovoid to elongated and sinuous, with electro-dense matrix, a result also observed by (Samanta et al., 2015) and (Paruruckumani et al., 2015). In contaminated places, these cells proliferate and the amount of mitochondria increases considerably, and in this study, this situation was reported for the fish collected in P2, which is correlated to the histology results when the fishes from this point presented high levels DTC (degree of tissue change) caused mainly by Epithelial lifting of the lamellae. Cadmium can also impair the homeostasis of other ions (LI et al., 2012). Due to the constant loss of sodium from the more concentrated body to the diluted environment, there is an absolute requirement for freshwater fish to absorb sodium at the gills (McRae et al., 2018), causing severe injuries in this organ as was observed at the gills from fishes collected at POINT 2.

Our results pointed that Hg was present at the ambient compartments, however according to (Borges et al., 2018), in Amazonia there is a great background of this metal in the environmental, especially at the rainy season. Since there is a variation in the physical-chemical characteristics depending on the hydrological period in tropical rivers, during long rainy periods, the temperature and conductivity of the water tend to be lower and deeper, speed and dissolved oxygen tend to be higher, which means, the scenario changes completely and consequently influences the bioavailability of the metallic elements. In this study, the concentration of this metal was below the permit limits.

The Itacaiúnas river presents hydrodynamic and hydrochemical features, typical of clear water rivers (Sahoo et al., 2019), as well as a pH close to neutrality. Studies indicate that metals such as Hg precipitate as oxides and hydroxides at alkaline pH; moreover, these metals become less bioavailable and less toxic (de Paiva Magalhães et al., 2015). The seasonal variations of heavy metals in water were potentially influenced by the physical and chemical properties of water, pH and dissolved oxygen levels (de Souza Machado et al., 2016).

Oxidative stress occurs when there is an imbalance between ROS production and the total antioxidant capacity of the cell (Birnie-Gauvin et al., 2017; Lushchak, 2016). This antioxidant capacity is due to the joint action of enzymes such as catalase, superoxide dismutase and glutathione peroxidase (Hermes-Lima et al., 1995). The metal contamination can increase the intracellular formation of ROS (Regoli and Giuliani, 2014; Regoli et al., 2011). Since the induction of antioxidants represents a cellular defense mechanism to compensate toxicity of ROS, it has been extensively used in several field studies to assess the extent of pollution (Pandey et al., 2003). Cadmium is not a redox-active metal and does not participate in the Fenton reaction, however, it is involved in the formation of reactive oxygen species (ROS) and inhibits the activity of antioxidants (Sandbichler and Höckner, 2016). These can react with biological molecules such as proteins or lipids, causing lipid peroxidation (LPO), antioxidant, and biotransformation systems alterations (Garcia-Santos et al., 2015; Pereira et al., 2016). This study verified the CAT and GST activity and lipid peroxidation; the results also show differences between fishes from different areas. Only the fishes in P2 showed modifications in the CAT activity and higher lipid peroxidation in the gill tissue. Lipid peroxidation is a free radical-induced oxidative degeneration of lipids; therefore an increase in the levels of LPO in the tissues could be attributed to the accumulation of heavy metals. Similar results were also verified by (Nunes et al., 2015), which reported that high levels of cadmium and copper decreased the catalase activity and increased the levels of LPO in *Gambusia holbrooki*.

GST acts in the second phase of biotransformation, conjugating toxic compounds or their metabolites with endogenous molecules to be eliminated from the organism (Hermes-Lima et al., 1995). In this study, there were decreases in the GST activity levels at POINT 2 on both seasons. This level of GST activity can be interpreted as a deleterious response to the exposure of the metals present in these environments. Previous studies have already reported decreases in the activity of GST activity in fish exposed to cadmium (Batool et al., 2018; Eroglu et al., 2015). In our study, LPO was also found in fishes from POINT 2 on both seasons, especially at the rainy season. The increased occurrence of LPO may show a significant increase in the responses of biochemical biomarkers such as biotransformation metabolism and oxidative stress. Lipid peroxidation is a well-known mechanism of cell injury in vertebrates and invertebrates, being an indicator of oxidative damage in cells and tissues (Birnie-Gauvin et al., 2017; Regoli and Giuliani, 2014). Therefore, the measurement of malondialdehyde (MDA), the end product of lipid peroxidation is widely used as an indicator of lipid peroxidation. Several studies have shown enhanced LPO in aquatic organisms exposed to high concentrations of metals (Regoli et al., 2011). In our findings, we observed a positive correlation with the results of LPO with the concentration of metal (Cd) in sediment and water.

Superoxide dismutase (SOD) is an antioxidant enzyme that can convert superoxide radical (O2●−) to hydrogen peroxide (H2O2) (Ighodaro and Akinloye, 2018). Catalase (CAT) is located in peroxisomes where it metabolizes H2O2, water and oxygen. In general, the activity of CAT is associated with SOD. These enzymes work together and take obligatory steps against oxidative stress (Fridovich, 1995). The study carried out observed the SOD and CAT activities in gills presented an antagonist behavior, where CAT decreased the activity meanwhile SOD increased at POINT 2 rainy season. Similarly (Xiong et al., 2011) observed decreasing activity of SOD and CAT in the gills of zebrafsh while comparing the acute toxic effects of metals. This reduction of antioxidant capacity could let this organ susceptible to suffer oxidative stress (Scandalios, 1993), generating harsh consequences to other biological levels, such as the tissue, a situation widely demonstrated in this study.

The seasonality is generally pointed as a fundamental factor in the behavior of the analyzed biomarkers since they are affected by the natural physiological cycle of organisms (Amira et al., 2018; de Oliveira et al., 2019). Also, the specie presented high viability to be a good biomonitor of the area since it presented physiological plasticity establishing itself along the metal pollution gradient with success; it demonstrated a responsive metabolic apparatus to environmental changes without, however, compromising its physiological functions; and a homogeneous response of biomarkers for seasonal changes interpreting data, in conditions of anthropogenic stress, clearer.

5. Conclusions

Biomarkers modulation is seasonal and the physical-chemical conditions did not reflect the sensitivity of the organisms to the seasonal changes. Besides, during the rainy period, there was a decrease in all biomarkers. This study was successful because we verified in a natural environment the quality of health status of the fishes using efficient tools. Therefore, we confirm that there is an environmental risk in the downstream region of the Itacaiunas river, once the health status of the fishes and the metal analysis reflects the actual scenario. Furthermore, the species selected in this study was considered an excellent bioindicator of pollution. The current study evidenced that the environmental quality of Itacaiúnas river is strongly influenced by geological and hydrological factors favoring metals entry in aquatic systems. However, the histological, ultrastructural, and biochemical changes observed in the gills of *S. rhombeus* individuals provide reliable information on the environmental conditions of the study area, evidencing a strong influence of cadmium in this river in the dry season, which suggests more target studies on this metal in this area.

Declarations

Author contribution statement

Caroline da Silva Montes: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maria Auxiliadora Pantoja Ferreira, Tommaso Giarrizzo, Lílian Lund Amado, Rossineide Martins Rocha: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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