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Histological and Ultrastructural Analysis of *Tilapia rendalli* Liver as an Environmental Assessment Tool for Cachoeira River, Bahia, Brazil

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Abstract: Due to the degradation of aquatic ecosystems, environmental monitoring studies have been developed with the aid of conventional and biological tools. The objective of this study was to evaluate the environmental quality of the Cachoeira River, Bahia, Brazil by physico-chemical analysis of water and histopathological and ultrastructural analysis of *Tilapia rendalli* (Boulenger, 1897) liver. Samples were collected at six points in the municipalities of Itapé, Itabuna and Ilhéus, between 2010 and 2012. Water samples were filtered through 0.7 µm GF/F glass-microfiber membranes, and ion analysis (Mg^{+2}, Ca^{2+}, K^+, Na^+, Cl^-, NH_4^+, NO_3^-, PO_4^{3-}, SO_4^{2-}) was performed by ion-exchange chromatography. Fish specimens were collected using a seine-type drag net and fixed in 10% formalin. For histological analysis, the samples were embedded in paraffin, sectioned and stained with hematoxylin-eosin. For ultrastructural analysis, the samples were embedded in Epon resin, and sections were prepared and contrasted with 0.5% uranyl acetate. Semi-quantitative histopathological analysis were performed based on the severity of alterations. There were high concentrations of nutrients in water samples, and at all points sampled, the specimens showed histopathological and ultrastructural changes. Points 1, 2, 3 and 4 were characterized as moderate to severe both for histopathological and ultrastructural changes, indicating that these points had greater degradation than the others.

Keywords: Histopathology, Environmental monitoring, Tilapia, Ultrastructure, Liver

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Introduction

Aquatic environments have been increasingly affected by human activities, resulting in damage to biodiversity (Moraes and Jordan, 2002; Abdel-Moneim and Abdel-Mohsen, 2010), mainly due to the exposure time and the type of toxic chemical pollutants to which these individuals are exposed. Accordingly, it is critical to conduct environmental monitoring studies involving consistent and effective measures to ensure that the quality of rivers is preserved (Buss *et al.*, 2003).

Studies related to physico-chemical analysis that assess water quality are of great importance, but the sole use of this tool can lead to misinformation about the ecological and biological conditions of an aquatic ecosystem, making it essential to use biological data as well (Jenkins, 2004). Biological indicators in studies of environmental quality assessment have been an excellent tool, since they allow us to make inferences about the environmental conditions through the responses of organisms (Goulart and Callisto, 2003).

Fish are considered excellent bio-indicator organisms of an aquatic ecosystem condition because they are abundant and sensitive to pollutants (Karr, 1981). When exposed to a stressor, fish exhibit biological changes in their organs, which allow us to assess the true status of their habitat. These changes are considered biomarkers (Nogueira *et al.*, 2009; Lins *et al.*, 2010). Jesus and Carvalho (2008) state that through the changes observed in the histopathological analysis of target organs, it is possible to identify the presence of pollutants in the water and the effects they have on fish.

The liver is considered an efficient target organ for detecting changes associated with the presence of pollutants in the environment, since it is involved in metabolism and excretion of xenobiotics (Figueiredo-Fernandes *et al.*, 2007) and perform the detoxification function (Perendija *et al.*, 2011). For this reason, changes in the liver have been related to environmental degradation (Stentiford *et al.*, 2003; Camargo and Martinez, 2007; Rocha *et al.*, 2010; Paulo *et al.*, 2012).

Cytological biomarkers are excellent indicators of the aquatic environment and, therefore, the organisms when exposed to a stressor, allow us to correlate the changes observed in their cells with the environment (Au *et al.*, 1999). Ultrastructural biomarkers have often been used to assess environmental quality (Gernhöfer *et al.*, 2001; Martins *et al.*, 2005; Abdel-Moneim and Abdel-Mohsen, 2010; Perendija *et al.*, 2011) because several studies using this tool have achieved satisfactory results.

The aim of this study was to evaluate the environmental quality of the Cachoeira River (Bahia, Brasil) by using physico-chemical analysis of water and histopathological, and ultrastructural liver analysis of the species *Tilapia rendalli* (Boulenger, 1896).

Materials and Methods

Study area

The hydrographic basin of the Cachoeira River is located in the southern region of Bahia State, Northeast Brazil, between the coordinates 14° 42’/15° 20' S and 39° 01’/40° 09' W) and has a drainage area of about 4830 square kilometers, encompassing thirteen municipalities distributed between the microregions of Ilhéus-Itabuna and Itapetinga (Schiavetti *et al.*, 2002). The basin is bounded on the north by the basins of the Contas and Almada Rivers, on the south by Pardo and Una rivers, to the west by the basins of the Pardo and Una Rivers and on the east by the Atlantic

Ocean (Cetra *et al.*, 2009) (Fig. 1). The Cachoeira River is of great economic, demographic and social importance to municipalities along its banks, since it is used for urban and industrial supply, fishing and as receivers of domestic discharges of sewage and solid waste, it is considered of great ecological system having great relevance to the region (Nacif *et al.*, 2000).

Human activities negatively affected the Cachoeira River basin in Bahia, where metals and high nutrient concentrations were observed (Pinho, 2001; Klumpp *et al.*, 2002; Lucio *et al.*, 2012.). To evaluate this basin, the fish *T. rendalli* (Boulenger, 1896) (Teleostei: Perciformes: Cichlidae) was chosen because it was constant and abundant throughout the sampling period. Known as Congo Tilapia, it is a freshwater fish quite resistant to diseases surviving in environments with little dissolved oxygen levels, which makes it an excellent sentinel species (Hauser-Davis *et al.*, 2010).

Sampling

Samples were collected quarterly over a period of three years (2010, 2011 and 2012), with a seine-type drag net (5.5 m × 1.5 m × 0.5 cm) (Malabarba and Reis, 1987). Seven collections were carried out at six points along the Cachoeira River, between the municipalities of Itapé and Ilhéus (Table 1).

In the field, the specimens were anesthetized with MS222 (methanesulfonate tricaine), killed, and then fixed in 10% formalin. The specimens were preserved in 70% ethanol and then identified to the species level. Standard length and total weight were measured with the aid of a digital caliper and a digital precision balance. The material analyzed was deposited in the scientific collection of the State University of Santa Cruz (UESC).

Physico-chemical analysis

In the field, we measured the abiotic variables: pH, temperature (C), dissolved oxygen (DO $mg.L^{-1}$) and electrical conductivity ($\mu S.cm^{-1}$) with the aid of a multiparameter apparatus (Horiba). The samples were collected in polyethylene bottles, washed with 1:1 HCl and distilled water, and transported in a cooler to the Laboratory of Marine Biogeochemistry at the State University of Santa Cruz (UESC).

In the laboratory, the samples were filtered through 47 mm diameter GF / F (0.7 μm) fiberglass membranes, previously calcined at 450 C, and subsequently, the filtered material (dissolved) was analyzed. The cations and anions (Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, NH_4^{+}, NO_3^{-}, PO_4^{3-}, SO_4^{2-}) were determined by DIONEX ICS 1000 ion chromatography.

Histopathological analysis

For light microscope analysis, ten individuals were randomly selected, when possible, at each sampling point, and fixed specimens were paraffin-embedded. Sections 5 to 7 μm thick were made with a microtome, and the slides were then stained with hematoxylin-eosin (HE) to get an overview of the affected tissues and organs.

Histopathological alterations in liver were characterized as follows: slight alteration (1) - changes that do not damage the liver tissues, so that an improvement in environmental conditions allows tissue restructuring and recovery of organ function; moderate alteration (2) - changes are more severe and lead to effects on tissues associated with the functioning of the organ; lesions are

Fig. 1: Location of sampling points along the Cachoeira River, Bahia, Brazil

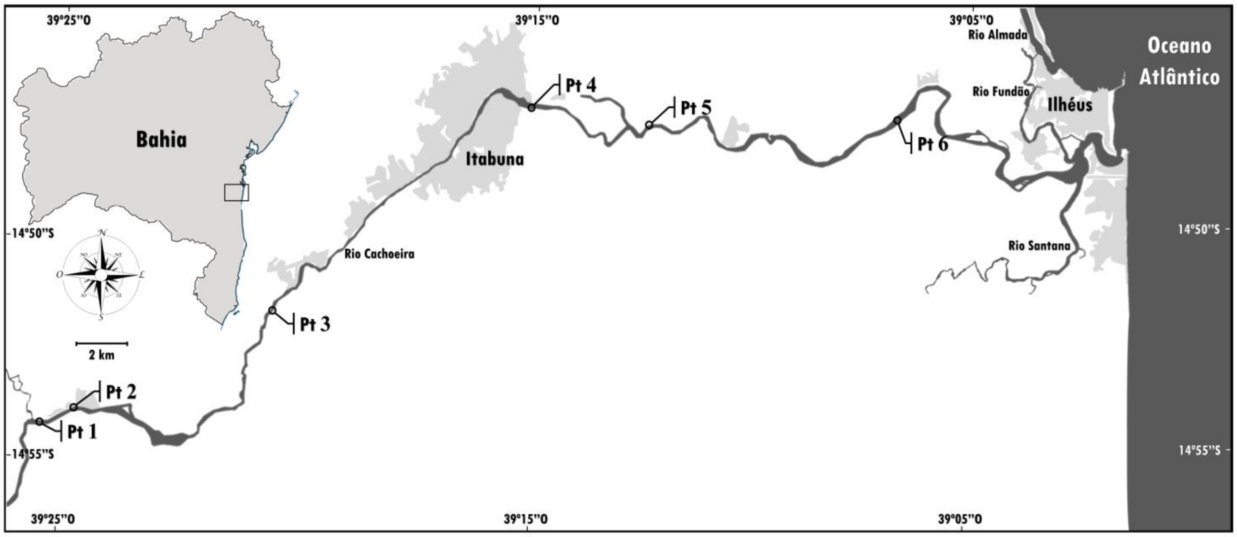

Table 1: Description of study area

Point	Location/City	Coordinates
P1	Upstream of a town (Itapé)	14° 53’ 934 "S and 39° 25' 781" W
P2	Downstream of a town (Itapé)	14° 53’ 934 "S and 39° 25' 781" W
P3	Upstream of a city (Itabuna)	14° 52’ 719 "S and 39° 24' 772" W
P4	Downstream of a city (Itabuna)	14° 47’ 20 "S and 39° 15' 41" W
P5	Downstream of urban area (Ilhéus)	14° 47’ 788 "S and 39° 12' 107" W
P6	Close to a cocoa plantation (Ilhéus)	14° 47’ 072 "S and 39° 06' 273" W

repairable and occur virtually throughout the liver; severe alteration (3) - the recovery of liver structure is not possible, even with improved water quality or stopping exposure to a toxic stimulus (modified from Poleksic and Mitrovic-Tutundzic, 1994).

The occurrence of histopathological changes in the liver of *T. rendalli* specimens was evaluated semi-quantitatively by the degree of tissue changes (Histopathological Alterations Index - HAI), based on the severity of damage. To calculate HAI, a modification of Poleksic and Mitrovic-Tutundzic (1994) was used, in which the alterations observed in each organ were classified in increasing stages of tissue damage: I = slight alterations, II =

moderate alterations and III = severe alterations (Table 2).

For each individual, HAI value was calculated and then the mean of each sampling point. This calculation was according to the following formula: HAI = (1X SI) + (10X SII) + (100X SIII), where S is the sum of alterations of a certain stage, and the numerals I, II and III represent the number of stage of alterations 1, 2 and 3, respectively. HAI indicates the conditions in which the specimen was found (Poleksic and Mitrovic-Tutundzic, 1994) (Table 3). Based on the capacity of the tissue to recover, changes were classified into three stages: I = recovery of tissue structure and function is possible; II = there are severe effects on tissue functionality; and III = tissue recovery is impossible. Some cases were selected and light microscopy images are presented.

Ultrastructural analysis

Selected specimens of *T. rendalli* were dissected in the field for ultrastructure analysis. Liver fragments were removed and fixed in 2% glutaraldehyde. In the laboratory, the tissues were washed 3 times with sodium cacodylate buffer and were subsequently post-fixed in 1% osmium tetroxide. The tissues were washed again with sodium cacodylate buffer, dehydrated in ascending series of acetone and embedded in Epon resin. Subsequently, they were sectioned with an ultra-microtome to obtain ultrathin sections of 70 nm, which were contrasted with 0.5% uranyl acetate and observed with a transmission electron microscope (TEM).

The ultrastructural changes seen in the liver cells were semi-quantitatively classified according to the severity of the abnormalities found. This characterization was based on work done by Gernhöfer *et al.* (2001) who classified the lesions with scores from 1 to 3 where: 1 = control, 2 = slight changes and/or visible responses, and 3 = severe responses and/or visible cell destruction.

Statistical analysis

The Kruskal-Wallis test was performed using the OriginPro 8 program to determine if there were any statistically significant differences between the physicochemical parameters and the sampling points. Principal component analysis (PCA) was performed to see which parameters influenced the quality of the Cachoeira River. This analysis was performed with the aid of the software Past 1:11. The data were first log-transformed and then subjected to PCA based on the correlation matrix. We verified whether there were statistically significant differences between the mean changes in degree of alterations using the nonparametric Mann-Whitney test for independent samples with $p<0.05$. The differences between the points for each parameter were also tested using a nonparametric ANOVA (Kruskal-Wallis). The significance level was 95%. These statistical analyses were performed in Past 1.11 and OriginPro 8.

Results

Physico-chemical analysis

Variations were found in abiotic variables between the collection points for pH, conductivity and temperature (Table 4). However, DO varied between the collection points with the lowest values occurring at points 4 and 5, located downstream of the urban area ($p<0.05$) (Table 4 - statistical analysis). Concentrations of NH_4^+ and PO_4^{3-} were significantly increased at point 4 and

Table 2: Classification of histopathological alterations observed in liver of *Tilapia rendalli* according to the extent of alteration (Modified from Poleksic and Mitrovic-Tutundzic, 1994)

Stage	Alteration
I	Melanomacrophage centers
	Vacuolated cytoplasm
	Irregular-shaped hepatocytes
	Eosinophilic granules in cytoplasm
	Nuclear hypertrophy
	Cellular hypertrophy
	Irregular nuclei
	Nucleus in lateral position
	Leukocyte infiltration
II	Cytoplasmic degeneration
	Nucleus degeneration
	Pyknotic nucleus
	Nuclear vacuolization
	Rectilineation of vessels
	Rectilineation of parenchyma
III	Focal necrosis
	Tumor

Table 3: Classification of degree of alterations observed in liver based on HAI values (Poleksic and Mitrovic-Tutundzic, 1994)

HAI	DESCRIPTION
0 to 10	Normal functioning
11 to 20	Slight damage
21 to 50	Moderate damage
50 to 100	Severe damage
Over 100	Irreparable damage

Table 4: Physico-chemical parameters observed in water from the six points sampled in the Cachoeira River

PARAMETER	POINTS					
	P1	P2	P3	P4	P5	P6
pH	6.86 ±0.99 5.76-7.70	6.87 ±1.4 5.30-8.20	7.0 ±1.1 5.80-8.10	6.65 ±1.1 5.34-7.50	7.13±0.3 6.84-7.45	7.26 ±0.2 7.08-7.60
O.D. ($mgO_2.L^{-1}$)	8.03 ±1.49 7.07-9.75	5.46 ±1.2 4.05-6.18	5.98 ±0.8 4.97-6.60	4.21 ±0.8 3.26-4.95	4.44 ±1.7 2.63-6.01	7.14 ±0.5 6.54-7.59
Temperature(°C)	28.2 ±1.97 25.7-30.0	28.5 ±2.4 26.3-31.3	28.4 ±1.5 26.7-30.1	29.1 ±2.7 26.2-32.8	29.5 ±1.2 28.3-31.1	29.8 ±1.1 28.5-30.6
Conductivity ($\mu s.cm^{-1}$)	496.5 ±1.27 406.0-587.0	464 ±164.9 310.0-638.0	338.3 ±25.9 310-361	441.6 ±41.2 413.0-489.0	456.6 ±28.1 425.0-479.0	453 ±87 377.0-548.0
Sodium (µM)	2188 ±775 1144.9 - 3304	1696.0±670.4 599.1 - 2694	1731.9±549.6 981.1 - 2377.9	2138.5 ±907 796.1 - 3.161.6	1758.2±934.4 541.6 - 3.097.4	2202.1 ±819.5 1475 - 3889
Ammonium (µM)	96.8 ±184.2 1.90 - 465.1	5.1 ±4.16 0.2 - 8.3	7.5 ±3.8 2.2 - 12.2	166.0 ±180.5 -4.1 - 354.6	211.7 ±269.2 3.9 - 750.0	22.3 ±4.0 0.2 - 103.5
Potassium (µM)	77.5 ±54.1 5.3 - 138.9	58.2 ±60.8 6.0 - 133.5	92.3 ±3.1 45.7 - 125.6	135.1 ±77.3 30.9 - 233.3	199.7 ±173.2 22.2 - 552.3	119.0 ±76.1 37.1 - 231.1
Magnesium (µM)	681.5 ±280.1 370.2 - 1125.8	556.7 ±212.9 255.0 - 859.3	577.1 ±156.2 349.4 - 723.9	524.6 ±156.5 284.7 - 674.5	500.5 ±158.3 216.6 - 668.8	506.0 ±147.3 340.0 - 768.0
Calcium (µM)	457.0 ±2.07 258.0 - 772.1	363.0 ±1.35 192.0 - 546.4	362.5 ±1.1 247.2 - 524.5	391.6 ±179.8 218.4 - 669.7	449.6 ±2.0 201.4 - 711.3	437.2 ±135.5 271.9 - 643.6
Chloride (µM)	3343.2±2124.8 461.5 - 6658.6	2081.0±1707.8 250.1 - 5466.5	1724 ±1400 112 – 4164	2265.9±2088.2 239.6 - 6371.5	2134 ±1638.2 397.5 - 4776	1949 ±1240 683 - 4242
Nitrate (µM)	4.9 ±4.5 0.6 - 12.3	10.4 ±9.3 0.9 - 10.9	6.4 ±8.1 0.4 - 19.7	15.0 ±17.8 0.5 - 42.0	107.9 ±59.4 60.6 - 222.1	125.6 ±108.9 21.0 - 336.2
Phosphate (µM)	6.9 ±576.7 1.4 - 6.2	5.2 ±4.2 1.7 - 12.6	6.5 ±4.8 0.6 - 12.8	20.7 ±15.5 3.1 - 50.1	28.1 ±16.3 8.9 - 50.6	15.7 ±9.3 7.0 - 29.4
Sulfate (µM)	553.2 ±289.1 222.8 - 1071.0	233.7 ±117.4 121.4 – 393.5	183,9 ±11.3 48.1 – 312.8	227.8 ±126.6 88.2 – 300.6	235.6 ±83.0 99.4 - 344.6	185.1 ±110.0 71.6 - 352.7

significantly decreased at point 6 ($p<0.05$). In the case of NO_3^-, concentrations were higher at points 5 and 6 which coincided with increased concentrations of dissolved oxygen of 4.4 (point 5) and 7.1 mg L^{-1} (point 6). Regarding the other ions, no variations were observed between the collection points, except sulfate which had the highest concentrations at point 1 ($p<0.05$).

Principal Component Analysis (PCA) demonstrated that the variations between sampling points and parameters analyzed were explained in two significant axes with a total variance of 44.07% (axis 1) and 26.39% (axis 2). Thus, axis 1 explained better the influence of physico-chemical variables on the six sampling sites (Fig. 2).

The analysis showed that axis 1 was directly correlated with the parameters pH, temperature, NO_3 (nitrate), potassium (K^+), phosphate (PO_4^{3-}) and turbidity, and inversely correlated with magnesium (Mg^{2+}), dissolved oxygen (DO) and biological data obtained by HAI. On the other hand, axis 2 was correlated with the parameters conductivity, nitrate (NO_3^-), calcium (Ca^{2+}), ammonium (NH_4^-), sulfate (SO_4^{2-}) and chloride (Cl). The results of this analysis showed that all parameters studied influenced water quality at the sampling points analyzed (Fig. 2). This indicated that besides the substances analyzed, probably other components present in the water could have affected the fish at these locations.

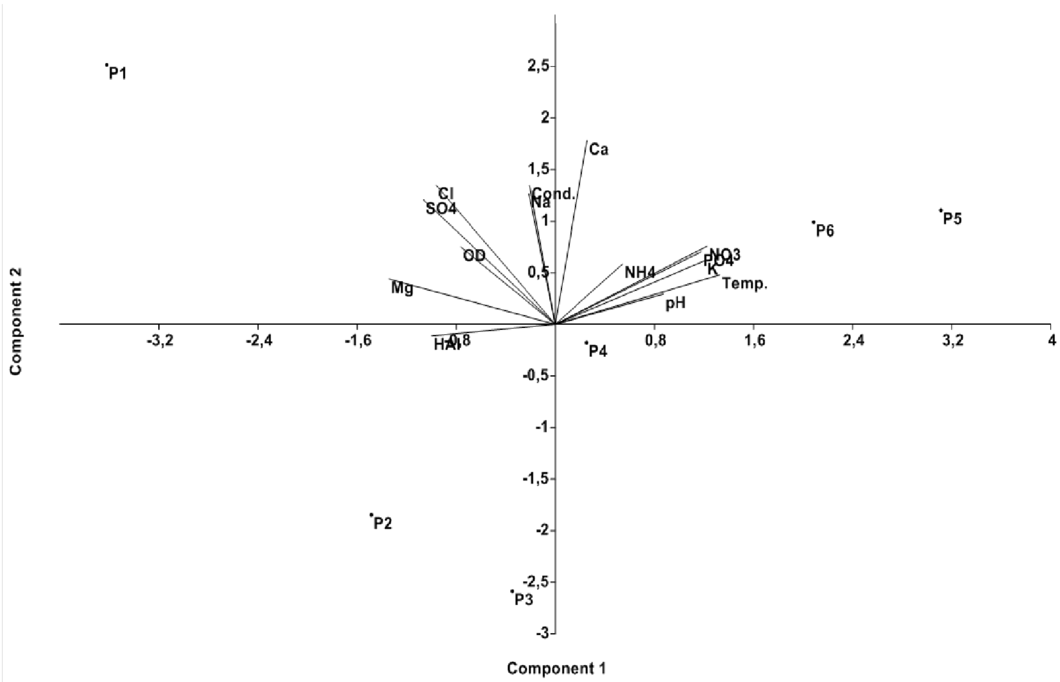

Fig. 2: Principal component analysis (PCA) of physico-chemical parameters of samples of Cachoeira River

Histopathological analysis

53 specimens of *T. rendalli* were used for histological analysis, ten individuals from each point, except points 2 and 6, where only eight and five individuals were collected, respectively. The total weight ranged from 0.3 to 5.21 g with a mean weight of 1.68 g and standard length ranged from 14.4 to 54.48 mm with a mean of 32.1 mm, indicating that the individuals were juveniles.

The liver showed the same structural pattern as other teleosts. Hepatocytes had a polyhedral shape, and the nuclei were mostly spherical, large and located in the center of the cell. The nucleolus was evident and the cytoplasm contained granules. The cells were irregularly distributed in the liver parenchyma and were separated by sinusoidal capillaries. We also observed the presence of small blood vessels, bile ducts and exocrine pancreas, which consisted of serous acinar cells, with eosinophilic staining at the apical part and basophilic staining near the base (Fig. 3A).

All individuals had histopathological changes, and the main lesions observed were: changes in the exocrine pancreas, rectilineation of blood vessels, pyknotic nuclei, cytoplasmic degeneration, irregular hepatocytes and nuclei, nuclei in the lateral position, cytoplasm vacuolization, leukocyte infiltration, nuclear hypertrophy and cellular and focal necrosis. These observed alterations were distributed in all classification stages (I, II and III) according to lesions severity (Table 2).

In individuals from point 1, alterations were observed as rectilineation of blood vessels (Fig. 3B), nucleus position and irregular hepatocytes in all specimens. Cytoplasmic degeneration and modifications in the exocrine pancreas were also observed (Table 5). In addition to these changes, we observed the presence of foreign-body giant cells in some individuals, and dilated blood vessels and arterioles, cell aggregates, small fibromas and unidentified parasites (Fig. 3D).

All specimens from point 2 had degenerated and vacuolated cytoplasm and rectilineation of blood vessels (Fig. 3C). A high percentage of individuals showed nuclei in the lateral position (87.5%) and irregular hepatocytes (87.5%) (Table 5). Aggregated hepatocytes, fibroblasts, foreign-body giant cells and dilated blood vessels were also observed.

At point 3, all liver specimens displayed vacuolated cytoplasm (Fig. 4A), nucleus in the lateral position and rectilineation blood vessels. The irregular shape of hepatocytes and cytoplasmic degeneration (Fig. 4B) were also often observed respectively in 90 and 80% of the specimens. All individuals from point 4 (Jardim das Acácias) had rectilineation vessels, vacuolated and degenerated cytoplasm, pyknotic nuclei, nucleus in lateral position and irregular hepatocytes (Table 5). The individuals from these two points showed blood vessel dilation, aggregated hepatocytes and foreign-body giant cells.

It was observed that the liver of all specimens from points 5 and 6 had vessel rectilineation, cytoplasmic degeneration and the nucleus in the lateral position. All individuals from point 6 also showed a vacuolated cytoplasm (Table 5). Some individuals from these points showed inflammation with blood immune cells (mainly lymphocytes), aggregated hepatocytes and very dilated blood vessels. In general, the most frequent alterations were rectilineation of parenchyma and blood vessels, present in 100% of the specimens analyzed, irregular-shaped hepatocytes (79.2%), nucleus in the lateral position (98.1%), cytoplasmic vacuolization (84.9%) and cytoplasmic degeneration (75.5%).

The frequency of histopathological alterations intensity observed in specimens from point 1 showed that 60% of changes were classified as moderate (2), and slight (1) and severe (3) changes were each 20%. Point 2 showed a high mean of moderate alterations (62.5%), followed by slight and severe alterations (25% and 12.5%, respectively). Moderate alterations were prevalent at points 3, 4, 5 and 6 (80, 80, 70 and 100%, respectively) (Fig. 5).

On the HAI analysis, points 4, 2, 1 and 3 had the highest means (67.3 ± 43.5; 66.25 ± 47.5, 63.5 ± 47.1 and 50.1 ± 37.4, respectively), indicating that the samples showed severe alterations in the liver. The HAI mean of points 5 and 6 were lower (49.8 ± 33.5 and 41.2 ± 5.8, respectively) (Fig. 6) but still showing the presence of moderate changes in the liver.

The high frequency of moderate alterations and high HAI mean values indicated that individuals from all six points analyzed were exposed to some stressor, but if environmental conditions were differences between any of the points according to alteration frequency or HAI mean.

Ultrastructural analysis

Eight livers of *T. rendalli* were used for ultrastructural analysis. The results showed that the cells had the same structural pattern described for hepatocytes of fish, consisting of nucleus, cytoplasm and organelles such as mitochondria, lysosomes, endoplasmic reticulum and Golgi apparatus. The hepatocytes showed a round nuclei, having granular and condensed chromatin located at the periphery. The hepatocytes contained glycogen granules spread throughout the cytoplasm and zymogen granules. Blood cells were observed adjacent to the hepatocytes (Fig. 7 A).

At all points analyzed, there were changes in the ultrastructure of cells, indicating that they were exposed to a stressor in the environment. The main changes were an

Table 5: Frequency of different types of histopathological alterations per sampling point

ALTERATION	POINTS					
	1	2	3	4	5	6
	N					
	10	8	10	10	10	5
	F (%)					
Rectilineation of blood vessels	100	100	100	100	100	100
Degeneration of cytoplasm	90	100	80	100	100	100
Nucleus in lateral position	100	87,5	100	100	100	100
Vacuolated cytoplasm	80	100	100	100	40	100
Pyknotic nucleus	70	62,5	50	100	40	60
Irregular hepatocytes	100	87.5	90	100	10	100
Irregular nucleus	60	50	30	40	50	40
Nuclear hypertrophy	70	62,5	50	40	40	80
Altered pancreas	90	75	50	80	90	40
Nuclear degeneration	30	12.5	30	30	20	40
Cellular hypertrophy	20	50	30	60	50	40
Leukocyte infiltration	20	12.5	10	0	0	0
Focal necrosis	20	25	10	20	10	0
Vacuolated nucleus	10	12.5	50	20	10	40
Melanomacrophage centers	10	37.5	0	0	0	20

N – sample number; F – frequency

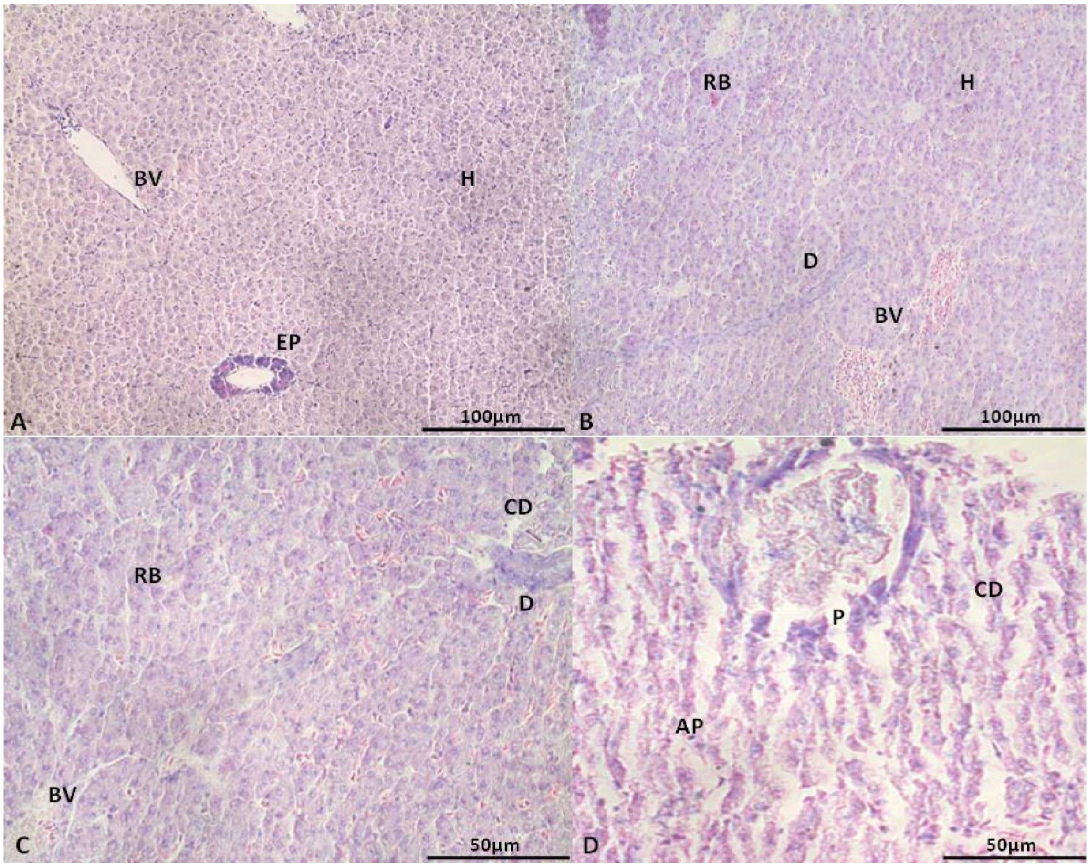

Fig. 3 Histological analysis of *Tilapia rendalli* liver, HE. (A) - Normal liver, HE. (B) - Liver with slight alterations, HE. (C)- Liver with moderate alterations, HE. (D) - Liver with severe alterations, HE. EP - Exocrine pancreas, H - Hepatocytes, BV - Blood vessels, RB - Rectilineation of blood vessels, D - Bile duct, CD – Cellular degeneration, AP - Altered parenchyma, P – Parasite

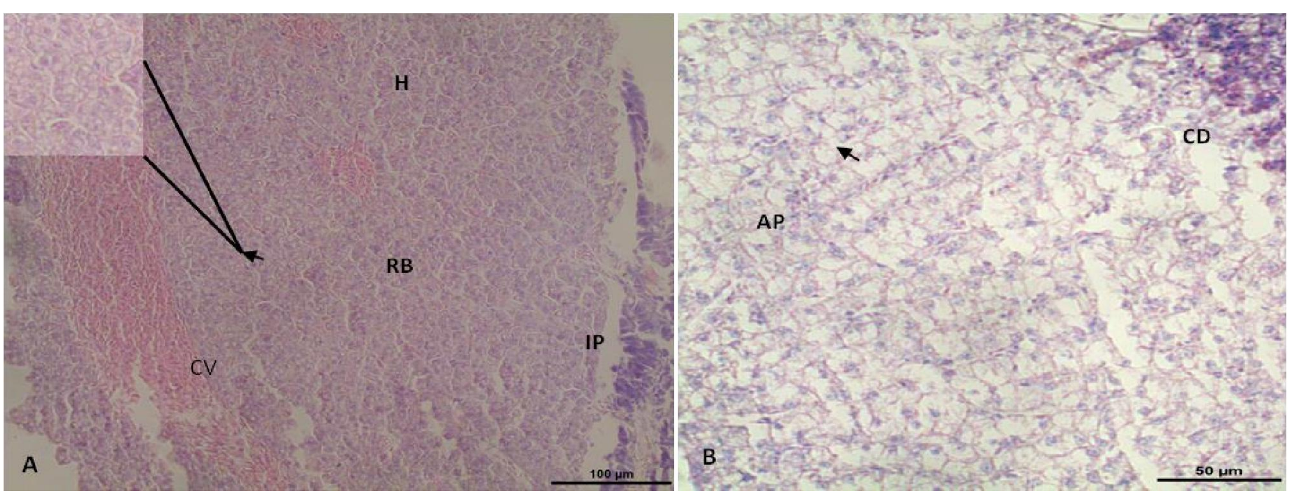

Fig. 4 (A) - Liver with moderate changes, HE. (B) - liver with severe alterations, HE. H - Hepatocytes, RB - Rectilineation of blood vessels, CV - Congested vessel, IP - Irregular pancreas, AP - Altered parenchyma, CD - Cellular degeneration, and in detail, Vacuolated cells (arrows)

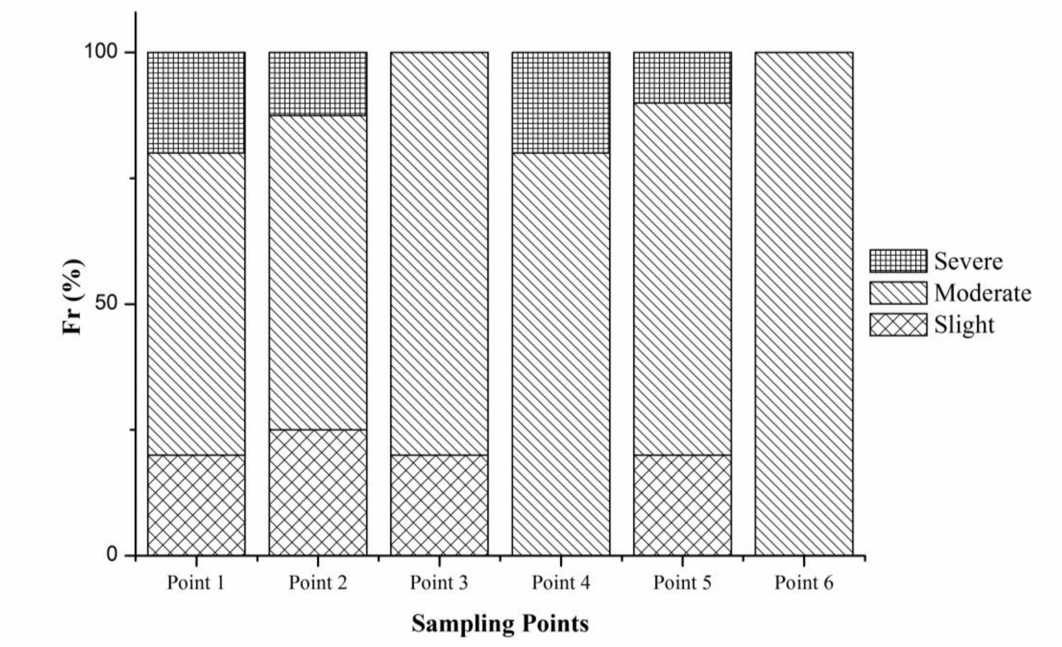

Fig. 5: Frequency of histopathological alterations intensity observed in *Tilapia rendalli* liver per sampling point on the Cachoeira River

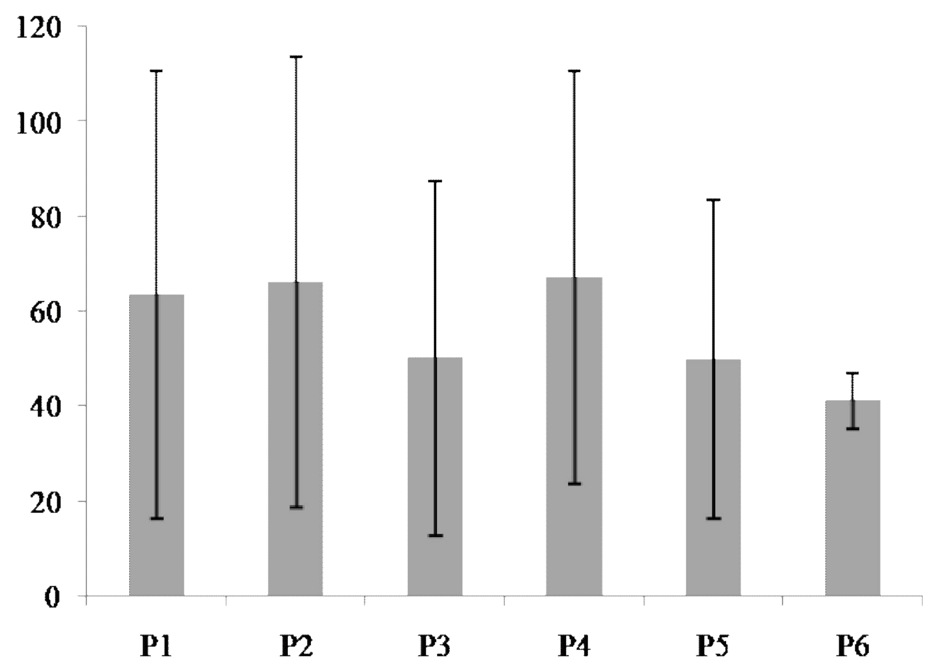

Fig. 6: Means and standard deviation of the histopathological alterations index (HAI) in *Tilapia rendalli* liver at sampling points on the Cachoeira River

increase in the amount of lipid droplets (Fig. 7 B) and secretory granules (Fig. 7 C), cell necrosis (Fig. 7 D), nucleus in the apoptotic process, organelles with undefined architecture, large zymogen granules composed of very electron-dense material (Fig. 8A). Other alterations were mitochondria with varied sizes and shapes (Fig. 8 B), mitochondrial proliferation and degeneration, excess condensed heterochromatin at the periphery of the nucleus and structural changes in the rough endoplasmic reticulum.

Accordong to semi-quantitative analysis based on the severity of the changes, it was not possible to identify any of the sampling points as in the normal or control stage. The ultrastructural alterations allowed to classify points 5 and 6 as moderate, since cells were in degeneration process. Points 1, 2, 3 and 4 were classified as severe, because many large secretory granules were observed, which in some cases were larger than the nuclei. Cells in necrosis or apoptosis process were also observed, indicating organ destruction (Figs. 8 C, D).

Discussion

This study demonstrated that the different methods used (physico-chemical, histological and ultrastructural) were efficient to evaluate the environmental quality of the Cachoeira River. Bruschi Jr. *et al.* (2000) stated that the study of the ecosystem integrity and the quantification of deterioration degree, along with its consequences on the ecosystem has been an ongoing challenge for scholars of environmental areas.

The liver of *T. rendalli* was shown to be sensitive to changes in the quality of the aquatic environment, as seen by the ultrastructural and histopathological alterations, making it an excellent environmental quality biomarker of the Cachoeira River. This study suggests that fish at sampling sites were suffering the effects of some stressor. Several authors have demonstrated that the cyto-histopathological alterations observed are an excellent response of the target organ and have pointed out that this is due to the fact that this organ responds satisfactorily when in contact with some chemicals, and performs vital functions for fish survival (Au *et al.*, 1999; Paris-Palacios *et al.*, 2000; Au 2004; Authman, 2011; Perendija *et al.*, 2011; Abdel-Moneim, *et al.*, 2012).

Histopathological alterations such as blood vessel rectilineation, cytoplasmic degeneration, nucleus in lateral position and cytoplasmic vacuolization were observed in the liver of *T. rendalli*. These alterations are normally associated with fish exposed to high concentrations of heavy metals such as copper (Figueiredo-Fernandes *et al.*, 2007), aluminum (Authman, 2011), and cadmium and zinc (Van Dyk *et al.*, 2005), herbicides (Peebua *et al.*, 2008), organophosphates (Srivastava *et al.*, 1990) and botanical pesticides (Kumar *et al.*, 2013). The high frequency of vacuoles observed in hepatocytes is also an indication that individuals have been exposed to a stressor (Authman, 2011) and these agents modify the hepatic parenchyma, giving the liver an irregular appearance (Kohler *et al.*, 1992). The results demonstrated a relationship between the occurrence of damage and environmental quality, so that they can be considered good biomarkers of the water quality at the site studied.

Changes observed in size of the hepatocyte nuclei in individuals of all sampling points were viewed as indicative of the presence of stressors in the environment. Figueiredo-

Fernandes *et al.* (2007) observed similar changes and Peebua *et al.* (2007) observed the presence of pyknotic nuclei in hepatocytes of *Oreochromis niloticus* exposed

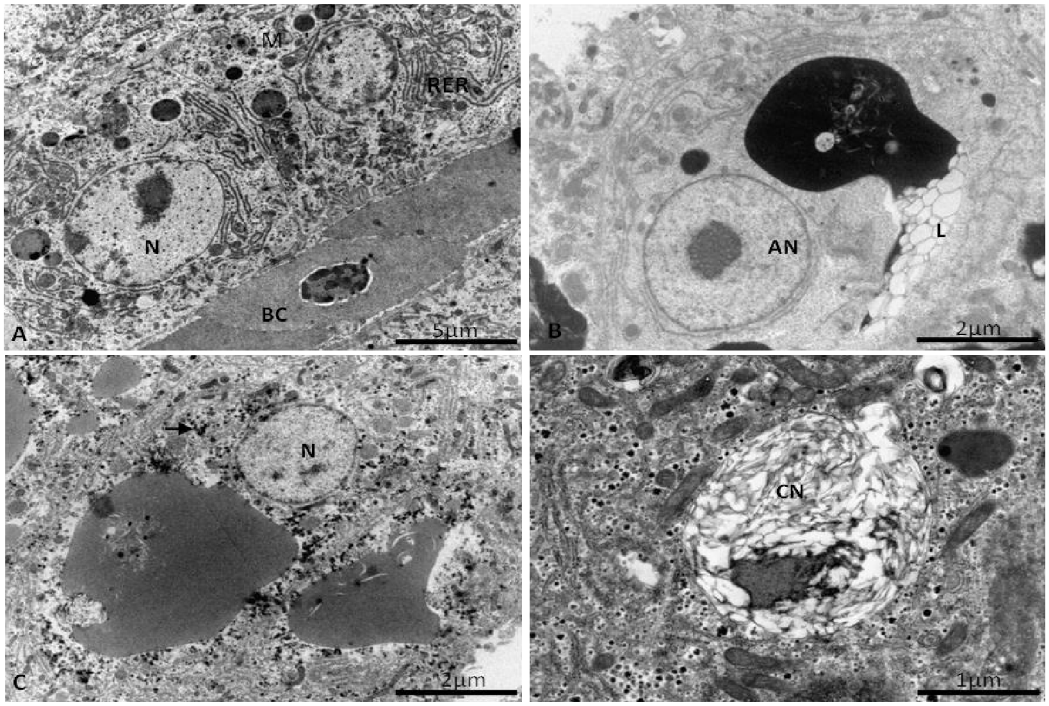

Fig. 7: Electron microscopy of *Tilapia rendalli* liver. N - nucleus, BC - blood cell, M - mitochondria, RER - rough endoplasmic reticulum, AN - apoptotic nucleus, L - lipids, CN - cellular necrosis and glycogen (arrow)

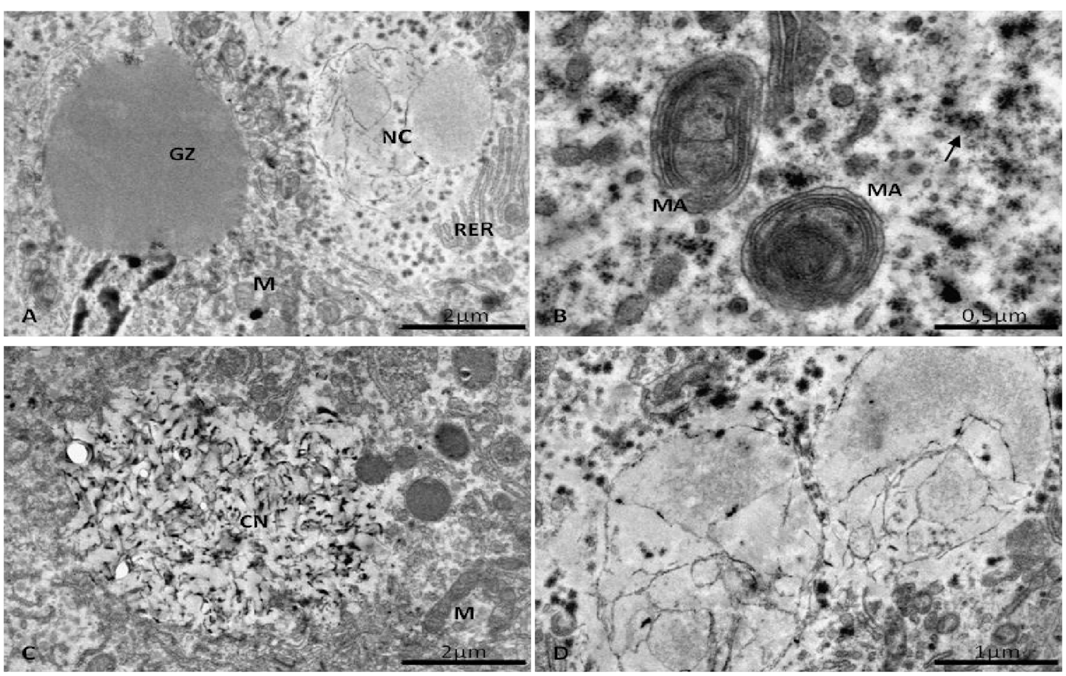

Fig. 8: Electron microscopy of *Tilapia rendalli* liver. GZ–enlarged zymogen granule, M - mitochondria, NC - cellular necrosis, RER - rough endoplasmic reticulum, MA - mitochondria with altered shape and glycogen (arrow)

to acute and subchronic concentrations of herbicides. Marigómez *et al.* (2006) noted that the occurrence of a pyknotic nuclei in fish hepatocytes is evidence that the organ is in a degenerative process, which can be caused by exposure to even at high sublethal concentrations of toxic substances.

Necrosis is considered a severe change and was observed in individuals at most points, except for points 3 and 6. According to Camargo and Martinez (2007), focal necrosis is a serious deterioration, in which tissue damage is irreparable. Kohler *et al.* (1992) also classified this alteration as severe, associating the occurrence of this damage with the presence of some toxic substance. The high amount of chemicals found in the samples of the Cachoeira River allowed us to infer that the presence of necrotic hepatocytes in the specimens analyzed was directly related to the physico-chemical water quality of this environment.

Among the ultrastructural changes, a high frequency of mitochondria with changes in shape and size was observed, and the occurrence of mitochondrial degeneration in fish liver can also be considered an indication that they are suffering from the effects of some stressor (Abdel-Moneim and Abdel-Mohsen, 2010). Authors such as Au *et al.* (1999) and Paris-Palacios *et al.* (2000) also found that fish exposed to elevated concentrations of heavy metals or nutrients had liver cells with altered mitochondria.

The occurrence of severe pathological and ultrastructural changes, associated with the presence of nutrients and heavy metals in the river water, are an indication that, probably, individuals were subjected to a low-quality environment. Flores-Lopes and Malabarba (2007) pointed out that the frequency of pathological changes in fish liver can be directly proportional to the degree of environmental contamination by heavy metals and pesticides. According to authors such as Au *et al.* (1999), Paris-Palacios *et al.* (2000), Gernhofer *et al.* (2001), Abdel-Moneim and Abdel-Mohsen (2010), Perendija *et al.* (2011) and Authman (2011), utrastructural lesions are commonly observed in fish livers exposed to chemicals or any adverse environmental conditions. Camargo and Martinez (2007) and Abdel-Moneim *et al.* (2012) observed these types of pathological changes and related them to the quality of river sampled, and Paulo *et al.* (2012) studied the liver of *Poecilia vivipara* from the Cachoeira River and revealed the occurrence of severe pathological changes, which they related to environmental quality.

By studying the chemical composition of Cachoeira River water, Klumpp *et al.* (2002) and Lucio *et al.* (2012) found high concentrations of aluminum, ammonium, magnesium, phosphate, potassium and sulfate, and the main degradation agents of this environment were the Itabuna sewage treatment, the replacement of native vegetation with crops, livestock breeding and geological formation, as well as discharge of domestic and industrial waste water.

The HAI results for points 1, 2 and 4 corroborated the frequency analysis results of alteration intensity and ultrastructural changes, since in both analysis, these points showed the highest values, plus point 3 showed the highest frequency of severe alterations. The presence of these severe alterations at the ultrastructural level indicates that individuals responded rapidly to the environmental stress to which they were exposed. In this study, point 2 was rated as having poor quality due to be presence of a higher frequency of specimens with cell death and moderate to severe histopathological alterations.

Paulo *et al.* (2012) also observed moderate and severe changes in the liver of *P. vivipara* at this point, which may be an indication that the fish at this site suffer from the effects of some stressors. These results are similar to those reported by Abdel-Moneim *et al.* (2010), who found that the occurrence of a high frequency of moderate and severe changes, coupled with the occurrence of a low frequency of slight changes, indicates that individuals have been exposed for a long period of time to the action of some stressors.

It can be inferred that high concentrations of sulfate and ammonium are one of the factors responsible for the severity of histopathological and ultrastructural alterations observed at points 1 and 4. In studying the Cachoeira River basin, Lucio *et al.* (2012) observed a large amount of ions, including chloride, sulfate, magnesium and ammonium, associating the occurrence of a high concentration of the last with the proximity between point 4 and some pastures, allowing the entrance of agricultural runoff. Pinho (2001) found a high phosphate content at this location, which was probably caused by the presence of industrial and agricultural runoff containing detergents and fertilizer. Pereira (2004) noted that the presence of excess sulfate in water could be related to the discharge of household sewage, industrial waste and also the geological formation of the water system.

Points 5 and 6 were described as moderate by ultrastructural and histopathological analysis, indicating that the fish are exposed to stressors. This can be explained by the high concentration of potassium, ammonium and phosphate at these points, which was also observed by Lucio *et al.* (2012). Klumpp *et al.* (2002) also observed a high phosphate content along the Cachoeira River, which according to Neto *et al.* (2012) is a concern, since this ion is one of the limiting nutrients in eutrophication.

The alteration intensity frequency and the HAI values showed no statistically significant difference between the points, indicating that they have a similar quality and that both measures are affected by the presence of stressors. The results showed that the points located between the municipalities of Itapé and Itabuna (1, 2, 3 and 4) are those with a lower environmental quality and that they have a similar physico-chemical quality of water, which indicates that all these parameters studied or some other environmental factor that was missed in the analysis may be affecting individuals that inhabit these aquatic environments.

Thus, both the physico-chemical data and histopathological and ultrastructural findings demonstrated that the Cachoeira River is experiencing a process of environmental degradation due to the presence of stressors, which has directly affected the health of organisms living there. This observation is supported by the occurrence of high frequency of moderate and severe changes at cyto-histopathological level.

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