AVALIAÇÃO DE ESPÉCIES DE PEIXES COMO INDICADORES DE INTEGRIDADE AMBIENTAL

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AVALIAÇÃO DE ESPÉCIES DE PEIXES COMO INDICADORES DE INTEGRIDADE AMBIENTAL

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RESUMO

A contaminação por metais em ecossistemas aquáticos representa uma séria ameaça para os peixes, tornando-se necessário o uso de técnicas rápidas e precisas para identificar esses contaminantes. O presente estudo foi realizado para (1) investigar os efeitos mutagênicos, genotóxicos e a bioacumulação de metais (Cd, Pb, Cr, Cu, Fe, Zn e Ni) em duas espécies de peixes (*Pterygoplichthys ambrosetti* e *Prochilodus lineatus*) no Rio Amambai (Alto Rio Paraná); (2) avaliar a frequência de micronúcleos e de anormalidades nucleares em eritrócitos e a concentração de metais na musculatura de *Astyanax lacustris*, *Hypostomus ancistroides* e *Rhamdia quelen* em locais com níveis distintos de integridade ambiental na Microbacia Tarumã (Alto Rio Paraná, MS); (3) analisar o uso e a ocupação da paisagem circundante dos córregos da Microbacia Tarumã e a contaminação do sedimento aquático por metais e a relação destes dois fatores com efeitos mutagênicos e genotóxicos em eritrócitos de *A. lacustris*; (4) identificar a presença de metais em escamas de *Prochilodus lineatus* e *Salminus brasiliensis*, usando espectroscopia LIBS, em três locais de amostragens ao longo do rio Amambai. A análise de micronúcleos indicou elevados potenciais mutagênicos nos peixes. Observou-se a concentração mais elevada de metais no fígado e a menor concentração na musculatura. A hierarquia das concentrações dos metais analisados neste estudo foi: Fe> Zn> Cu> Pb>Cr> Cd> Ni. Os resultados demonstraram que o uso do solo de maneira não planejada, com a expansão de atividades antrópicas, pode estar prejudicando a integridade ambiental da Microbacia Tarumã. O bioensaio realizado demonstrou claramente que a água dos locais mais impactados da microbacia Tarumã induziu efeitos mutagênicos e genotóxicos em eritrócitos de *A. lacustris*. Por meio da técnica de LIBS foi constatada a presença de metais nas escamas das espécies analisadas, sendo uma ferramenta preditiva de monitoramento ambiental. Conclui-se que as espécies estudadas podem ser consideradas boas indicadoras da qualidade do ambiente onde vivem e que os métodos utilizados na pesquisa se mostraram satisfatórios para responder aos objetivos propostos.

PALAVRAS-CHAVE: Ambientes aquáticos, metais, bioacumulação, genotoxicidade, mutagenicidade, LIBS.

ABSTRACT

Contamination by metals in aquatic ecosystems poses a serious threat to fish, making it necessary to use rapid and accurate techniques to identify these contaminants. The present study was carried out (1) to investigate the mutagenic, genotoxic effects and bioaccumulation of metals (Cd, Pb, Cr, Cu, Fe, Zn and Ni) in two species of fish (*Pterygoplichthys ambrosetti* and *Prochilodus lineatus*) on the Amambai River (Upper Paraná River); (2) to evaluate the frequency of micronuclei and nuclear abnormalities in erythrocytes and the concentration of metals in the musculature of *Astyanax lacustris*, *Hypostomus ancistroides* and *Rhamdia quelen* in sites with distinct levels of environmental integrity in Tarumã Microbasin (Upper Rio Paraná, MS); (3) to analyze the use and occupation of the surrounding landscape of Tarumã Microbasin and the contamination of the aquatic sediment by metals and the relation of these two factors with mutagenic and genotoxic effects in *A. lacustris* erythrocytes; (4) to identify the presence of metals in scales of *Prochilodus lineatus* and *Salminus brasiliensis* using LIBS spectroscopy at three sampling sites along the Amambai River. Micronucleus analysis indicated high mutagenic potential in fish. The highest concentration of metals in the liver and the lowest concentration in the musculature were observed. The hierarchy of the concentrations of metals analyzed in this study was: Fe> Zn> Cu> Pb> Cr> Cd> Ni. The results demonstrated that the unplanned use of the soil, with the expansion of anthropic activities, may be damaging the environmental integrity of Tarumã Microbasin. The bioassay carried out clearly demonstrated that water from the most impacted sites of the Tarumã Microbasin induces mutagenic and genotoxic effects on *A. lacustris* erythrocytes. Using the technique of LIBS, we were able to verify the presence of metals in the scales of the analyzed species, being a predictive tool of environmental monitoring. It is concluded that the studied species can be considered good indicators of the quality of the environment where they live and that the methods used in the research proved satisfactory to respond to the proposed objectives.

KEY WORDS: Aquatic environments, metals, bioaccumulation, genotoxicity, mutagenicity, LIBS.

CAPÍTULO 1. CONSIDERAÇÕES GERAIS

A redução da cobertura vegetal local ao redor das margens de rios e córregos, decorrente da expansão de atividades agrícolas e da ocupação urbana, facilita a entrada de contaminates que são carreados para os ambientes aquáticos (Zhang et al., 2012; Jindal; Verma, 2015). A contaminação por metais em ecossistemas aquáticos pode representar uma grave ameaça para a comunidades de peixes, podendo resultar na perda de diversidade de espécies (De Jonge et al., 2008; De Jonge et al., 2015). Neste caso, os metais, incluindo tanto elementos essenciais como não essenciais, têm um significado particular em ecotoxicologia, por causa de sua toxicidade, longa persistência e bioacumulação nos peixes (Ebrahimpour; Mushrifah, 2010; Yousafzai et al., 2010). Mesmo os metais essenciais, acima de certas concentrações, podem ser tóxicos para as atividades biológicas dos organismos (Merciai et al., 2014).

O grau de contaminação depende do tipo de poluente, da espécie de peixe, do seu nível trófico, do seu modo de alimentação e do local de amostragem (Asuquo et al., 2004). Desta forma, o uso de peixes como bioindicadores dos efeitos da poluição vem tendo crescente importância, pois pode indicar precocemente a poluição no ambiente aquático (Van Der Oost et al., 2003). Em particular, espécies de peixes são adequadas como bioindicadores de qualidade do ambiente, uma vez que desempenham um papel importante nos ecossistemas aquáticos, ocupando diferentes níveis tróficos, além de serem explorados para consumo humano (Lemos et al., 2007; Jesus et al., 2016).

Os peixes são organismos adequados para monitorar a poluição ambiental no meio aquático, visto que concentram alguns poluentes provenientes diretamente da água, e também através da sua dieta, nos seus tecidos, pois sofrem bioacumulação e biomagnificação por contaminantes químicos (Ayas et al., 2007). Sendo assim, o uso de peixes como bioindicadores de impacto ambiental tem sido cada vez mais frequente, pois estes organismos são eficientes para alertar sobre o perigo de substâncias químicas derivadas da poluição ambiental (Schulz; Martins, 2001; Van Der Oost et al., 2003). A utilização destes organismos para bioindicação é capaz de fornecer informações que descrevam as alterações nos ecossistemas aquáticos (Smith et al., 1997; Magurran; Phillip, 2001; Lima-Junior et al., 2006; Souza; Lima-Junior, 2013).

Neste sentido, biomarcadores em peixes, como o teste de micronúcleos, tem sido utilizados, com grande sucesso, para avaliar a mutagenicidade dos poluentes ambientais e de diferentes compostos químicos (Klobucar et al., 2003; Hoshina et al., 2008; Jesus et al., 2016). Os peixes que estão submetidos a toxinas aquáticas apresentam uma frequência mais elevada de micronúcleos e de alterações nucleares. Micronúcleos são fragmentos cromossômicos, ou cromossomos inteiros, que estão fora do núcleo celular, fenômeno que ocorre após a mitose (Udroiu, 2006). Estes micronúcleos são marcadores úteis para biomonitoramento ambiental aquático de contaminantes químicos, pois este dano tende a ser irreversível e continua se manifestando em gerações futuras através da hereditariedade, e as espécies afetadas tendem a ser drasticamente reduzidas (Obiakor et al., 2012).

As alterações nucleares em eritrócitos (ENA) também são indicadores de danos com potencial genotóxico que afetam a integridade do DNA, resultantes de contaminantes no ambiente aquático (Stankeviciute et al., 2016). Estas anormalidades podem se manifestar de diversas formas: célula binucleada, núcleo vacuolizado, brotamento nuclear, invaginação nuclear, núcleo lobulado, ponte nuclear e picnose (Carrasco et al., 1990). Estas anormalidades nucleares são indicadores de divisão celular anormal, devido a alguma substância presente no ambiente aquático que ocasiona danos mutagênicos e genotóxicos (Stankeviciute et al., 2016).

A análise de metais em peixes tem sido investigada em vários estudos, sendo uma importante ferramenta de monitoramento ambiental para se diagnosticar e quantificar efeitos tóxicos diretos e indiretos que afetam os peixes (Lins et al., 2010). A análise quantitativa da absorção de metais pesados, em diferentes órgãos e tecidos de peixes, é valiosa na biologia ambiental (Wan; Wang, 2015).

As escamas de peixes são consideradas bons bioindicadores ambientais, podendo bioacumular contaminantes, como uma impressão digital química dos eventos de poluição do ambiente em que o peixe habita (Kaur; Dua, 2012; Almeida et al., 2016; Santana et al., 2016). As escamas estão diretamente expostas a vários poluentes e tem o primeiro contato com os contaminantes. As informações sobre metais nas escamas de peixes podem dar uma visão do grau de poluição da água no período de vida do peixe (Nováková et al., 2010).

Por meio da técnica de Espectroscopia de Plasma Induzida por Laser (*Laser-induced breakdown spectroscopy* – LIBS), com junção do infravermelho por Transformada de Fourier, podemos determinar a presença de Fe e Pb presente nas escamas de peixes. LIBS é uma técnica de espectroscopia de emissão atômica que utiliza um laser pulsado com uma fonte de excitação (Wan; Wang, 2015). Esta técnica tem características proeminentes, sendo altamente seletiva, sensível, rápida e direta (Cremers et al., 2006; Li et al., 2012; Yu et al., 2014).

Neste contexto, o foco principal deste estudo foi utilizar diferentes espécies de peixes como bioindicadores ambientais para avalição da integridade ambiental do Rio Amambai e da Microbacia Tarumã (Alto Rio Paraná, Brasil) e a possibilidade de utilização das espécies analisadas como indicadores da qualidade ambiental no ecossistema aquático tendo, como objetivos, (1) avaliar os efeitos mutagênicos e genotóxicos e verificar a acumulação de metais no fígado e na musculatura de *Pterygoplichthys ambrosetti* e *Prochilodus lineatus* do rio Amambai a fim de estimar o potencial de cada uma como bioindicador ambiental; (2) avaliar a frequência de micronúcleos e de anormalidades metanucleares em eritrócitos e a concentração de metais na musculatura de *Astyanax lacustris*, *Hypostomus ancistroides* e *Rhamdia quelen* da Microbacia Tarumã; (3) investigar o uso e a ocupação da paisagem circundante dos córregos da Microbacia Tarumã e a contaminação do sedimento aquático por metais e a possível relação destes dois fatores com efeitos mutagênicos e genotóxicos em eritrócitos de *A. lacustris*; (5) determinar a eficiência da técnica de LIBS para detectar a presença de Fe e Pb bioacumulados em escamas de *S. brasiliensis* e *P. lineatus* e comparar os resultados obtidos a partir da técnica de LIBS com a técnica convencional de espectrofotômetro de absorção atômica para detecção de metais nas escamas de *S. brasiliensis* e *P. lineatus*.

A tese está estruturada em quatro capítulos redigidos na forma de artigos científicos e um capítulo final com as considerações gerais do trabalho.

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CAPÍTULO 2. MUTAGENIC AND GENOTOXIC EFFECTS AND METAL CONTAMINATIONS IN FISH OF THE AMAMBAI RIVER, UPPER PARANÁ RIVER, BRAZIL

Artigo submetido à Environmental Science and Pollution Research

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ABSTRACT. The present study evaluated mutagenic and genotoxic effects and metal accumulation in the liver and musculature of *Pterygoplichthys ambrosetti* and *Prochilodus lineatus* in the Amambai River, a tributary of the Upper Paraná River in Brazil. We also evaluated the potential for these fish species as environmental bioindicators. We found that *P. ambrosetti* had a higher frequency of micronuclei compared to *P. lineatus* ($p<0.0001$). There were no significant differences between species in other erythrocyte nuclear abnormalities (ENA) ($p> 0.05$). For both species, the liver contained a higher concentration of metals (Cd, Pb, Cr, Cu, Fe, Zn and Ni) than did the musculature ($p <0.0001$). Of the two species, *P. ambrosetti* was more suitable to evaluate mutagenic and genotoxic effects and metal accumulation in the liver and musculature, likely due to its resident behavior, hence we highlight the potential of this species for use as an environmental bioindicator. The concentrations of non-essential metals observed in the fish confirm conditions of environmental stress in the Amambai River, possibly related to the discharge of pollutants and exacerbated by lack of native vegetation cover along the watercourse.

KEYWORDS. Contaminants, aquatic environments, ichthyofauna, bioindicators, biomarkers, micronuclei.

2.1. INTRODUCTION

The reduction of vegetation cover around river banks and streams causes negative consequences because, among other problems, it compromises the filtering of toxic contaminants that end up being carried from the terrestrial environment to the aquatic environment. The aquatic environment suffers constant exposure to pollution from industrial, urban, and agricultural sources, and can receive high input of toxic substances. This can cause bioaccumulation of metals in fish tissues that induce mutagenic and genotoxic damage, potentially resulting in loss of biodiversity (Linde-Arias et al. 2008; De Jonge et al. 2008; Sunjog et al. 2012; De Jonge et al. 2015).

Fish are model organisms for aquatic genotoxicity evaluation because they occupy different trophic levels and exhibit inter-population differences in behavior; they are also bioconcentrators that metabolize chemical contaminants, and are sensitive to low concentrations of genotoxic substances (Lemos et al. 2007; Rocha et al. 2009; Jesus et al. 2016). Prior to death or obvious disease, fish can react to stress by changing biochemical and physiological responses, which can then serve as biomarkers of environmental pollution (Linde-Arias et al. 2008; Freire et al. 2015). The ability to recognize and measure these biomarkers may allow prediction of more serious future consequences on the aquatic community (Azevedo et al. 2011).

One of the most useful biomarkers for evaluation of mutagenic effects in fish is the micronucleus test, which is an effective tool for diagnosis of environmental stress (Klobucar et al. 2003; Hoshina et al. 2008; Jesus et al. 2016). Micronuclei are whole chromosomes or chromosomal fragments that remain outside of the cell nucleus after mitotic division (Udroiu 2006; Jesus et al. 2016). Changes in normal nucleic morphology are also considered to be indicators of genotoxic damage, such as erythrocyte nuclear abnormalities (ENA) related to cell division failure or apoptosis (Carrasco et al. 1990; Furnus et al. 2014). Analysis of metal bioaccumulation in fish tissues is another essential environmental monitoring tool for diagnosis and quantification of direct and indirect toxic effects (Van Der Oost et al. 2003; Lins et al. 2010; Weber et al. 2013; De Jonge et al. 2015; Wan and Wang 2015; Yamamoto et al. 2016).

The Upper Parana River Basin provides water to the most densely populated region of Brazil, where high population density has reduced the integrity of the aquatic environment. The section of the Upper Paraná River in Mato Grosso do Sul state flows through regions with lower population density than São Paulo and Paraná states. However, strong agricultural activity and the absence of adequate sewage treatment in riverside cities may lead to pollutants being carried into rivers, negatively impacting the aquatic biota (Message et al. 2016).

This study utilized two detritivorous fish species as environmental bioindicators: *Pterygoplichthys ambrosetti* (Holmberg 1893) (gray armored catfish), a (Siluriformes: Loricariidae), and *Prochilodus lineatus* (Valenciennes 1837) (‘curimba’), a medium-sized (Characiformes: Characidae) (Souza et al. 2008; Cazenave et al. 2014). These species are suitable for environmental monitoring due to their feeding habits, as detritivores are able to incorporate contaminants from the water column and sediment (Bacchetta et al. 2011). The objective of this study was to estimate the potential of these two species as environmental bioindicators in the Amambai River by evaluating mutagenic and genotoxic effects and measuring metal bioaccumulation in the liver and musculature.

2.2. MATERIALS AND METHODS

2.2.1. Study Area

The Amambai River has an extension of approximately 290 km (Vasconcelos and Paranhos Filho 2010) and is part of the Upper Parana River Basin, located in the southern part of Mato Grosso do Sul state, in Brazil. Sampling was carried out between December 2014 and July 2015 at three sampling points, chosen to best represent the extent of the Amambai watercourse: the upper portion (Point 1), the middle portion (Point 2) and the mouth (Point 3) (Figure 1). The Amambai River Basin area is associated with diverse types of land use and degrees of human occupation, and includes agricultural areas (e.g., pastures, croplands dominated by sugar cane monoculture) and areas with urban/suburban development (e.g., industrial buildings, urban infrastructure), as well as rural properties, and consequently have reduced native vegetation cover (Silva et al. 2014).

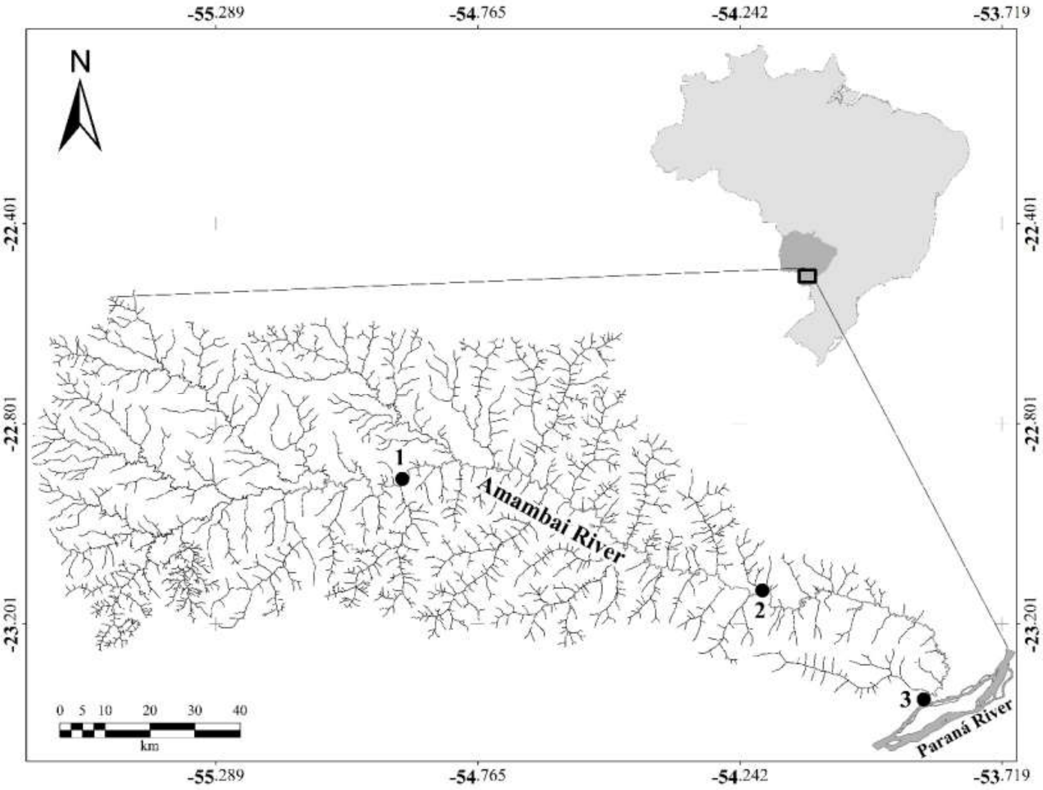

Figure 1. Location of sampling points along the Amambai River in the Upper Parana River Basin, Brazil.

2.2.2. Environmental data

Limnological conditions of the study sites was carried out by measuring the following parameters: water electrical conductivity (µS.cm^{-1}), turbidity (NTU) and dissolved oxygen (mg L^{-1}). Sediment from each site was collected using a dredger, stored in previously decontaminated plastic bags, and maintained under refrigeration (-4 ºC). The samples were oven dried at 60 ºC for 4 hours in order to avoid drag of the metals by water vapor. The samples were then sieved and only the fraction < 53 µm was used for the chemical extraction. For extraction, about 1 g of each sample was added to a test-tube along with 4 mL of 50% aqua regia (3:1 $HCl:HNO_3$) and 5 mL of $HClO_4$. Samples were heated for 30 min at 90 °C in a water bath. The resulting solution was filtered and brought to 0.5 ml volume with ultrapure water (Hortellani et al. 2008; Betemps and Sanches Filho, 2012). Concentrations of Cd, Pb, Cr, Cu, Fe, Zn and Ni in samples were then estimated using Atomic Absorption Spectrometry (Shimadzu, model AA7000) with flame atomization.

2.2.3. Collection of Biological Material

Fish were collected by use of casting nets and waiting nets with mesh of various sizes (1.5 - 8.0 cm between adjacent nodes). Soon after the collection, the fish were immersed in ice water *in situ* to reduce activity. After blood extraction for the mutagenicity analyses animals were sacrificed for the analysis of metal accumulation in tissues. This project was approved by the Committee on Ethics for the Use of Animals at UEMS (011/2014) and authorized by IBAMA (SISBIO 11156-1).

2.2.4. Analysis of mutagenicity

Blood samples were collected from fish using heparinized syringes. We obtained enough blood to prepare two smears per individual, which were air dried for 15 min, fixed in absolute alcohol for 10 min, and stained with 10% Giemsa solution for 20 min (Schmid 1975; Jesus et al. 2016). We analyzed 2,000 blood cells per slide using on an optical microscope at 1,000X magnification. Micronuclei (MN) were identified following the criteria proposed by Fenech et al. (2003). Analysis of erythrocytes nuclear Abnormalities (ENA) followed the classifications proposed by Carrasco et al. (1990).

2.2.5. Analysis of metal in fish tissues

Individuals were frozen for a maximum period of 30 days until analysis of metal concentration. Briefly, part of the muscle tissue was separated between the dorsal fin and the end of the caudal peduncle, then the liver was removed. The samples were dehydrated at 50 ºC for 3 hours and macerated. About 2.5 ml of 65% HNO_3 were added to 0.5 g of each tissue sample. Muscle and liver samples were transferred to digestion tubes and allowed to react for 1 hour using a digester block apparatus heated at 60 °C. After addition of 2.5 mL of 65% HNO_3 to each sample, they were again heated to 100 °C until almost completely dry. The samples were then filtered and re-suspended to a final volume of 10 mL of 0.5N HNO_3, then stored in a freezer at -4 °C for a maximum of 15 days until analysis (Seixas et al. 2009; Eneji et al. 2011). Flame atomic absorption spectrometry (Shimadzu, model AA7000) was used to estimate concentrations of Cd, Pb, Cr, Cu, Fe, Zn and Ni in fish tissues.

2.2.6. Statistical analysis

We performed two discriminant analysis (DA) to compare the three sampling sites for the limnological conditions of the water and the concentration of metals in the sediment. We also used a DA to compare the sampled sites in relation to metal concentrations in tissues from *P. lineatus*, and a non-parametric Mann-Whitney test (since the DA was not possible due small n) to compare the two sites where *P. ambrosetti* was captured (site 2 and site 3), using the metal concentrations in tissues from this species as dependents variables. We also used a Mann-Whitney test to compare MN and ENA between fish species. Comparisons of metal concentrations between tissue types and species were done using a t test. All analyses were performed using the R platform (R Development Core Team 2016) ($\alpha = 0.05$).

2.3. RESULTS

The Discrimant Analysis (DA) did not show significant differences among the sampled sites considering the limnological variables (electrical conductivity, turbidity and dissolved oxygen) (Wilks' Lambda=0.0199; p=0.3667). In relation to metal concentrations in sediment (Cd, Pb, Cr, Cu, Fe, Zn and Ni), only copper did have significant difference among sites (Wilks' Lambda=0.0024; p= 0.0191). Beside this, we not found spatial differences concerning to metal concentrations in tissues from *P. lineatus* (Wilks' Lambda=0.0178; p= 0.3225), and only hepatic Cu of *P. ambrosetti* showed difference between sites 2 and 3 (Z=2.2048; p=0.0275). From these results, we have chosen to group the data of the different sites in the subsequent analyzes.

Eight *P. ambrosetti* and 19 *P. lineatus* specimens were collected in total. We identified seven nuclear abnormalities among the species. The most frequent nuclear abnormality in both species was the presence of micronuclei, and all types of evaluated nuclear abnormalities occurred in both species (Figure 2). *P. ambrosetti* had higher occurrence of micronuclei than did *P. lineatus* ($p < 0.0001$) (Figure 3). There were no significant differences between species ($p > 0.05$) in the frequencies of the other types of nuclear abnormalities (Figure 4A- F).

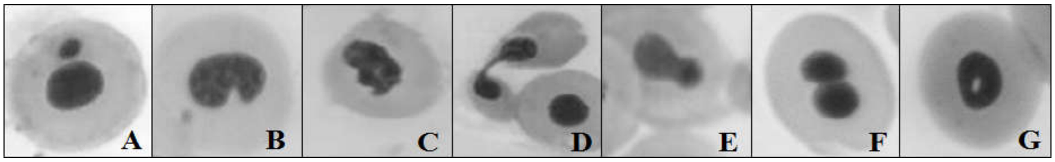

Figure 2. Erythrocyte nuclear abnormalities (ENA) in fish from the Amambai River. A. Micronucleus, B. Notched nuclei, C. Lobed nuclei, D. Nuclear bridge, E. Nuclear budding, F. Binucleated cell, and G. Vacuolated nuclei. Magnification: 1000x.

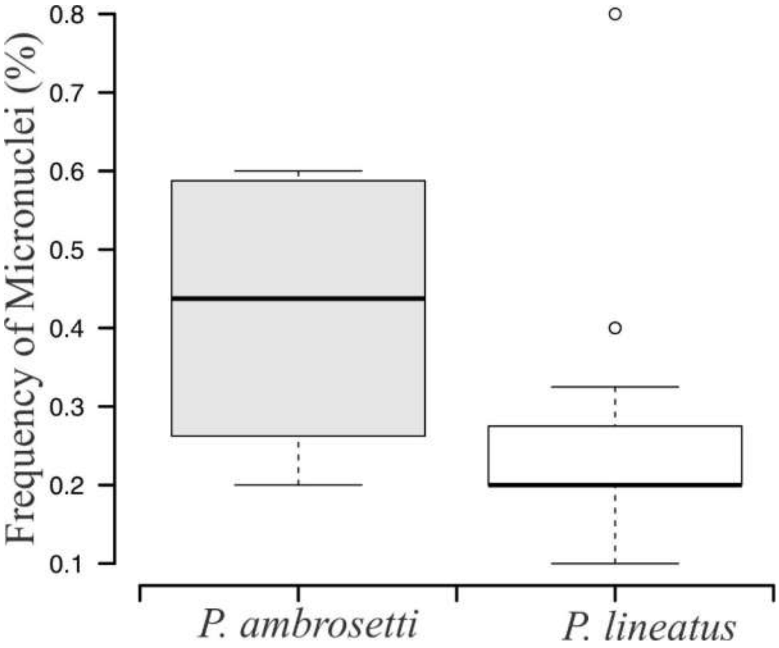

Figure 3. Percentual frequency of micronuclei (median, quartiles, maximum and minimum) for *P. ambrosetti* and *P. lineatus* collected in the Amambai River in the Upper Paraná River Basin between December 2014 and July 2015.

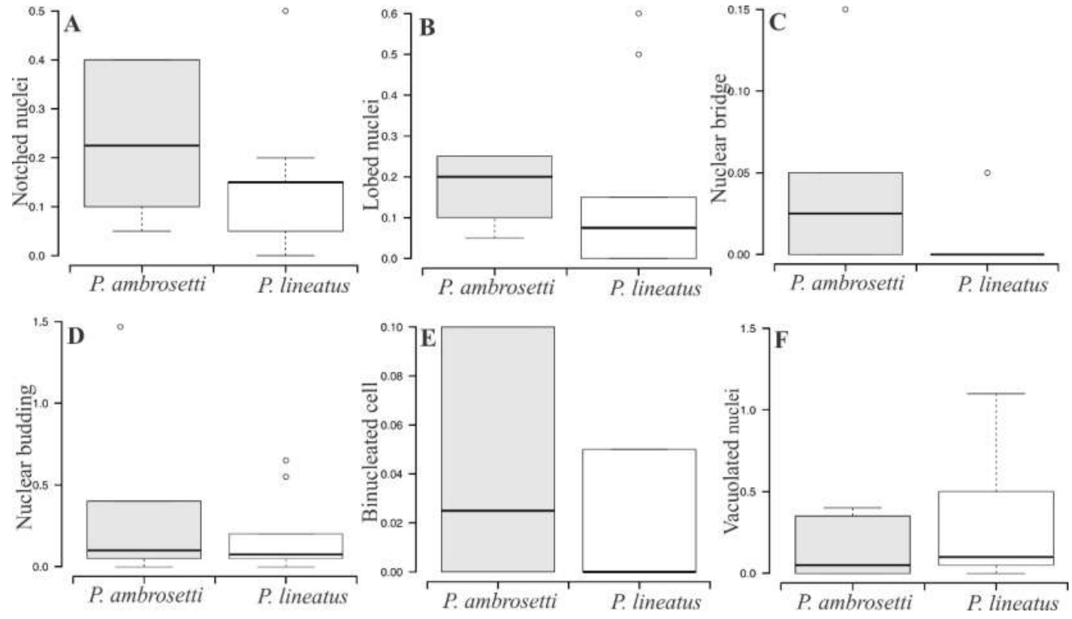

Figure 4. Percentual frequency of ENA (median, quartiles, maximum and minimum) in *P. ambrosetti* and *P. lineatus* collected in the Amambai River in the Upper Paraná River Basin between December 2014 and July 2015.

Both fish species had higher metal concentrations in the liver than in the musculature ($p < 0.0001$ for all comparisons) (Figure 5A-G). The average metal concentrations found in the musculature were within the limit established for consumption by the National Sanitary Surveillance Agency (Anvisa 1998; 2013) and were as follows: Cd (0.05 µg g^{-1}); Pb (0.30 µg g^{-1}); Cr (0.10 µg g^{-1}); Cu (30 µg g^{-1}); Zn (50 µg g^{-1}) e Ni (5.0 µg g^{-1}). For Fe, there is no maximum concentration stipulated by the law.

P. ambrosetti had higher Cd and Fe concentration in the liver ($p<0.05$) compared to *P. lineatus* (Figure 5A and 5E). We did not find significant interspecific differences in hepatic bioaccumulation of Pb, Cr, Cu, Zn and Ni ($p>0.05$) (Figure 5). *P. lineatus* had higher Cd concentrations in the musculature than did *P. ambrosetti* ($p=0.0219$) (Figure 5A). There were no significant differences between species for the concentrations of other analyzed metals in muscle tissues (Figure 5A-G).

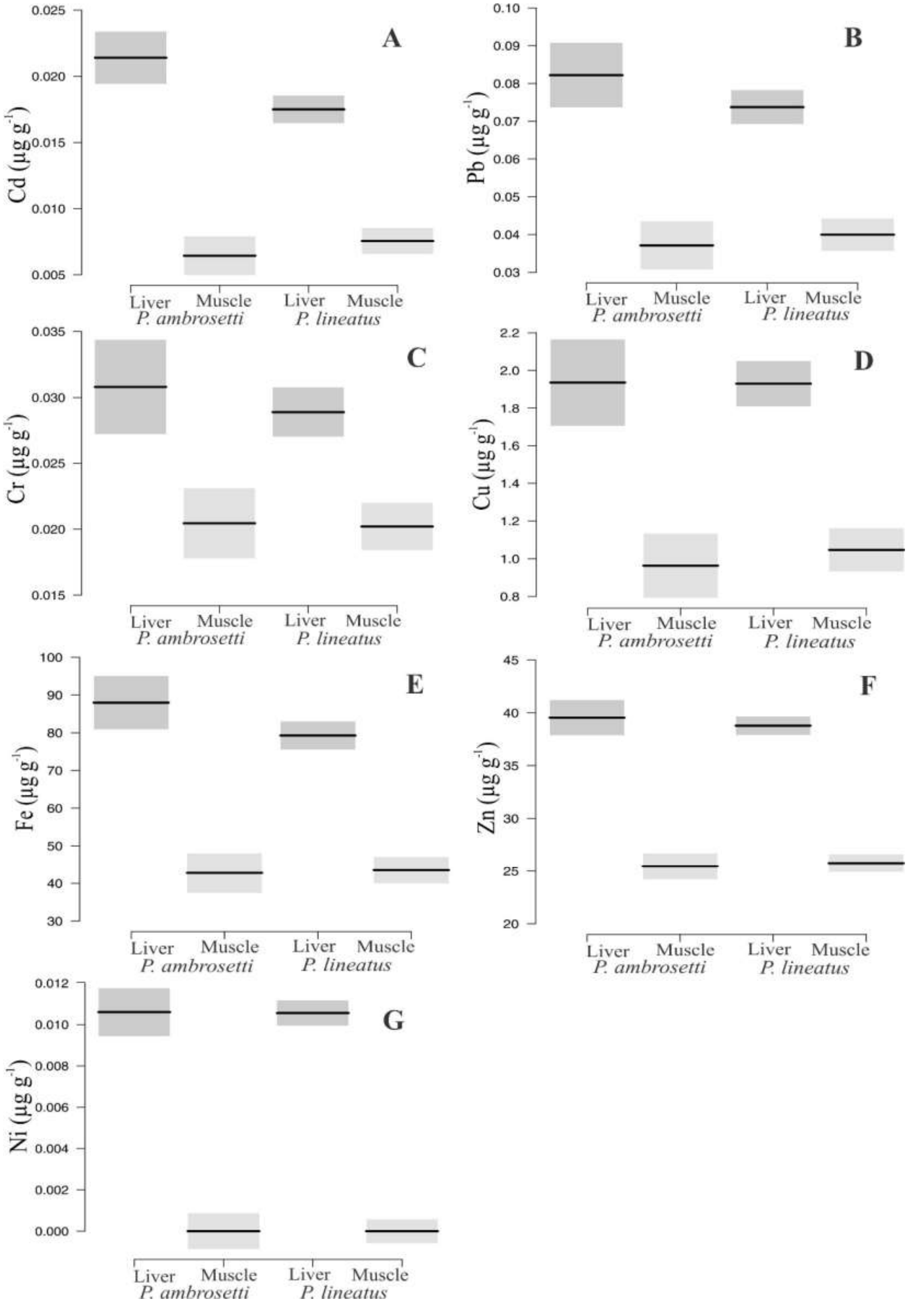

Figure 5. Muscular and hepatic metal concentrations (mean ± Confidence Interval) in *P. ambrosetti* and *P. lineatus* collected in the Amambai River in the Upper Paraná River Basin between December 2014 and July 2015.

2.4. DISCUSSION

This study represents the first evaluation of the ecotoxicological effects on the ichthyofauna of the Amambai River. Specimens showed evidence of contamination with non-essential metals (Cd, Pb, and Cr), and blood cells had abnormalities related to mutagenic and genotoxic effects, possibly due to the discharge of pollutants. Reduction of riparian vegetation may also be a factor, as it facilitates the entry of contaminants into the aquatic environment. We observed a higher frequency of micronuclei in *P. ambrosetti* compared to *P. lineatus*. This may be due to *P. ambrosetti* being a more resident species, and thus more frequently exposed to toxic substances dissolved in the water and accumulated in the sediment near areas with anthropogenic activities. This result suggests that *P. ambrosetti* may be used as an indicator of environmental quality at sub-lethal levels, in agreement with Sunjog et al. (2012).

On the other hand, *P. lineatus* is species that travels long distances (200 to 600 km) in search of food and for reproductive purposes. This habit presents a disadvantage for use as a bioindicator of local conditions (Oldani 1990; Lombardi et al. 2010; Message et al. 2016). The health of local fish fauna should thus be investigated through investigation of biomarkers in resident species (Zhou et al. 2008; Sanchez and Porcher 2009; Freire et al. 2015).

Variation in the frequency of micronuclei occurrence is likely related to intrinsic ecological characteristics of the fish that affect ingestion, accumulation, metabolism, and excretion (Jha 2004; Furnus et al. 2014). Our results are consistent with other studies from the literature indicating mutagenic potential for fish species, and are even more evident for the resident species (*P. ambrosetti*). This may be related to the presence of toxic chemical components in the water and sediment (Hoshina et al. 2008; Matsumoto et al. 2006; Duarte et al. 2012; Jesus et al. 2016). However, there were no significant differences between species in the frequency of other nuclear anomalies, which indicates that micronuclei assays may have higher sensitivity for detection of altered water quality in aquatic environments. Nuclear abnormalities in fish erythrocytes are more frequent after long periods of exposure to genotoxic agents (Osman et al. 2010; Vicari et al. 2012), and several studies have shown higher frequency of ENA in erythrocytes of fish contaminated by metals such as Cr, Ni, Pb and Zn (e.g., Barbosa et al. 2010). Among the nuclear abnormalities, binucleated erythrocytes had high frequency for both species this may be an important biomarker of cell division failure due to blocking of cytokinesis (Thomas et al. 2009; Batista et al. 2016; Stankeviciute et al. 2016).

Metals are absorbed by the fish through four main routes: water, food, gills and skin. The blood then carries them to the liver for processing and storage, where they may also bioaccumulate in other organs (Jabeen and Chaudhry 2010). The metals analyzed in this study were more concentrated in the liver of the specimens that in the muscle tissue, in agreement with the literature. Because the liver is an organ of continuous accumulation, biotransformation, and detoxification, the response to environmental pollutants is more rapid (Jarić et al. 2011; Weber et al. 2013; Ghisi et al. 2016). The heavy metals concentrations in fish musculature did not exceed the established limits acceptable for human consumption in Brazil (ANVISA 1998, 2013). However, our results for hepatic bioaccumulation of metals are worrisome, especially for non-essential metals (Cd, Pb and Cr).

We emphasize that cadmium has no known biological role and is considered to be one of the most toxic metals even at low levels, with potential to cause damage to the genetic material of fish (Kosanovic et al. 2007; Jayaprakash et al. 2015; Velusamy et al. 2014). The high hepatic Cd concentrations found in this study are thus particularly worrying, because by having a higher coefficient of toxicity Cd presents higher ecological risks than other metals (Fu et al. 2009; Yi et al. 2011; Effendi et al. 2016).

The two species did not differ from each other in hepatic and muscular concentrations of Pb, Cr, Cu, Zn and Ni. It is possible that these metals are being accumulated by biomagnification through feeding (since the metals decant in the sediment) and accumulation due to exposure to metals in the water (Jayaprakash et al. 2015).

Lead (Pb) is also highly toxic to fish and has no known biological role. It can damage organs, has carcinogenic effects, and bioaccumulation occurs gradually in the long term (Kosanovic et al. 2007; Velusamy et al. 2014). Chromium (Cr) is a nonessential element that plays a critical role in glucose metabolism, partially affecting fish at high concentrations (Krishna et al. 2014). It can reduce the life expectancy of fish, and negatively impacts growth and reproduction (Outridge and Scheuhammer 1993; Jesus et al. 2014). Chromium is present in several agricultural fertilizers (Pedrazzani et al. 2012), and its presence in fish tissues is likely due to the intense agrochemical use in the area surrounding the river.

Copper (Cu), on the other hand, is an important element for fish due to its interactions with proteins and enzymes for synthesis of hemoglobin. However, it can also have toxic effects at 30 $\mu g\ g^{-1}$ (Filipović-Marijić and Raspor 2014; Velusamy et al. 2014). Its extensive use for control of agricultural pests (Wei and Yang 2015; Carvalho et al. 2015) partially explains its occurrence in the fish tissues analyzed.

Iron (Fe) was observed in all samples, and was the most abundant metal in tissues from both species. It tends to accumulate in hepatic tissues due to the physiological role of the organ in maintaining blood cells, however excess levels can be harmful (Gorur et al. 2012).

Zinc (Zn) was the second most common metal in the analyzed samples, and is an essential element for growth and development in fish. Its absorption from the environment is regulated according to nutritional demand through homeostatic control (Shinn et al. 2009). The highest concentrations of Zn in the liver occur in species that feed on or are associated with the substrate – such as the species analyzed in this study – because they have an intestinal structure that promotes greater absorption of lipophilic substances found in the sediment (Furnus et al. 2014). Nickel (Ni) is also an essential micronutrient for most of aquatic life, however at high concentrations it can be harmful due to mutagenic and genotoxic effects (Bielmyer et al. 2013; Jesus et al. 2014).

The presence of high concentrations of metals in aquatic ecosystems is a major concern due to toxicity and genotoxicity in fish (Florea and Büsselberg 2006; Hoshina et al. 2008). Bioaccumulation of metals in fish tissue also induces biochemical and physiological changes in cells (Fernandes et al. 2007; Paulino et al. 2014).

As a final consideration, *P. ambrosetti* seems to be a more adequate species than *P. lineatus* for evaluation of mutagenic and genotoxic effects, as well as metal accumulation in the liver and musculature. This is probably due to *P. ambrosetti* having resident behavior, which also makes the species useful as an environmental bioindicator for local conditions. Further, we may infer that the mutagenic and genotoxic effects observed in this study were caused by metals present in the water, or possibly by a mixture of other chemical components in the river. The concentrations of non-essential metals observed in the fish confirm a situation of environmental stress in the Amambai River, likely associated with the discharge of pollutants, and exacerbated by lack of native vegetation cover along the watercourse. Although the metals found in the fish tissues did not exceed the limits deemed acceptable by Brazilian legislation, their consumption by the population (mainly *P. lineatus*, which is a species of great commercial interest) may cause bioaccumulation of these metals in the bodies of consumers, potentially leading to anomalies such as mutagenic and genotoxic effects.

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CAPÍTULO 3. CELLULLAR BIOMARKERS RELATED TO METAL BIOACCUMULATION IN NEOTROPICAL FISH SPECIES, BRAZIL

Artigo submetido à Ecological Indicators

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ABSTRACT. This study evaluated the frequency of micronuclei (MN) and nuclear abnormalities in erythrocytes (ENA) and the concentration of metals in musculature of three native fish species – *Astyanax lacustris*, *Hypostomus ancistroides* and *Rhamdia quelen* – in sites with different levels of ecological integrity in the Tarumã Microbasin, Upper Paraná River, Brazil. We ran principal components analysis using twelve limnological variables to classify sites into one of two levels of environmental integrity: less impacted (LI) and impacted (IMP). *A. lacustris* showed a higher frequency of MN at IMP sites compared to LI sites ($p < 0.0001$). We found no significant differences in MN frequency between site classes for *H. ancistroides* and *R. quelen* ($p > 0.05$). There were no significant differences between site classes with respect to ENA frequencies ($p > 0.05$). *A. lacustris* from IMP sites had higher muscle tissue concentrations of Pb, Cu, Fe, Zn, and Ni ($p < 0.05$), while *H. ancistroides* from IMP sites had higher concentration of Cr, Cu and Ni ($p < 0.0001$) and *R. quelen* showed higher concentration of Cd, Fe and Ni at these sites ($p < 0.0001$). The results indicate that fish in the system are currently subjected to environmental stress, which is more evident in the lower drainage portion due to anthropogenic input.

KEYWORDS. Environmental quality; Contaminants; Biomonitoring

3.1. INTRODUCTION

The ecological integrity of many rivers and streams has been broadly threatened and degraded by unprecedented levels of pollution from agricultural, industrial, and other anthropogenic activities. This has contributed to the reduction of vegetation cover on and around stream banks, resulting in increased flow of residues and toxic substance into the rivers and streams and negatively impacting aquatic biota (Vieira et al., 2016; Topal et al., 2017). Pollution in aquatic environments has serious consequences for fish, including mutation, tumors, and cell death (Beyersmann and Hartwig, 2008; Bogoni et al., 2014).

Fish are widely used to assess the health of aquatic systems, and are commonly used as bioindicators of environmental pollution due to strong responses to biochemical and physiological changes in the system (Lavado et al., 2006; Cazenave et al., 2009). Fish metabolize and accumulate xenobiotics over time, are sensitive to even low concentrations of metals in the water, and are distributed among various habitats in the aquatic environment (Lemos et al., 2008; Bogoni et al., 2014; Bebianno et al., 2015; Silva et al., 2015; Batista et al., 2016; Cáceres-Vélez et al., 2016). Assessment of fish responses to environmental contamination can be achieved by integrating a set of biomarkers associated with pollution and metal concentration, and characterizing subsequent genetic alterations (Guiloski et al., 2013; Bueno-Krawczyk et al., 2015; Ghisi et al., 2016). Beside this, the use of biomarkers may help to provide early warning signals of possible damage to aquatic ecosystems (Van Der Oost et al., 2003; Cazenave et al., 2009).

Various biomarkers have promising applications for evaluation of degradation in aquatic environments (Lemos et al., 2008; Cantanhêde et al., 2016). This can be evaluated using a micronucleus (MN) test, which evaluates damage to DNA at the chromosome level. This test is a vital component of routine genetic toxicology tests, because chromosomal mutation is the primary cause of cancer formation (De Flora et al., 1993; Fenech, 2000; Arslan et al., 2015). Erythrocyte nuclear abnormalities (ENA) are also widely used as biomarkers in toxicological examination of fish because they can provide estimates of DNA damage; they are also associated with failure of cell division and cell death (Lemos et al., 2008; Basiene et al., 2013; Hemachandra and Pathiratne, 2016; Ossana et al., 2016).

The concentrations of metals in fish tissues can also be used as an important environmental indicator, as bioaccumulation can lead to several genetic alterations and can result in reproductive inhibition, potentially reducing species diversity (Ghisi et al., 2014; Simonato et al., 2016).

The streams and rivers of the Upper Paraná River Basin have been seriously affected by anthropogenic impacts of high population density around the basin area, primarily due to urban, industrial, and agricultural waste disposal (Ghisi et al., 2014; Carvalho et al., 2015; 2016; Silva et al., 2016). Using *in situ* fish species as bioindicators for ecotoxicological evaluation of the basin area may provide more accurate information of environmental conditions at specific sites (Castro et al., 2004; Camargo and Martinez, 2006).

In this study, we evaluated MN and ENA biomarkers and metal bioaccumulation in three native fish species: *Astyanax lacustris* (Lutken, 1875) (Characiformes: Characidae), *Hypostomus ancistroides* (Ihering, 1911) (Siluriformes: Loricariidae), and *Rhamdia quelen* (Quoy and Gaimard, 1824) (Siluriformes: Heptapteridae). *A. lacustris*, known as the 'yellow tail lambari' are opportunistic omnivores with a high degree of phenotypic plasticity; this species can be useful as an environmental bioindicator (Viana et al., 2013; Siqueira-Silva et al., 2015). *H. ancistroides*, popularly known as 'armored catfish', is a benthic detritivore that exhibits non-migratory nesting behavior and nocturnal activity. It occurs in urban streams, making it a good candidate as an indicator species for environmental quality (Casatti, 2002; Oliveira and Bennemann, 2005; Sofia et al., 2008). This species also has strong economic importance due to human consumption (Ghisi et al., 2016). *R. quelen*, a catfish species, is a benthic opportunist whose diet varies according to environmental characteristics; it feeds on terrestrial and aquatic insects, crustaceans, and plant remains, as well as fish such as lambaris (Casatti et al., 2001; Casatti, 2002; Casatti and Castro, 2006; Gomiero et al., 2007; Villares Junior and Goitein, 2013). This species was chosen as a bioindicator because it is native to freshwater and has economic importance due to human consumption.

Our objective was measure the frequency of micronuclei and nuclear abnormalities in erythrocytes, and the concentration of metals in the musculature in fish collected from sites with distinct levels of ecological integrity in the Tarumã Microbasin, Upper Paraná River Basin, Mato Grosso do Sul State (MS), Brazil, in order to compare the sites and to verify the influence of environmental degradation on these biomarkers.

3.2. MATERIALS AND METHODS

3.2.1. Study area

The streams of Tarumã Microbasin are tributaries of the Amambai River in the Upper Parana River Basin, Brazil. Sampling was carried out between September 2014 and December

2015 at six sites along the microbasin (Fig. 1), chosen according to the position in the drainage network and to represent different levels of environmental integrity.

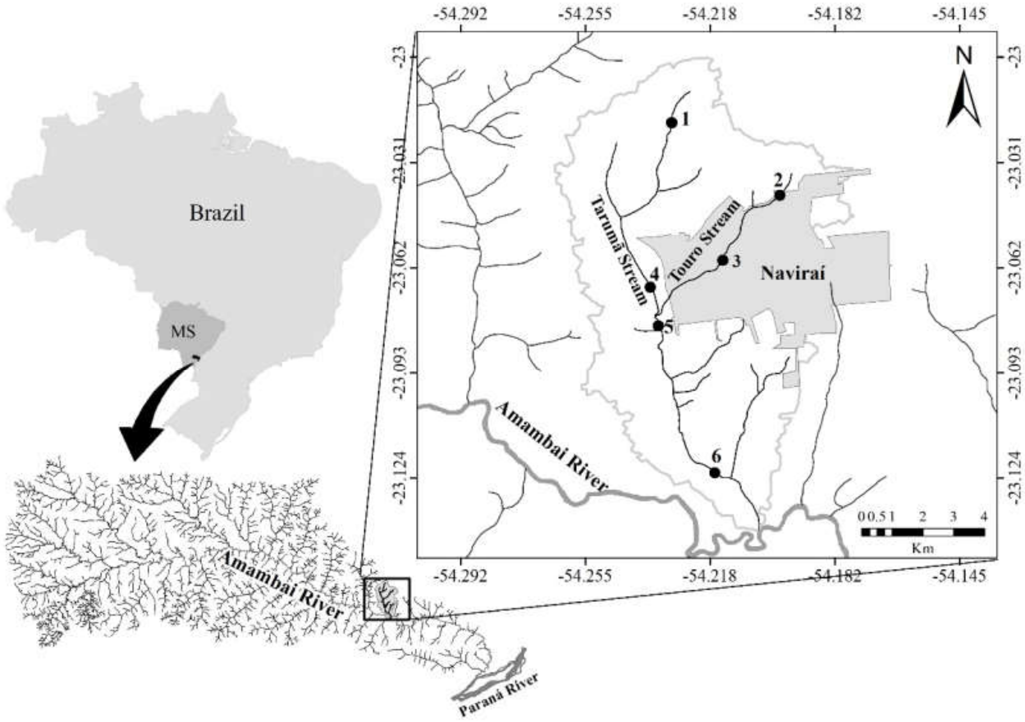

Fig. 1. Location of sampled sites along the Tarumã Microbasin (Upper Paraná River, Brazil).

Most of the collection sites are located in rural areas of the municipality of Naviraí (sites 1, 2, 4-6), while site 3 lies within an urban region. Sites 1 and 2 are located in the springs of the Tarumã and Touro streams, respectively, while site 4 is situated in the middle portion of the Tarumã stream. At these three sites, there is practically no native vegetation cover and the soil is mainly used for pasture and sugar cane and eucalyptus cultivation. Site 3 (middle portion of the Touro stream) is located within the urban perimeter and is characterized by degraded native forest, residential sewage disposal, litter on the banks, and silted bed. Sites 5 and 6 are located in the Tarumã stream in the lower portion of the microbasin, thus receiving contaminants from the upper portion of the basin, in addition to evident silting. The stream banks also present degraded native forest, mainly occupied by pasture and sugarcane monoculture, as well as some industrial plants (slaughterhouse, tannery, and sugar and alcohol mill).

3.2.2. Data collection

Limnological conditions of the study sites was carried out in triplicated samples (using a Horiba u53 multiparameter) by measuring the following parameters: water electrical conductivity (µS cm^{-1}), turbidity (NTU), dissolved oxygen (mg L^{-1}), pH and temperature (°C). Metal concentrations (Cd, Pb, Cr, Cu, Fe, Zn and Ni) were measured in water samples, which were collected from the basin sites, stored in polyethylene containers (500 mL), and transported on ice to the laboratory. Performed in triplicates, 10 mL of each sample was withdrawn and plated, after which 2 mL of PA nitric acid was added and the plates were heated to 100 °C for half an hour (to evaporate 6 mL of solution). After reaching room temperature, we added 4 mL of aqua regia (HNO_3 and HCl, 1: 3 by volume) to obtain a final solution of 10 mL (Ferreira et al., 2010). The filtered samples were analyzed using Atomic Absorption Spectrometry (Shimadzu, model AA7000) with flame atomization to produce estimates of Cd, Pb, Cr, Cu, Fe, Zn and Ni concentrations.

Fish were collected during the day using a rectangular metal sieve (0.8 x 1.2 m) with 2 mm aperture. The collection procedures were approved by the Ethics Committee on the Use of Animals at UEMS (011/2014) and authorized by IBAMA (SISBIO 11156-1).

Immediately after capture of fish, blood samples were obtained vie caudal puncture with heparinized syringes. For each sampled specimen, two thin smears were prepared on slides using a single drop of blood. The smears were air-dried for 15 min, fixed in absolute alcohol for 10 min, then stained with 10% Giemsa solution for 20 min (Schmid, 1975; Jesus et al., 2016). We analyzed 2,000 blood cells per slide using a 1000x magnification optical microscope, for a total of 4,000 cells analyzed per individual. Micronuclei (MN) were identified following the criteria proposed by Fenech et al. (2003). For the analysis of erythrocyte nuclear abnormalities (ENA), we followed the classifications proposed by Carrasco et al. (1990) and Jesus et al. (2016).

For determination of metal concentrations in fish muscle tissues, specimens were frozen for a maximum of 30 days prior to analysis. Upon thawing, a portion of the muscle tissue was separated between the dorsal fin and the end of the caudal peduncle. The samples were dehydrated at 50 ºC for three hours and macerated. About 2.5 mL of 65% HNO_3 was added to 0.5 g of the sample. Samples were then transferred to digestion tubes and allowed to react using a digester block apparatus heated at 60 °C for 1 h. Another 2.5 mL of 65% HNO_3 was then added and the block was again heated to 100 °C until near total dehydration. The samples were then filtered and re-suspended to a final volume of 10 mL of 0.5 mol L^{-1} HNO_3 solution and kept in a freezer (-4 °C) for a maximum of 15 days until analysis (Seixas et al., 2009; Eneji et al., 2011). The filtered samples were then analyzed to produce estimates of the same metals in fish tissues, using the same methods used for water samples.

3.2.3. Data Analysis

We used a Principal Component Analysis (PCA) from all limnological variables in order to obtain an ordination of the sampled sites by environmental quality class. As the data had no normal distribution, a non-parametric Mann-Whitney test ($\alpha = 0.05$) was used to compare MN, ENA, and metal concentrations in fish tissues among species in each environmental quality class. Correlations among metal concentrations in musculature and MN and ENA frequencies were quantified by Spearman index, for each species. All analyses were performed using the R platform (R Development Core Team 2016).

3.3. RESULTS

The PCA results from limnological data analysis allowed us to split the sampled sites in two categories with respect to environmental integrity. The first axis of the PCA explained 66.68% of variation in the data, while the second axis explained 20.20%. Metal concentration, turbidity, pH, and water temperature were negatively correlated with the first PC. Electrical conductivity showed a positive correlation with the second PC, while the O_2 concentration was negatively correlated with the second PC (Fig. 2). Sites 1, 2, and 4 were considered less impacted (LI) because in general, they presented lower concentrations of metals in the water, lower turbidity, pH, temperature, and electrical conductivity, and higher O_2 concentrations. Sites 3, 5, and 6 were considered impacted (IMP), because in general, they presented higher concentrations of metals in the water, higher values for turbidity, pH, temperature and electrical conductivity, and lower concentrations of O_2 (Fig. 2).

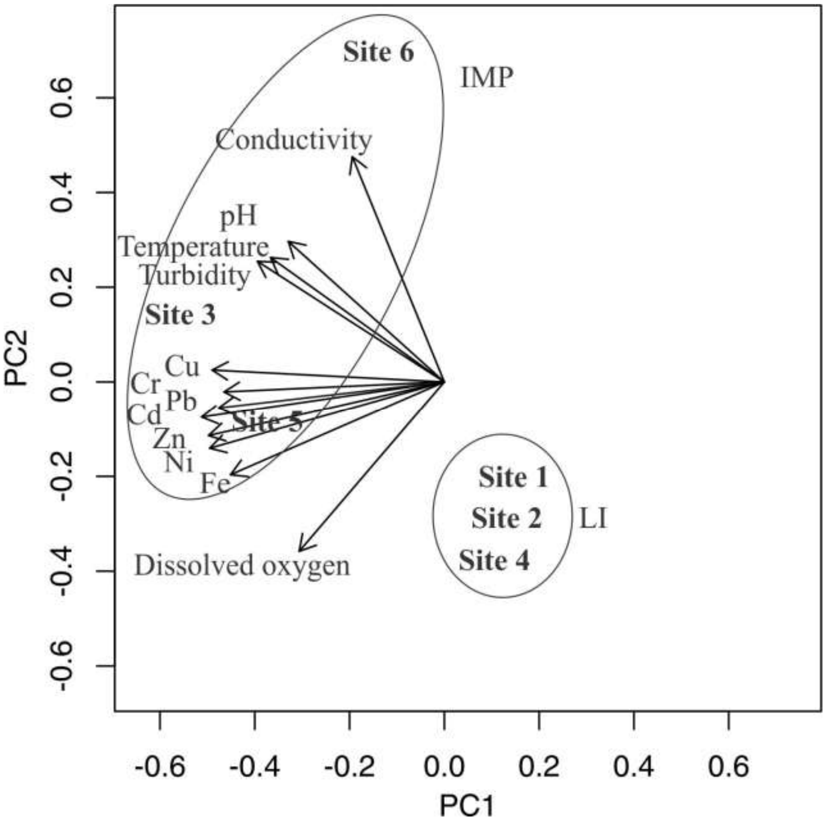

Fig. 2. Distribution of sites scores along the first two axes produced from PCA analysis of limnological variables in the Tarumã Microbasin (Upper Paraná River, Brazil).

The water analysis of the LI sites of the microbasin indicates concentrations of metals below the maximum limit allowed by Brazilian legislation (Resolution CONAMA 357/2005), except for Ni. For the IMP sites, we found concentrations that exceeded the limits established by the legislation for Cu and Ni (Table 1).

The number of collected individuals of each species is showed in Table 2. We identified MN and ENA from the three species from LI and IMP sites (Fig. 3).

Table 1. Concentration of metals in water (mean ± S.D) in less impacted (LI) and more impacted (IMP) sites of the Tarumã Microbasin. Bold values indicate concentration above the maximum limits established by Brazilian legislation (CONAMA 357/2005).

Metal	Concentration in the water (mg L^{-1})		CONAMA 357/2005 (mg L^{-1})
	LI sites	IMP sites	
Cd	0.0006±0.0005	0.0072±0.0010	0.0100
Pb	0.0082±0.0004	0.0112±0.0029	0.0330
Cr	0.0088±0.0008	0.0108±0.0032	0.0500
Cu	0.0090±0.0001	**0.0153±0.0063**	0.0130
Fe	3.6000±0.2688	3.6983±0.2847	5.0000
Zn	1.0100±0.0791	1.1583±0.2539	5.0000
Ni	**0.0764±0.0143**	**0.0902±0.0209**	0.0250

Table 2. Species and abundances of fish sampled by sites classes (LI and IMP) in the Tarumã Microbasin (Upper Paraná River, Brazil).

Species	LI sites	IMP sites	Total
A. lacustris	11	21	32
H. ancistroides	14	33	47
R. quelen	13	12	25
Total	38	66	104

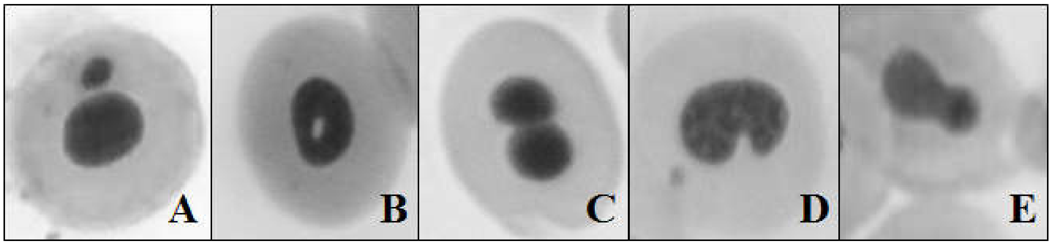

Fig. 3. Erythrocyte nuclear abnormalities (ENA) in fish from the Tarumã Microbasin. A. micronucleus; B. vacuolated nucleus; C. binucleated cell; D. notched nucleus, E. nuclear budding. Magnification: 1000x.

A. lacustris from IMP sites had higher frequency of micronuclei than LI sites ($p < 0.0001$). *H. ancistroides* and *R. quelen*, however, did not differ between sites ($p > 0.05$) (Fig. 4). There were no significant differences in the frequencies of the various forms of ENA between the sites classes for any of the fish species ($p > 0.05$) (Fig. 5A-D).

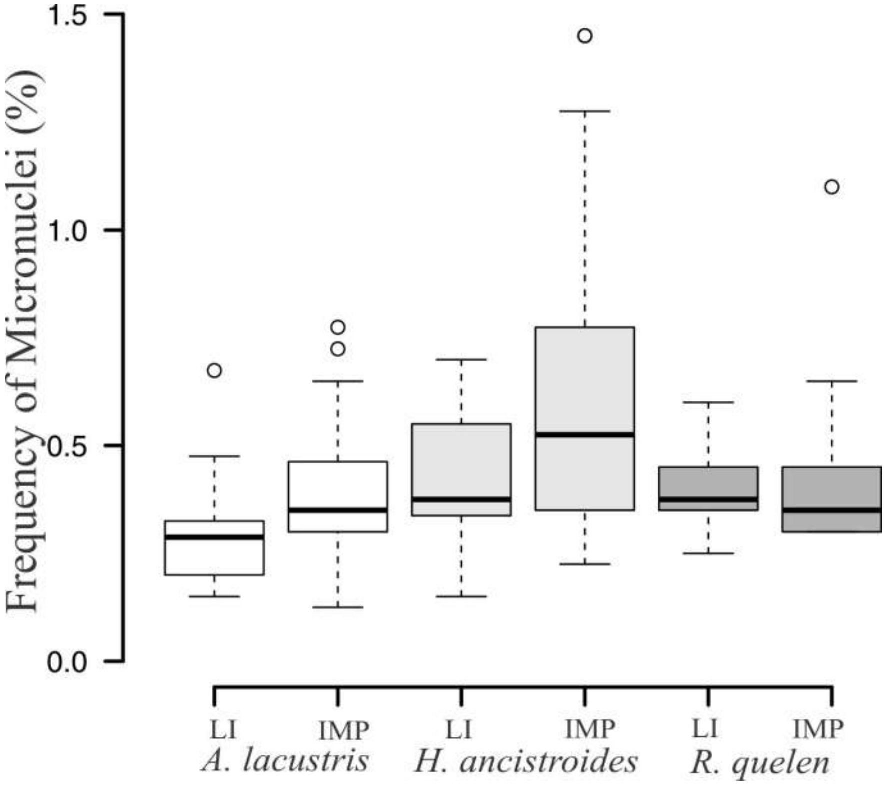

Fig. 4. Percentual frequency of micronuclei (median, quartiles, maximum and minimum) in erythrocytes from *A. lacustris*, *H. ancistroides* and *R. quelen* collected in the Tarumã Microbasin, Upper Paraná River, Brazil.

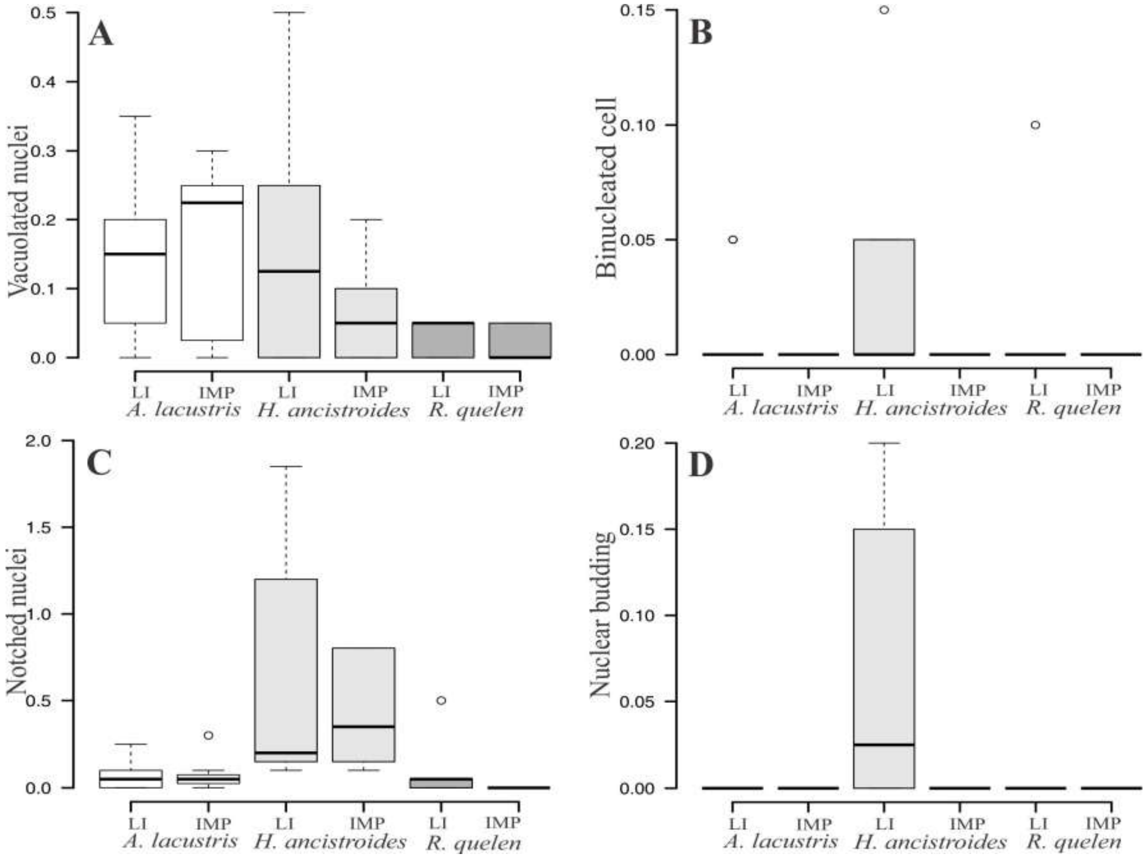

Fig. 5. Percentual frequency of ENA (median, quartiles, maximum and minimum) in *A. lacustris*, *H. ancistroides* and *R. quelen* collected in the Tarumã Microbasin, Upper Paraná River, Brazil.

Analysis of metal concentrations in fish musculature revealed that *A. lacustris* from IMP sites had higher concentrations of Pb, Cu, Fe, Zn and Ni ($p < 0.05$), but found no differences in Cd or Cr concentrations between site classes ($p > 0.05$) (Fig. 6A-G). *H. ancistroides* from IMP sites had higher concentrations of Cr, Cu, and Ni ($p < 0.0001$), but did not differ in Cd, Pb, Fe, or Zn concentrations between site classes ($p > 0.05$) (Fig. 6A-G). *R. quelen* from IMP sites had higher concentration of Cd, Fe, and Ni ($p < 0.001$) in muscle tissue. However Pb, Cr, Cu, and Zn did not differ between LI and IMP site classes ($p > 0.05$) (Fig. 6A-G).

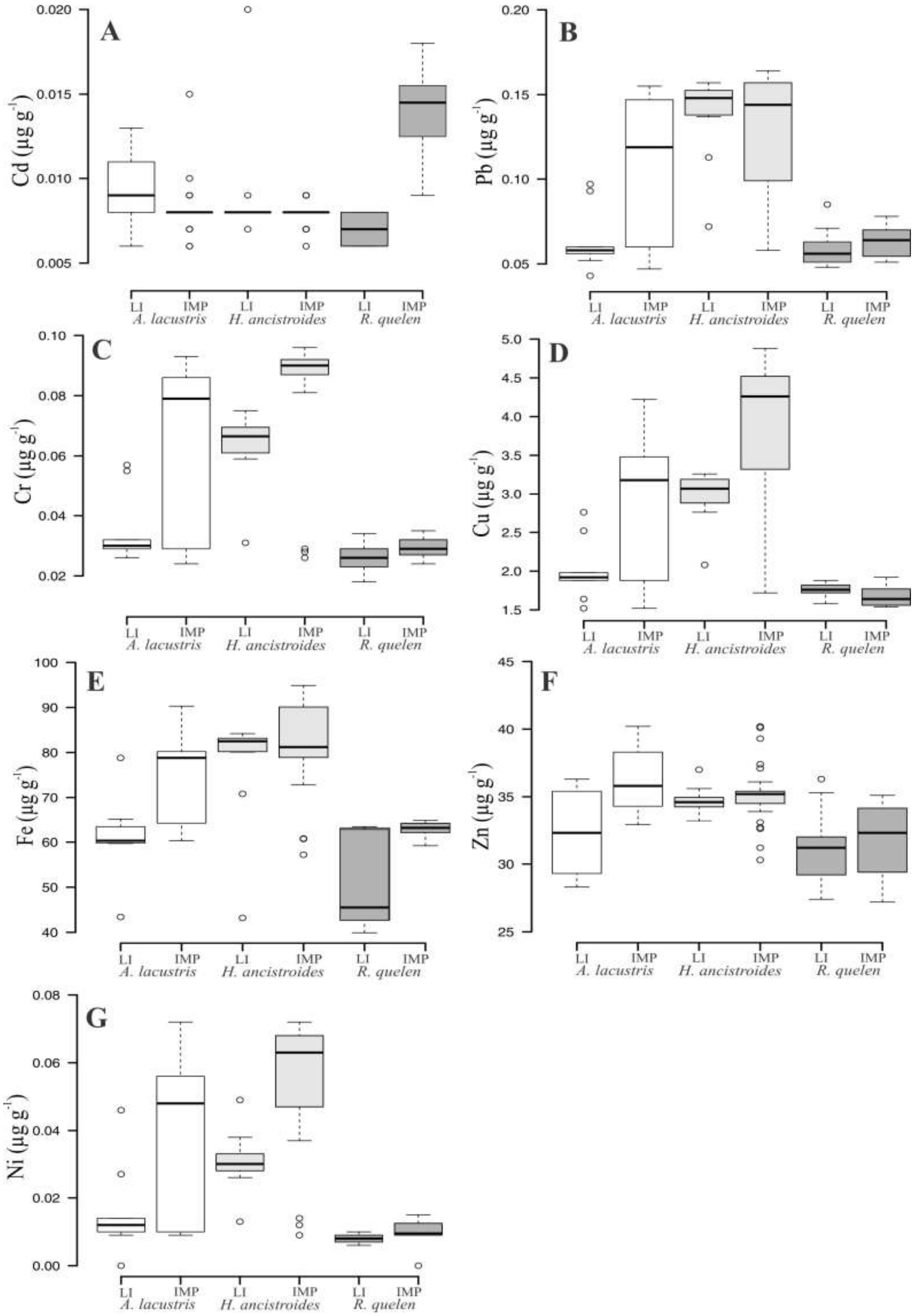

Fig. 6. Metal concentrations in musculature (median, quartiles, maximum and minimum) of *A. lacustris*, *H. ancistroides* and *R. quelen* collected in the Tarumã Microbasin, Upper Paraná River, Brazil. Anvisa (2013) established the legal limits for concentrations of Cd (0.05 µg g^{-1}), Pb (0.30 µg g^{-1}), Cr (0.10 µg g^{-1}), Cu (30 µg g^{-1}), Zn (50 µg g^{-1}) and Ni (5.0 µg g^{-1}) in fish musculature.

A. lacustris showed a significant positive correlation between the frequency of micronuclei and muscular concentration of four metals (Pb, Cr, Cu and Ni). *R. quelen* exhibited significant correlations between MN and two metals (Pb – positive – and Fe – negative), and between ENA and Cu (positive). For *H. ancistroides*, on the other hand, we found no correlation between bioaccumulation of metals and the cellular biomarkers (Table 3).

Table 3. Spearman correlations among metal concentrations in musculature and MN and ENA frequencies in *A. lacustris*, *H. ancistroides* and *R. quelen* collected in the Tarumã Microbasin, Upper Paraná River, Brazil. Bold values indicate significant correlations ($p < 0.05$).

Metals	*A. lacustris*		*H. ancistroides*		*R. quelen*	
	MN	ENA	MN	ENA	MN	ENA
Cd	-0.2369	-0.0632	-0.1657	0.0474	-0.4123	-0.0435
Pb	**0.4943**	0.0985	-0.0947	-0.1160	**0.5107**	0.3023
Cr	**0.5431**	-0.1377	-0.0814	-0.0922	0.3524	0.1189
Cu	**0.5185**	-0.0606	-0.2655	-0.0428	0.0803	**0.4659**
Fe	0.5407	-0.0152	-0.2005	0.2065	**-0.5727**	0.0754
Zn	0.3120	0.1139	-0.0490	0.0215	-0.3671	-0.1313
Ni	**0.5123**	-0.0245	-0.0647	-0.2474	-0.2664	-0.0348

3.4. DISCUSSION

Spatial variation in limnological parameters made it possible to assign sampled sites to one of two classes of environmental integrity (LI and IMP). High turbidity, conductivity, and metal concentrations in the water are relevant variables for indication of environmental degradation, and this situation is exacerbated by having low bank vegetation cover. As a consequence of these factors, oxygen concentration in the water decreases and temperature increases, resulting in decline of environmental quality (Minello et al., 2009; Viana et al., 2014).

For both IMP and LI sites, the Ni concentration in water exceeded acceptable values as established by Brazilian Resolution 357/2005 (CONAMA, 2005) for Class III freshwater environments. In IMP sites, Cu concentrations were also above the established legal limit. High concentrations of Ni in the aquatic environment may be a consequence of sewage input into water bodies, which poses a serious threat to fish due to carcinogenic, genotoxic, and immunotoxic effects (Costa et al., 2005; Cempel and Nikel, 2006; Vijayavel et al., 2009;

Adjroud, 2013; Kubrak et al., 2013; Topal et al., 2017). On the other hand, Cu has fungicidal and bactericidal properties that make it useful in agriculture. Sugarcane cultivation is common in areas surrounding in Tarumã Microbasin, thus agricultural input is likely the main source of water contamination. Bioaccumulation of this metal in fish tissues can affect DNA integrity (Diop et al., 2016; Dourado et al., 2016; Simonato et al., 2016).

We found higher concentrations of Pb, Cu, Fe, Zn, and Ni in *A. lacustris* from IMP sites compared to the LI sites. At IMP sites *H. ancistroides* had higher muscle concentrations of Cr, Cu, and Ni, while *R. quelen* had higher concentrations of Cd, Fe and Ni. Nickel can significantly impact fish physiology and behavior by interfering with antioxidant defenses, acting as a mutagen and genotoxin, and blocking the gill filaments, potentially causing asphyxia (Dourado et al., 2016).

The bioaccumulation of essential or non-essential metals such as Cd, Pb, Cr, Cu, Fe, Zn, and Ni in fish musculature is cited as one of the main contamination-associated stressors. Negative impacts include genetic damages, reproductive disturbance, reduction in growth rate, potentially fatal pathologies, and reduction or elimination of sensitive native species, which may affect ecosystem ecological balance (Cantanhêde et al., 2016; Ghisi et al., 2016). We emphasize that Cd, Pb, and Cr are heavy metals that do not present any physiological function and that are toxic to fish even at low levels, causing damage of genetic material and increased frequency of micronuclei (Ossana et al., 2009, 2016; Tsekenis et al., 2015). Some studies report that the toxic effects of Pb on fish are associated with oxidative stress (Zhang et al., 2007; Monteiro et al., 2011). In summary, the results obtained in the current study indicate bioaccumulation of metals, especially non-essential, in fish musculature, although heavy metal concentrations did not exceed the acceptable limits established in Brazil for human consumption (Anvisa, 2013).

The micronuclei analysis showed more evident genetic damage in *A. lacustris* from IMP sites compared to LI sites, a result in agreement with other studies indicating higher frequency of micronuclei in individuals from polluted waters (Omar et al., 2012; Obiakor et al., 2014; Gutiérrez et al., 2015; Colin et al., 2016). In addition, this species showed a significant positive correlation between MN and muscular concentration of four metals (Pb, Cr, Cu and Ni). These results suggest that *A. lacustris* has a more discriminant response to genetic damage evoked by pollutants than *H. ancistroides* and *R. quelen*, which presented higher concentration of metals in the musculature than *A. lacustris* in the two site classes (LI and IMP), but did not present such evident correlations between metals and MN. This hypothesis is supported by other studies showing that different species may respond in completely different ways to specific contaminants (Grisolia et al., 2009; Leung et al., 2014; Silva et al., 2015).

The occurrence of micronuclei is evidence of damage caused by chemical contaminants in the aquatic environment, and highlights the need for environmental biomonitoring (Lemos et al., 2008; Bolognesi and Hayashi, 2011; Bogoni et al., 2014). Our results are consistent with other studies showing the relationship between urban, agricultural, and industrial pollution and an increase in mutagenic effects in fish species (Geremias et al., 2012; Bianchi et al., 2015; Tabet et al., 2015; Batista et al., 2016). These mutagenic effects occur partially due to the intimate interactions of fish with environment contaminants (Hemachandra and Pathiratne, 2016).

As stated previously, there were no significant differences in the frequency of micronuclei between site classes for *H. ancistroides* and *R. quelen*. Metals carried into the aquatic system are mainly incorporated into the bottom sediments through adsorption, flocculation, and precipitation in the water (Botte et al., 2007; Cheng et al., 2015; Tang et al., 2016). As a consequence, benthic species are considered more prone to environmental contamination, because they tend to accumulate more elements than the other species with different feeding habits (Terra et al., 2008; Wei et al., 2014; Alamdar et al., 2017). *H. ancistroides* is a bottom scraping species, and *R. quelen* is known to feed near the bottom of the stream (Casatti, 2002; Gomiero et al., 2007).

The frequency of ENA, in our results, can be considered as a less effective indicator of genotoxicity than frequency of micronuclei, as no significant differences were found between the IMP and LI sites for any of the fish species. Besides this, considering all the correlations tested between ENA and metal bioaccumulation, only one was significant (between ENA and Cu, for *R. quelen*). However, the simple occurrence of these abnormalities is an indicator of loss of environmental integrity in the microbasin, as they arise as a response to contaminants that are known to cause genotoxic and cytotoxic effects in fish cells (Cavalcante et al., 2008; Omar et al., 2012; Bueno-Krawczyk et al., 2015). The Tarumã microbasin includes extensive areas devoid of native vegetation that were replaced by pasture or agricultural plots (mainly sugar cane monoculture), and areas with intense urban occupation. Consequently, the occurrence of ENA in the present study may be associated with increased agricultural and urban pollution associated with the reduction or absence of barrier vegetation.

3.5. CONCLUSIONS

This study is one of the pioneers in the evaluation of the ecotoxicological effects, integrating biomarkers and bioaccumulation of metals, on the Neotropical ichthyofauna. For this reason, it can serve as a model for studies in other systems. As a final consideration, the biomarkers analyzed in this study is suitable for evaluation of environmental quality in the Tarumã Microbasin, indicating that fish in this system are currently subjected to environmental stress, which is more evident in the lower portion of microbasin due to anthropogenic effects. Thus, we emphasize the importance of implementing environmental recovery policies in the microbasin for preservation of the natural balance in these streams, in order to ensure the maintenance of biodiversity in the fish community.

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CAPÍTULO 4. MUTAGENIC AND GENOTOXIC RESPONSES OF *Astyanax lacustris* TO LANDSCAPE CHARACTERISTICS AND METAL CONTAMINATION IN A NEOTROPICAL STREAM

Artigo a ser submetido à Science of the Total Environment

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ABSTRACT. The objective of this research was to evaluate land use and occupation in the landscape surrounding the Tarumã Microbasin streams of the Upper Paraná River in Brazil, and whether land use and aquatic contamination by metals can induce mutagenic and genotoxic effects in *A. lacustris*. We collected water and sediment from six sites along the microbasin for use in laboratory bioassays. Three sites were grouped in the category "Less impacted" (LI) and three sites were grouped in the category "Impacted" (IMP). High resolution aerial images were used to map land use and land cover. We found that the area surrounding the Tarumã microbasin is predominantly used for agricultural purposes (73.46% of the total area), followed by buildings (15.21%). Only 8.78% of the area consisted of forest fragments. We observed higher concentrations of Cd, Pb, Cu, Zn, and Ni in sediment from IMP sites compared to LI sites ($p < 0.05$). We also observed higher frequencies of micronuclei in erythrocytes of *A. lacustris* exposed to water from IMP sites compared to LI sites and the negative control (NC) ($p < 0.05$). Analysis of nuclear alterations in *A. lacrustis* erythrocytes showed significant differences in the frequency of notched nuclei and nuclear pyknosis only between the NC and the IMP sites ($p < 0.05$). The results show that unplanned land use along with the expansion of anthropogenic activities may be damaging the environmental integrity of Tarumã Microbasin. Our results also indicate that water from the most impacted sites of the microbasin can induce mutagenic and genotoxic effects in *A. lacustris*.

KEYWORDS. Aquatic environments, contaminants, mutagenicity, fish.

4.1. INTRODUCTION

Riparian vegetation becomes increasingly fragmented due to the expansion of anthropic activities and unplanned soil use. These actions can affect the structure and functioning of aquatic environments, and have potential to modify interactions in ecological systems (Vitousek et al., 2008). Reduction of vegetation cover near streams can cause erosive processes to occur in stream margins, thereby facilitating the entry of sediments and residues in the aquatic environment, causing siltation and contamination and, consequently, loss of local diversity (Lorion and Kennedy, 2009; Rothwell et al., 2010; Casatti, 2010; Silva et al., 2012; Burrel et al., 2014; Dusman et al., 2014). In this context, analysis of the landscape structure around streams is critical for assessment of the integrity of aquatic ecosystems. As human population growth continuously increases demand in the agricultural, industrial, and urban sectors, more detailed studies are needed evaluating stream quality.

One way to assess the environmental quality is by analyzing the concentrations of metals that accumulate in the sediments, as these metals contribute to the deterioration of water quality and threaten aquatic biota (Wang et al., 2015; Torres et al., 2015; Melo Gurgel et al., 2016). Since the sediment provides nutrients and habitat for a wide variety of benthic organisms, the analysis of this material is relevant for understanding relationships between the environment and the aquatic biota (Paixão et al., 2011; Peluso et al., 2013). Another form of environmental monitoring is through the use of *in vivo* fish bioassays, which are effective for environmental biomonitoring for possible contaminants in water. Fish used as biological models respond to stressful conditions by adjusting cellular metabolism and various defense mechanisms, and thus, may reveal the mutagenic and genotoxic potential of the environment (Tank et al., 2016; Hemachandra and Pathiratne, 2017). Fish bioassays have been widely used as indicators for evaluation of environmental mutagenicity and genotoxicity, primarily through observations of micronuclei and nuclear alterations (Osman et al., 2012; Fuzinatto et al., 2013).

Astyanax lacustris (Characiformes: Characidae) has characteristics that make it particularly useful as a bioindicator species, because it is an omnivorous, opportunistic, species that is abundant and broadly distributed throughout the Upper Paraná Basin. It can be easily captured, has a body size suitable for experimentation, and is well-adapted to laboratory conditions (Carrasco-Letelier et al., 2006; Ghisi et al., 2014; Viana et al., 2013).

Streams in the Upper Paraná River basin receive waste from anthropogenic activities, which causes serious damage to the aquatic environment (Dusman et al., 2014). Some streams of the basin have banks devoid of riparian vegetation, and present several erosive processes on their slopes. In this context, the objective of this work was to evaluate (1) the use and occupation of the surrounding landscape of the Tarumã microbial streams of the Upper Paraná River; (2) metal contamination in the aquatic sediment; and 3) mutagenic and genotoxic effects of these factors on erythrocytes in *A. lacustris*.

4.2. MATERIALS AND METHODS

4.2.1. Study area

The streams of Tarumã Microbasin are tributaries of the Amambai River in the Upper Parana River Basin in Mato Grosso do Sul (MS), Brazil. Six sites were selected along the microbasin (Fig. 1), chosen according to the position in the drainage network and representing different levels of environmental integrity. Most of the collection sites are located in rural areas of the municipality of Naviraí (Points 1, 2, 4-6), while site 3 lies within an urban region. Sites 1 and 2 are located in the springs of the Tarumã and Touro streams, respectively, while site 4 is situated in the middle portion of the Tarumã stream. At these three sites, there is practically no native vegetation cover and the soil is mainly used for pasture and sugar cane and eucalyptus cultivation. The water in these sites is relatively unpolluted (Souza and Lima-Junior, 2013), and these three sites were grouped for later analyses into the category "Less Impacted Sites" (LI).

Site 3 (middle portion of the Taurus stream) is located within the urban perimeter and is characterized by degraded native forest, residential sewage disposal, litter on the banks, and silted bed. Sites 5 and 6 are located in the Tarumã stream in the lower portion of the microbasin, thus receiving contaminants from the upper portion of the basin, in addition to evident silting. The stream banks also present degraded native forest, mainly occupied by pasture and sugarcane monoculture, as well as some industrial plants (slaughterhouse, tannery, and sugar and alcohol mill). The water from sites 3, 5 and 6 can be considered as having a high degree of pollution (Souza and Lima-Junior, 2013) and these three sites were grouped for later analyses in the “Impacted Sites” category (IMP).

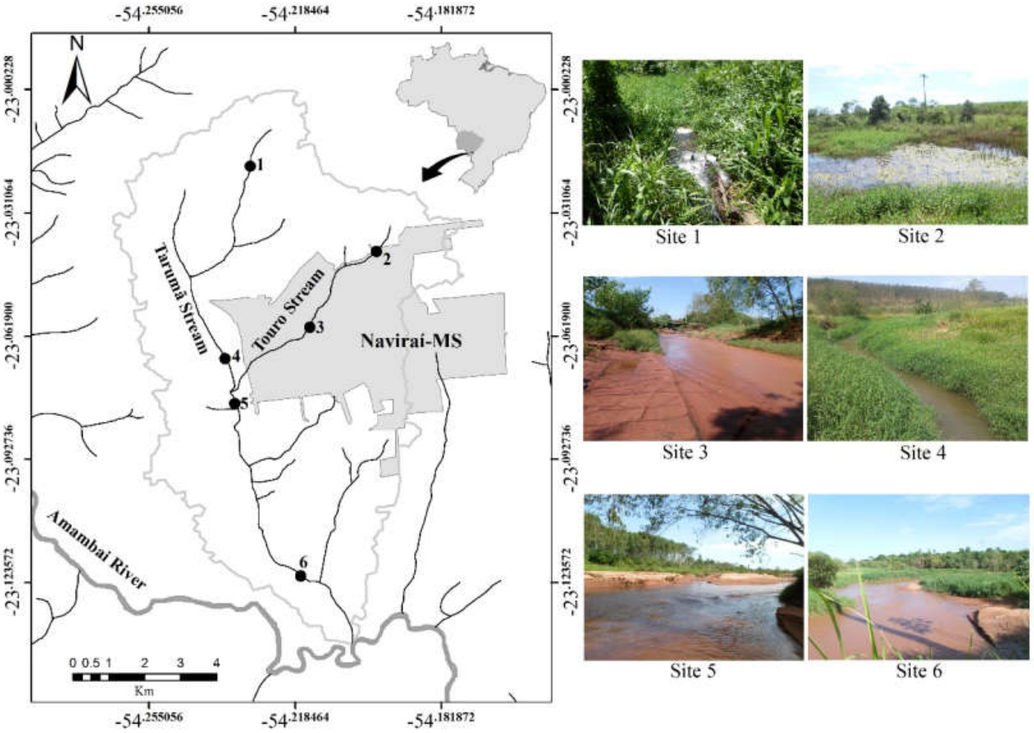

Fig. 1. Location and images of the collection sites in the Tarumã microbasin of the Upper Paraná River in Brazil.

4.2.2. Analysis of land use and occupation

We used high resolution aerial images (years 2014 and 2015) from Google Earth Pro™ with resolution of 1 meter (DigitalGlobe, 2017) to map land use and coverage in the Tarumã Microbasin. We generated buffers of 1 km radius around each sampling site, and land use was classified as agriculture, forest fragments, forest plantation, exposed soil, water bodies, or buildings, as defined by IBGE (2013). For the interpretation of the images, an unsupervised classification (clustering) was used using the classification tools provided by the ArcGIS™ program trial version 10.3 (ESRI, 2015), by calculating the areas and percentages of each land occupation category based on the buffer areas.

4.2.3. Water and sediment collection

Water samples were collected in July 2014 and December 2015 at the six sample sites and stored in polyethylene containers previously washed with distilled water. Sediment from each site was collected using a dredger, stored in previously decontaminated plastic bags, and maintained under refrigeration (-4 ºC). The samples were oven dried at 60 ºC for 4 hours in order to avoid drag of the metals by water vapor. The samples were then sieved and only the fraction < 53 µm was used for the chemical extraction. For extraction, about 1 g of each sample was added to a test-tube along with 4 mL of 50% aqua regia (3:1 $HCl:HNO_3$) and 5 mL of $HClO_4$. Samples were heated for 30 min at 90 °C in a water bath. The resulting solution was filtered and brought to 0.5 ml volume with ultrapure water (Hortellani et al., 2008; Betemps and Sanches Filho, 2012). Concentrations of Cd, Pb, Cr, Cu, Fe, Zn and Ni in samples were then estimated using Atomic Absorption Spectrometry (Shimadzu, model AA7000) with flame atomization.

4.2.4. Fish bioassays

Six independent glass aquariums (40 x 30 x 20cm) were used for bioassays, one for water from each sampling site. For the negative control (NC), drinking water was placed in a seventh aquarium. In each aquarium, 10 *A. lacustris* were added, all of them from the same breeding stock. The standard length of individuals did not differ among treatments (F=1.50; p=0.21). Before the fish were submitted to the bioassays, a previous analysis was carried out for each individual for mutagenicity and genotoxicity. The fish were well-fed throughout the exposure period, and the temperature (26 °C), pH (7.15), and dissolved oxygen (7.5 mg L^{-1}) were kept within limits appropriate for the animals. The collection procedures were approved by the Ethics Committee on the Use of Animals at UFGD (10/2014).

After a period of 72 hours, blood samples were obtained from fish by caudal puncture with heparinized syringes. For each specimen, blood was drawn two thin layer slides were prepared using a drop of blood. The smears were air-dried for 15 min, fixed in absolute alcohol for 10 min, and then stained with Panotic LB (Schmid, 1975; Jesus et al., 2016). We analyzed 2,000 erythrocytes per slide, resulting in a total of 4,000 cells for each individual. Cells were analyzed using 1,000x magnification on an optical microscope. Micronuclei (MN) were identified following the criteria proposed by Fenech et al. (2003). For the analysis of erythrocytes nuclear abnormalities (ENA), we used the classifications proposed by Carrasco et al. (1990). The collection procedures were approved by the Ethics Committee on the Use of Animals at UEMS (011/2014) and authorized by IBAMA (SISBIO 11156-1).

4.2.5. Statistical analysis

We used a partition chi-square test for contingency tables to verify if LI and IMP sites have significant differences related to land occupation categories. A non-parametric Mann-Whitney test was applied to analyze for differences in sediment metal concentration between the LI and IMP sites. We also used a non-parametric Kruskall-Wallis test to compare MN and ENA frequencies in *A. lacustris* erythrocytes among LI, IMP, and control treatments. All tests were performed using the R platform (R Development Core Team, 2016), with a significance level of 0.05.

4.3. RESULTS

Land use in the Tarumã Microbasin is primarily associated with anthropogenic activities, with land cover being predominantly related to agriculture (73.46% of the microbasin area) followed by buildings (15.21%), and with only 8.78% of the total area categorized as forest fragment (Fig. 2). In the comparison between the LI and IMP sites we found a significant difference in relation to the higher proportion of buildings in the IMP sites ($p<0.0001$). For the other categories (forest fragments, forest plantations, agriculture and exposed soil) we did not observe differences in the proportion of land occupation between the sites ($p> 0.05$) (Table 1).

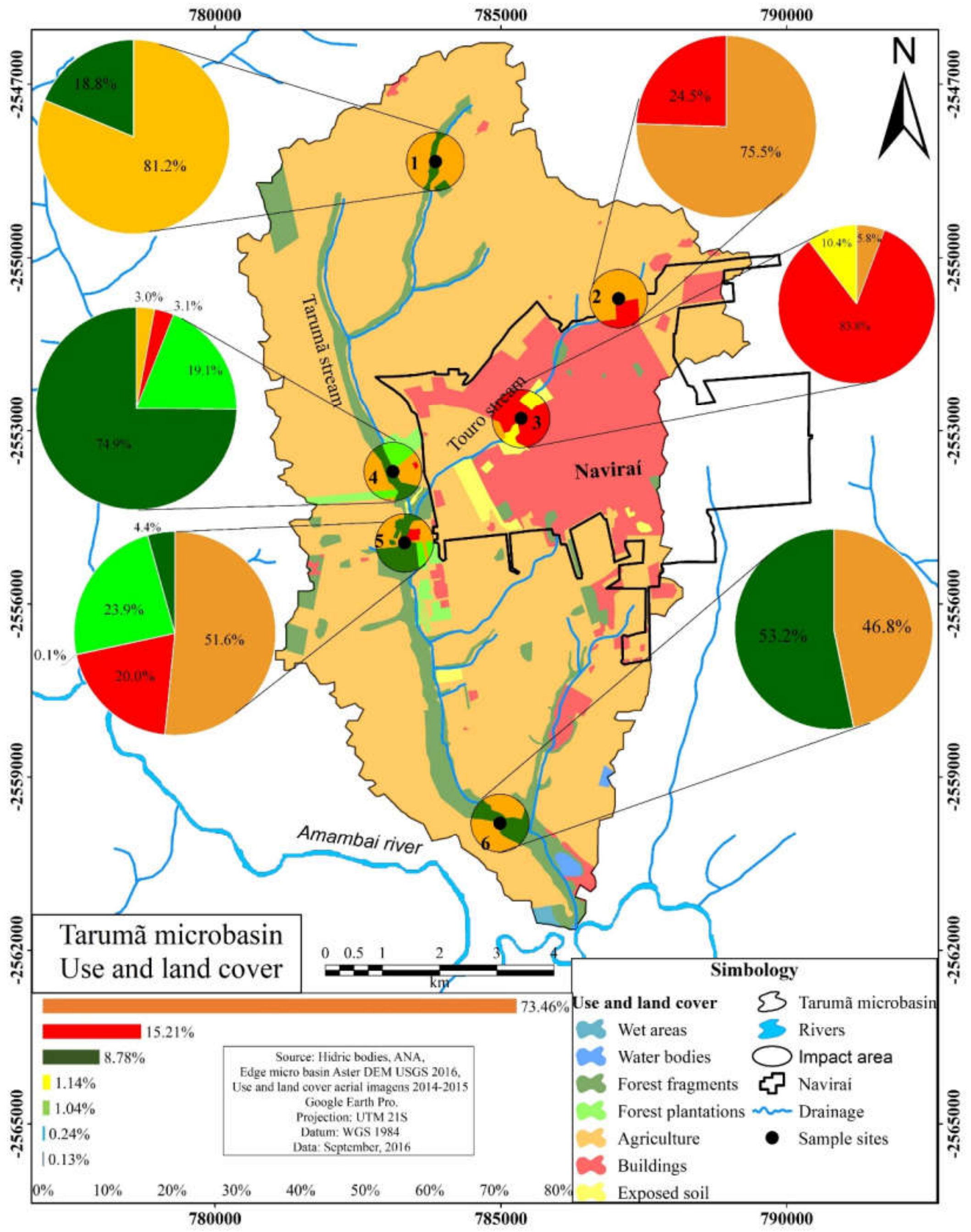

Fig. 2. Land use and location of the sites sampled in Tarumã Microbasin in the Upper Paraná River, Brazil. Sites 1, 2, and 4 were considered less impacted (LI) and sites 3, 5, and 6 were considered impacted (IMP).

Table 1. Partition chi-square test for contingency tables comparing LI and IMP sites concerning to land occupation categories. Bold values indicate significant difference ($p < 0.05$).

Partitions	**X^2**	**DF**	**p**
Forest fragments and Forest plantations	1.3728	1	0.2413
Agriculture	0.0912	1	0.7626
Buildings	20.1370	1	**< 0.0001**
Exposed soil	3.5623	1	0.0591
Total	25.1633	4	**< 0.0001**

The analysis of metals in the sediment of the sampled sites revealed higher concentrations of Cd, Pb, Cu, Zn, and Ni in the IMP sites compared to the LI sites ($p < 0.05$). We did not observe differences between the sites for Cr and Fe ($p > 0.05$) (Fig. 3A-G).

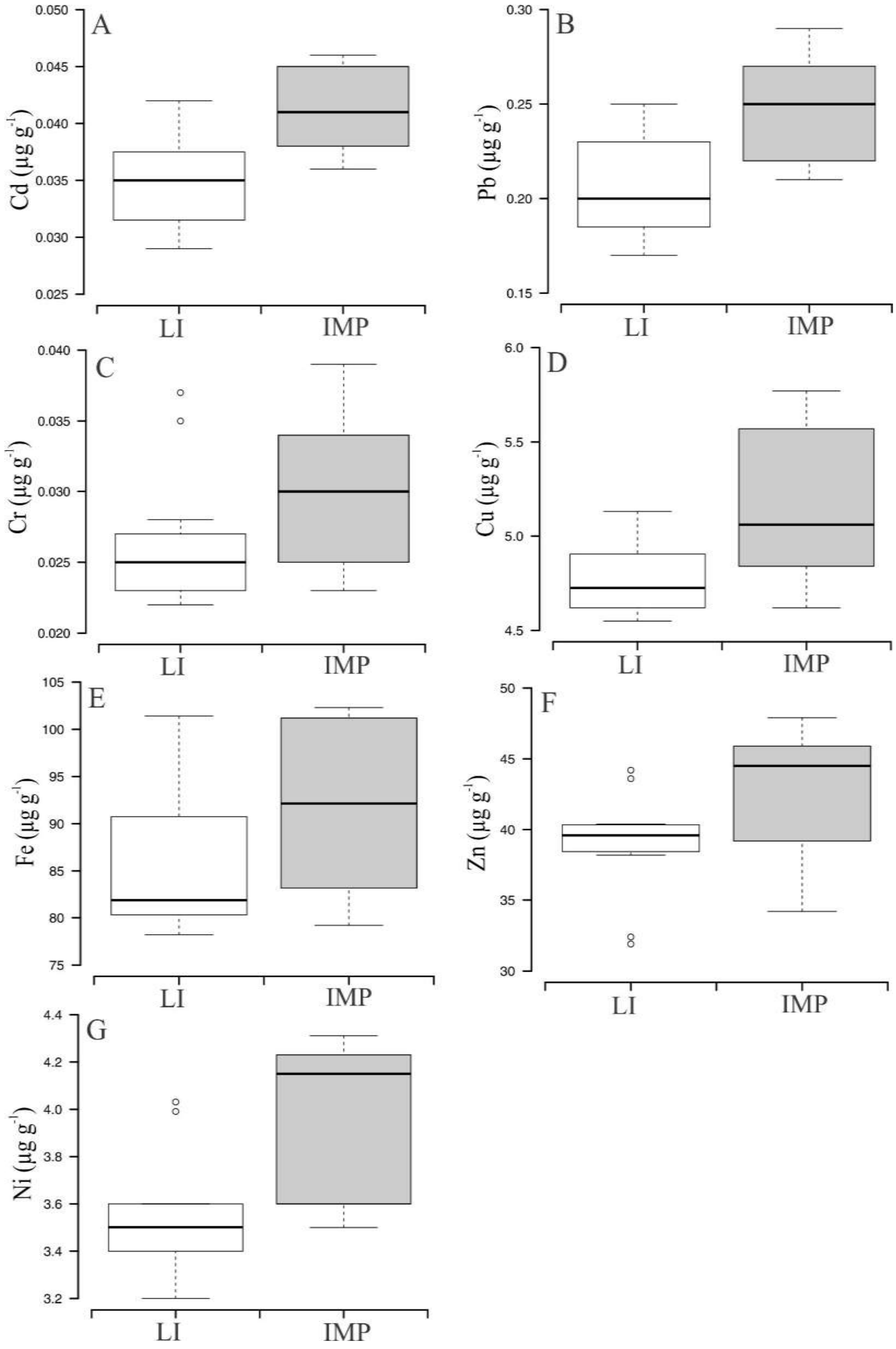

Fig. 3. Sediment metal concentrations (median, quartiles, maximum and minimum) in the less impacted (LI) and impacted (IMP) sites of the Tarumã Microbasin in the Upper Parana River Basin, Brazil.

Analysis of micronuclei frequency in erythrocytes of *A. lacustris* revealed higher frequencies in the IMP sites compared to the LI sites and controls (p <0.05). There were no significant differences between LI sites and controls (p> 0.05) (Fig. 4).

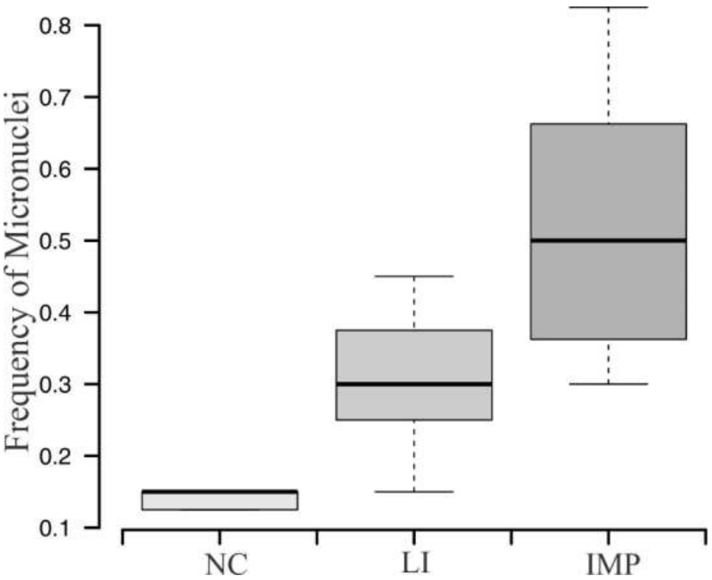

Fig. 4. Percentual frequency of micronuclei (median, quartiles and interquartile deviation) in erythrocytes of *A. lacustris*. NC: negative control. LI: less impacted sites. IMP: impacted sites.

Analysis of nuclear abnormalities showed no significant differences in nuclear budding, vacuolated nuclei, lobed nuclei, and binucleated cells in *A. lacustris* among treatments (NC, LI, and IMP; p> 0.05). We did, however, find significant differences between the negative control and IMP sites (p <0.05) for notched nuclei and nuclear pyknosis (Fig. 5A-F).

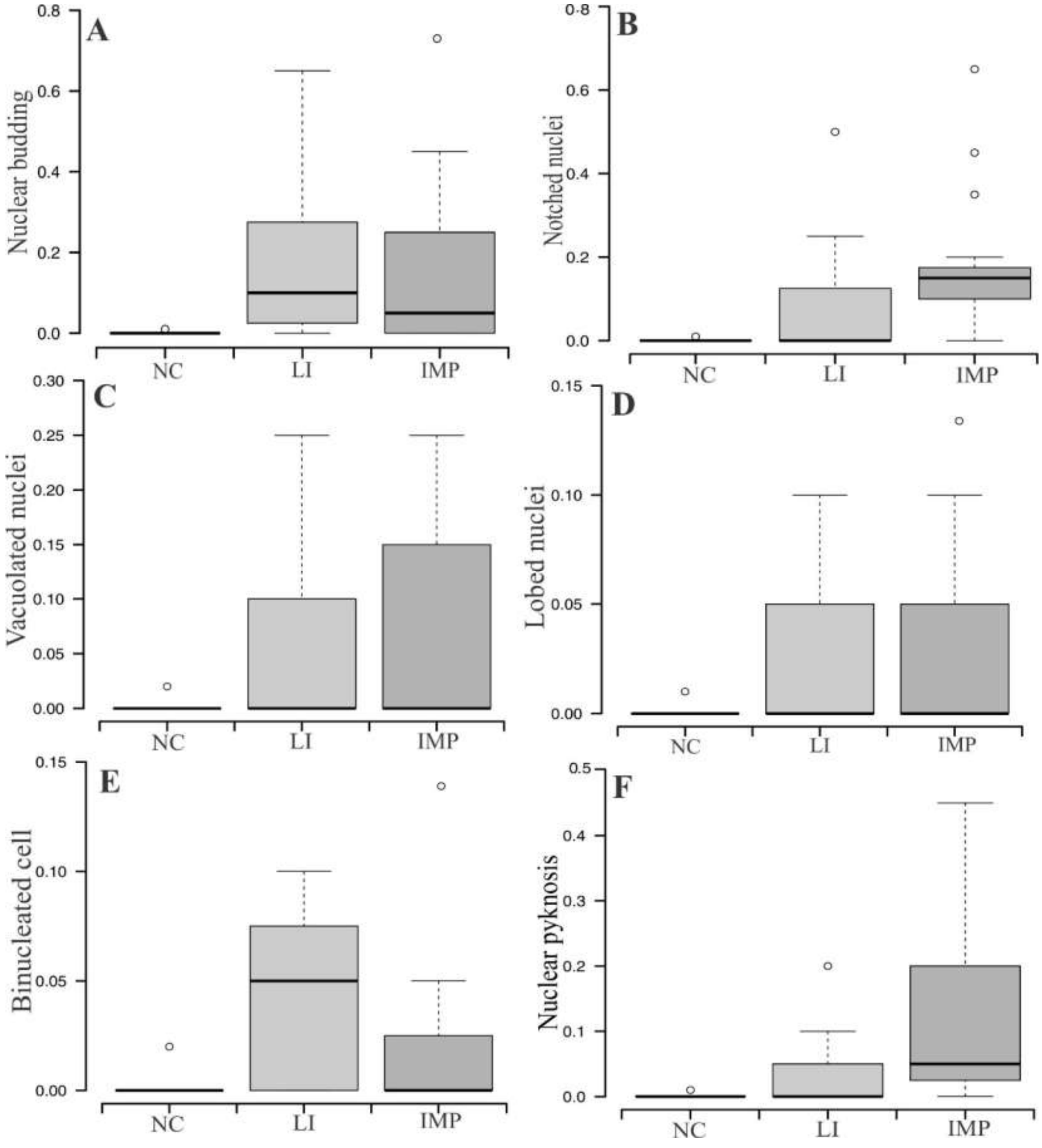

Fig. 5. Percentual frequency of nuclear abnormalities (median, quartiles and interquartile range) in *A. lacustris* erythrocytes. NC: negative control. LI: less impacted sites. IMP: impacted sites.

4.4. DISCUSSION

The analysis of land use and occupation in the Tarumã Microbasin revealed a large proportion of area used for agricultural activities, including areas near the springs and banks of the watercourse. Agricultural waste can be transported through the course of streams and deposited into sediments via adsorption, flocculation, and precipitation in the water (Cheng et al., 2015; Tang et al., 2016).

In addition, IMP sites are characterized by a higher proportion of occupancy by buildings (residential and industrial) and consequently more subject to the discharge of contaminants due to increasing urbanization (Cunico et al., 2011). In addition, urban occupation contributes to the reduction of native vegetation cover. These factors together represent an important source of impacts for streams, compromising water quality and facilitating the entry of contaminants and sediments that are carried to the aquatic environment, negatively affecting aquatic biota (Araújo et al., 2013; Dusmam et al., 2014; Torres et al., 2015; Wang et al., 2015; Melo Gurgel et al., 2016).

Analysis of the sediment in the microbasin watershed showed concentrations of Cd, Pb, Cr, Cu, Fe, Zn, and Ni below the limits established for level II water bodies (i.e., the lowest level of pollution) according to Resolution 454/2012, which pertains to water quality of rivers and streams in Brazil (CONAMA, 2012). Nevertheless, the bottom of the river bottom provides nutrients and habitat for a wide variety of benthic organisms, and the continuous ingestion of these sediments by fishes that exploit the bottoms of rivers may lead to bioaccumulation of contaminants, potentially causing mutagenic or genotoxic effects (Paixão et al., 2011; Jesus et al., 2012; 2014; Voigt et al., 2016). Further, non-essential metals such as Cd, Pb and Cr are considered toxic to aquatic biota even in small doses, and may have harmful effects on fish due to biomagnification in the food chain, leading to physiological changes (Adam et al., 2010; Galunin et al., 2014; Govind and Madhuri, 2014). In fact, Viana et al. (unpublished data) in a study carried out in the same microbasin found higher concentrations of Pb, Cu, Fe, Zn, and Ni in musculature and higher frequency of micronuclei in erythrocytes from *A. lacustris* captured in IMP sites compared to individuals from LI sites.

The presence of micronuclei in erythrocytes is considered an indicator of genetic damage (Samanta and Dey, 2012), and are more frequent in fish exposed to polluted waters (Obiakor et al., 2014; Ribeiro et al., 2014). Some contaminants, mainly non-essential metals, may alter formation of the mitotic spindle or cause chromosomal damage or loss, and consequently, may result in formation of micronuclei (Matsumoto et al., 2006; Barbosa et al., 2010; Dourado et al., 2016). In this study, the frequency of micronuclei in individuals exposed to water from LI sites was not significantly different from that observed in the negative control. However, we observed a higher frequency of micronuclei in individuals exposed to water from IMP sites compared to the other two treatments. This demonstrates the greater mutagenic and genotoxic potential of conditions in impacted sites of the microbasin. Similar results were found by Dourado et al. (2016), who also observed genetic alterations (e.g., chromosomal alterations, micronuclei, DNA damage) in erythrocytes of *A. lacustris* exposed to polluted waters.

Exposure to water from the IMP sites of the Tarumã Microbasin also induced a higher frequency of nuclear alterations such as notched nuclei and nuclear pyknosis compared to the negative control. These abnormalities have been used by several authors as indicators of genotoxic damage and cytogenetic toxicity (Ayllon and Garcia-Vazquez, 2000; Cavaş and Ergene-Gözükara, 2003; Rivero-Wendt et al., 2013). Notched nuclei can be attributed to changes in the cytoskeletal protein responsible for maintaining nuclear shape (Alberts et al., 2002; Ghisi et al., 2014). Nuclear pyknosis is an indicator of cell death due to DNA degradation and failure of the repair system (because it is either inefficient or not activated), and is strongly associated with the presence of contaminating chemical substances in the environment (Adam et al., 2010; Palermo et al., 2015; Zheng et al., 2014).

In summary, our data show that unplanned land use in combination with the expansion of anthropogenic activities may be damaging the environmental integrity of the Tarumã Microbasin. The *A. lacustris* bioassay clearly demonstrated that exposure to water from the most impacted sites of the watershed produces mutagenic and genotoxic effects. Considering that Tarumã Microbasin is a "nursery" for fish species occupying the Amambai and Paraná Rivers, it is crucial that we adopt environmental recovery and preservation policies in the system. These plans should include the introduction of efficient tools for monitoring the health of fish fauna.

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CAPÍTULO 5. DETECÇÃO DE Fe e Pb EM ESCAMAS DE PEIXES POR *LASER-INDUCED BREAKDOWN SPECTROSCOPY* – LIBS

Artigo a ser submetido (periódico não definido)

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RESUMO. O estudo teve como objetivo determinar, por meio da técnica de LIBS, se há presença de Fe e Pb bioacumulados em escamas de *S. brasiliensis* e *P. lineatus* e comparar os resultados obtidos a partir da técnica de LIBS com a técnica convencional de espectrofotômetro de absorção atômica. O estudo foi realizado no rio Amambai, Alto Rio Paraná, localizado na parte Sul do Estado do Mato Grosso do Sul. A técnica de LIBS detectou nas escamas de *S. brasiliensis* a presença de Fe e para a espécie *P. lineatus* Fe e Pb, em diferentes regiões de absorção nas escamas. Em relação à concentração de Fe em escamas determinada por espectrofotômetro de absorção atômica, constatamos que houve diferença significativa entre as duas espécies analisadas, com maior concentração para *S. brasiliensis* (p=0.0004). O elemento Pb não foi detectado por espectrofotometria de absorção atômica, em nenhuma espécie, devido ao fato de estar abaixo do limite de detecção. A técnica de LIBS foi eficiente para detectar Fe e Pb nas escamas de *S. brasiliensis* e *P. lineatus*, demostrando ser uma importante ferramenta preditiva de monitoramento ambiental.

PALAVRAS-CHAVE. Contaminantes, bioacumulação, bioindicadores

5.1. INTRODUÇÃO

As modificações na paisagem em torno de rios e córregos decorrentes de atividades humanas, com a expansão de atividades agropecuárias e de edificações, resultam na redução da cobertura vegetal local e na formação de fragmentos florestais. Isso compromete a integridade do ambiente, pois acarreta o maior escoamento de contaminantes para os sistemas hidrológicos, resultando em ameaças à biodiversidade aquática (Cunico et al., 2012; Message et al., 2016). Em peixes, esses contaminantes podem alterar suas atividades fisiológicas e seus parâmetros bioquímicos normais, provocando efeitos mutagênicos e genotóxicos, podendo, inclusive, levar os indivíduos à morte (Vinodhini; Narayanan, 2008; Hussain et al., 2016). Neste sentido, os peixes são amplamente utilizados para avaliar a saúde e as condições nos ecossistemas aquáticos e são considerados excelentes bioindicadores ambientais (Shikha; Sushma, 2011; Brraich; Jangu, 2013).

A análise de metais nas escamas de peixes é uma importante ferramenta para o monitoramento ambiental (Van Der Oost et al., 2003; Lins et al., 2010; Weber et al., 2013; De Jonge et al., 2015; Wan;Wang, 2015; Yamamoto et al., 2016), pois as escamas estão diretamente expostas a vários agentes tóxicos e têm o primeiro contato com os contaminantes (Sultana et al., 2017). Neste sentido, as escamas podem acumular contaminantes, como uma impressão digital química dos eventos de poluição do ambiente ao qual o peixe está exposto (Kaur; Dua, 2012; Almeida et al., 2016; Santana et al., 2016).

A técnica *Laser Induced Breakdown Spectroscopy* (LIBS) é uma ferramenta de espectroscopia de emissão atômica que utiliza um laser pulsado com uma fonte de excitação (Wan;Wang, 2015; Avila et al., 2015). Com este método e a junção do infravermelho por Transformada de Fourier, pode ser determinada a presença de metais presentes nas escamas de peixes. Esta técnica tem características proeminentes, baseada na análise das linhas espectrais emitidas a partir do plasma gerado por interação do pulso do laser e da amostra, sendo altamente seletiva, sensível, rápida, direta e não polui o ambiente com a sua execução (Cremers et al., 2006; Li et al., 2012; Yu et al., 2014). Além disso, para ser aplicada às escamas de peixes, não exige o sacrifício dos animais.

Neste estudo utilizamos as escamas de duas espécies de peixes Characiformes, com hábitos alimentares distintos, como modelos de bioindicadores ambiental: uma espécie detritívora, *Prochilodus lineatus* (Valenciennes, 1837) (Souza et al., 2008; Cazenave et al., 2014), e uma espécie carnívora, *Salminus brasiliensis* (Cuvier, 1816) (Volkof et al., 2016), ambas consideradas migradoras de longa distância. Neste contexto, esse trabalho tem como objetivo (1) determinar a eficiência da técnica de LIBS para detectar a presença de Fe e Pb bioacumulados em escamas de *S. brasiliensis* e *P. lineatus* e (2) comparar os resultados obtidos a partir da técnica de LIBS com a técnica convencional de espectrofotometria de absorção atômica para detecção de metais nas escamas dessas duas espécies.

5.2. MATERIAIS E MÉTODOS

5.2.1. Área de estudo

Este estudo foi desenvolvido com peixes coletados no rio Amambai, Alto Rio Paraná, localizado na parte sul do Estado do Mato Grosso do Sul, Brasil. A bacia do rio Amambai é caracterizada por diversos tipos de uso do solo, com o predomínio da agricultura (pastagens e culturas agrícolas, com a dominância da monocultura da cana-de-açúcar) e edificações (indústrias, áreas urbanas e propriedades rurais). Consequentemente, ocorreu a redução da cobertura vegetal nativa, resultando em fragmentos florestais. Assim, os rios da bacia acabam recebendo maior quantidade de contaminantes carreados junto com materiais alóctones no período de pluviosidade, contaminando a água, sendo depositados nos sedimentos e afetando a biota aquática (Cunico et al., 2012; Message et al., 2016).

As amostragens foram realizadas entre dezembro de 2014 e julho de 2015, em três pontos de amostragens, visando investigar toda a amplitude do curso do rio Amambai: porção superior (ponto 1), porção média (ponto 2) e foz (ponto 3) (Figura 1).

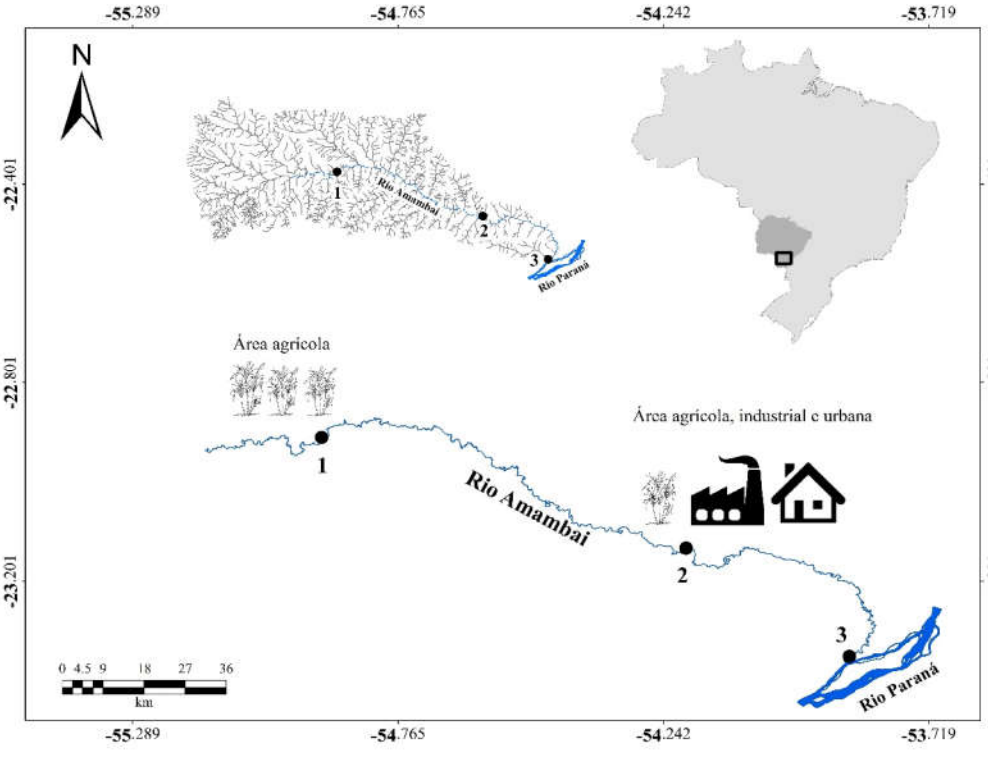

Figura. 1. Localização dos pontos amostrados ao longo do Rio Amambai (Alto rio Paraná, Brasil).

5.2.2. Coleta do material biológico

Os peixes foram coletados com redes de espera, com malha variando entre 1,5 a 8,0 cm entre nós adjacentes, e tarrafas. Para determinar a presença de metais nas escamas de peixes, foram removidas escamas da região umeral de 11 indivíduos de *S. brasiliensis* e de 13 exemplares de *P. lineatus*, sempre do mesmo lado do corpo dos animais. O projeto foi aprovado pela Comissão de Ética no uso de Animais / UEMS (011/2014).

5.2.3. Análise das escamas por LIBS

A espectroscopia de plasma induzida por laser (LIBS) foi utilizada para verificar a existência de Fe e Pb nas estruturas das escamas dos peixes, através de um laser de Nd:YAG (Neodimiumdoped Ytrium Aluminium Garnet), operando em 1064 nm, com o ajuste na forma pulsada para que houvesse a geração do plasma ao incidir na amostra.

No laboratório foi realizada medidas de metais na amostra de Fe e Pb, no comprimento de onda de 200 a 600nm, a fim de obter um referencial de comprimentos de onda com relação às intensidades de emissão e os correspondentes elementos químicos encontrados nas amostras. Dessa maneira, ao realizar medidas com as escamas já era possível ter um padrão com relação ao comprimento de onda desses metais. Para LIBS é recomendável que sejam utilizados materiais de referências para obtenção das curvas analíticas de calibração.

Para análise de LIBS três escamas foram removidas dos peixes, em seguida foram lavadas com água destilada para remoção de impurezas e acondicionadas entre lâminas de vidro, para que se mantivessem planas. Para padronização das análises foram retiradas as escamas das lâminas para obtenção da altura, largura e espessura de cada escama. Em uma mesma amostra de escama foi tirada três medidas correspondendo a diferentes regiões na face externa das escamas (Figura 2A-C), com energia de 16.2% e rede de 2400nm. As linhas de emissões foram identificadas de acordo com os dados das amostras padrão de Fe e Pb. Todas as leituras foram obtidas nas mesmas condições experimentais.

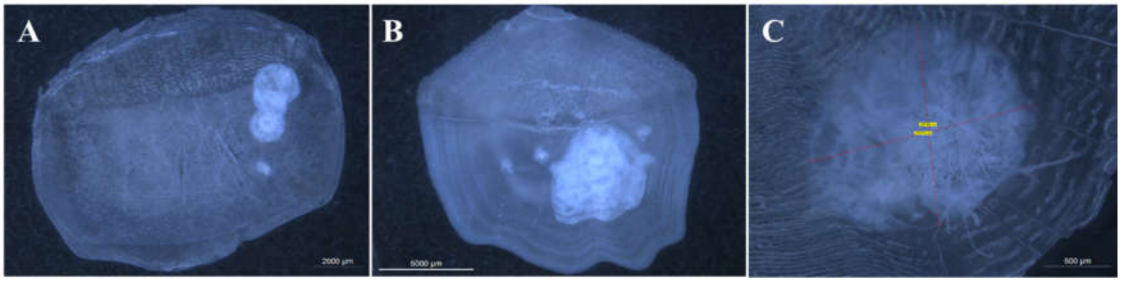

Figura 2. Imagens de escamas de *S. brasiliensis* (A) e de *P. lineatus* (B-C) após a emissão de LIBS.

5.2.4. Análise das escamas por Espectrofotometria de Absorção Atômica

Para estimar as concentrações de Fe e Pb nas escamas por espectrofotometria de absorção atômica (espectrofotômetro Shimadzu, modelo AA7000), com atomização em chama, foram utilizadas as escamas dos mesmos indivíduos analisados pela técnica de LIBS. A partir de um conjunto de escamas com massa total de 0.05g, cerca de 0.5 mL de HNO_3 65% foi adicionado à amostra. Cada amostra foi então transferida para tubos de digestão e deixada para reagir em um aparelho de bloco digestor, a 60ºC por 1h. Após esta etapa, adicionou-se 0.5mL de HNO_3 65% a cada amostra e estas foram novamente aquecidas a 100ºC, até quase a secura total da amostra. Em seguida, as amostras foram filtradas e ressuspendidas a um volume final de 5 mL de solução de HNO_3 0,5 N e mantidas em freezer (-4ºC) por no máximo 15 dias até a sua análise (Seixas et al., 2009; Eneji et al., 2011**)**.

5.2.5. Análises estatísticas

Utilizamos o teste não-paramétrico de Kruskall-Wallis (α=0.05) para comparar as intensidades dos espectros de LIBS observados nas três diferentes regiões definidas nas escamas dos peixes. A comparação entre as espécies com relação às intensidades de Fe obtidas na análise de LIBS e à concentração de Fe determinada por espectrofotômetro de absorção atômica foi processada a partir do teste não-paramétrico de Mann-Whitney (α=0.05). Os testes citados foram realizados usando a plataforma R (R Development Core Team, 2016).

5.3. RESULTADOS

Nas escamas de *S. brasiliensis* constatamos a presença de dois picos para o elemento Fe na região de (510.61 e 510.93 nm) no espectro obtido no intervalo de 510 a 511.5 nm (Figura 3A). Para a espécie *P. lineatus* observamos a presença de dois picos para Fe na região de (260.27 e 261.36 nm) e de dois picos correspondentes ao Pb (260.77 e 260.94 nm), em diferentes regiões de absorção na face externa das escamas (Figura 3B).

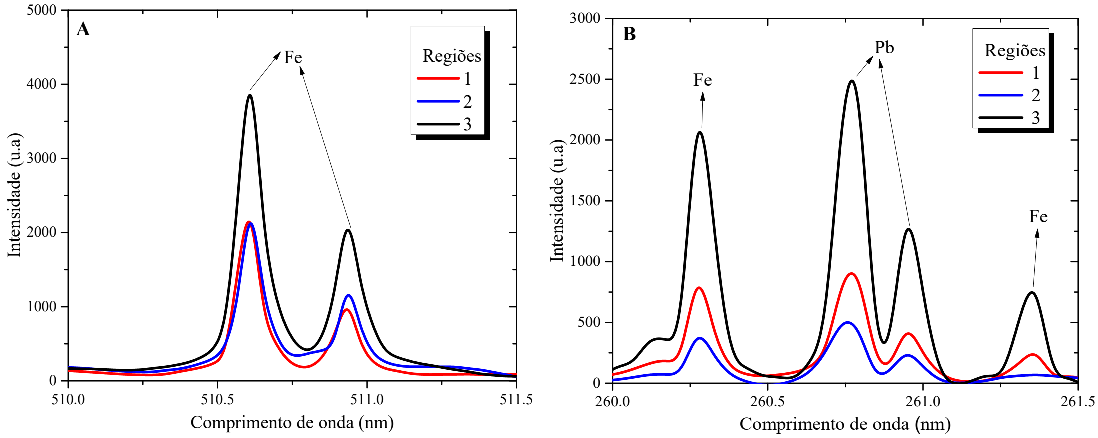

Figura 3. Espectros de LIBS obtidos a partir de escamas de *S. brasiliensis* (A) e de *P. lineatus* (B) coletadas no Rio Amambai, Alto Rio Paraná.

Constatamos que não houve diferença significativa, em nenhuma comparação, entre as três diferentes regiões definidas nas escamas com relação às intensidades de Fe e de Pb registradas nas análises de LIBS ($p>0.05$) (Figura 4A-E).

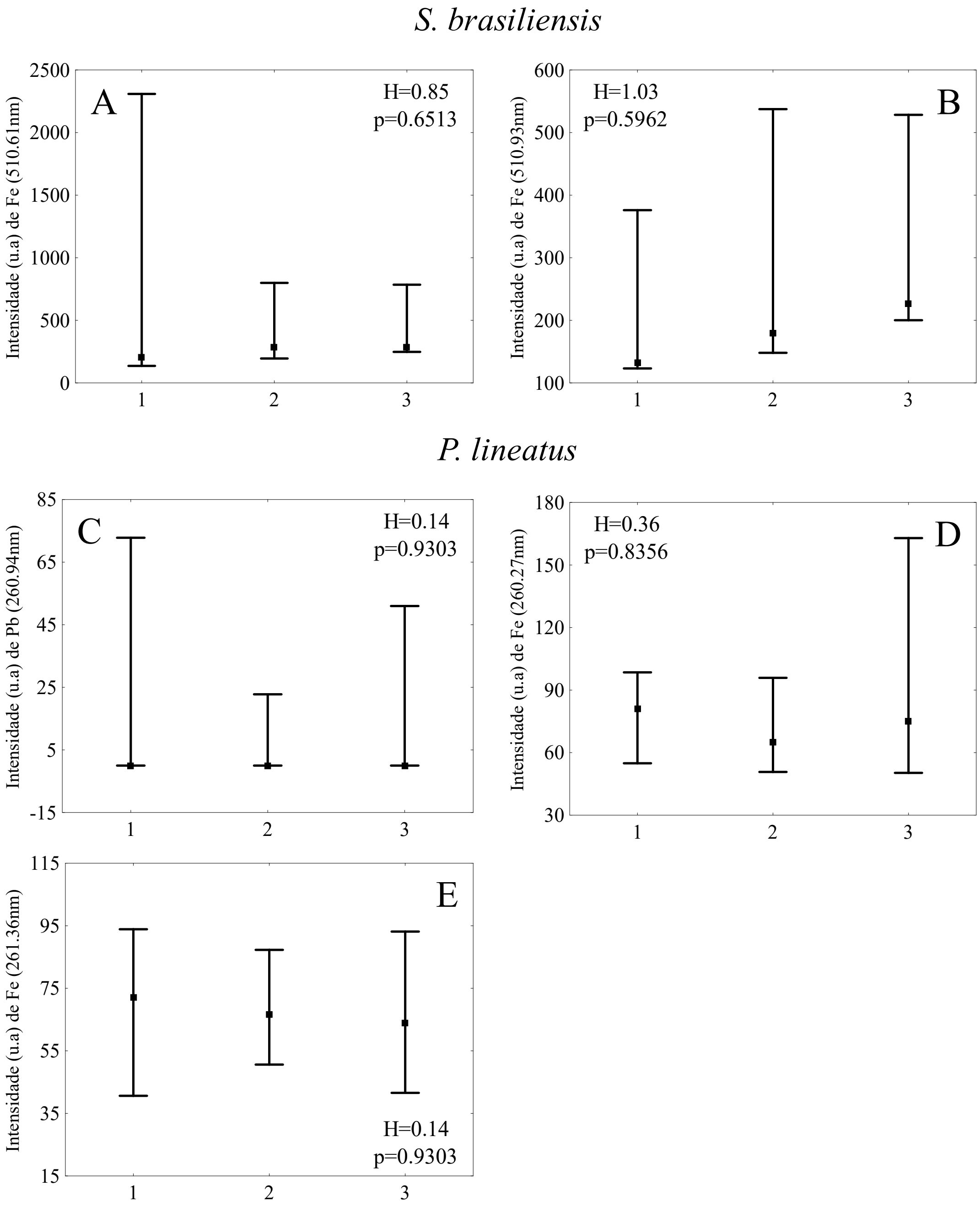

Figura 4. Intensidades de Fe e Pb determinadas por LIBS (mediana e quartis) em três regiões distintas (numeradas de 1 a 3) de escamas de *S. brasiliensis* e *P. lineatus* coletadas no Rio Amambai, Alto Rio Paraná.

Nas análises de LIBS o elemento Pb foi observado somente nas escamas de *P. lineatus*, fato que impossibilitou a comparação entre as espécies. Para o Fe, entretanto, foi possível realizar a comparação interespecífica, e os resultados indicaram intensidades significativamente maiores desse elemento nas escamas de *S. brasiliensis*, para os dois picos ($p<0.05$) (Figura 5A-B).

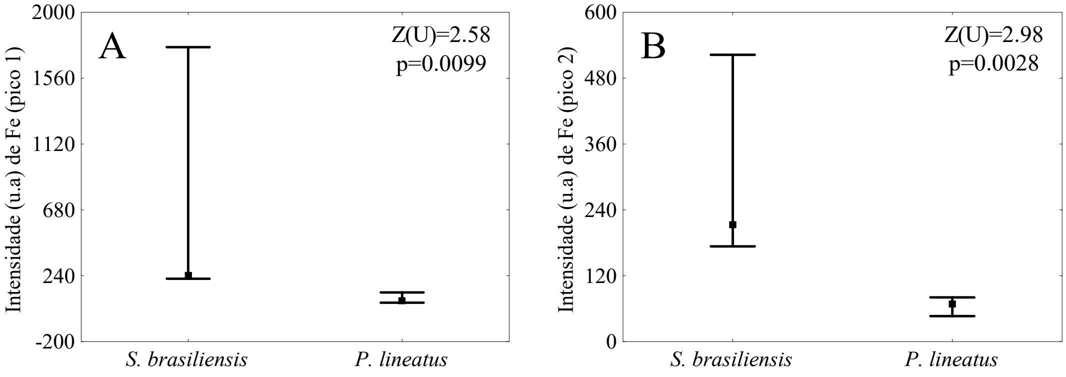

Figura 5. Intensidades de Fe determinadas por LIBS (mediana e quartis) em escamas de *S. brasiliensis* e *P. lineatus* coletadas no Rio Amambai, Alto Rio Paraná.

Em relação à concentração de Fe em escamas determinada por espectrofotometria de absorção atômica, constatamos que houve diferença significativa entre as duas espécies analisadas, com maior concentração para *S. brasiliensis* ($p=0.0004$) (Figura 6). O elemento Pb não foi detectado por esta técnica, em nenhuma espécie, devido ao fato de estar abaixo do limite de detecção.

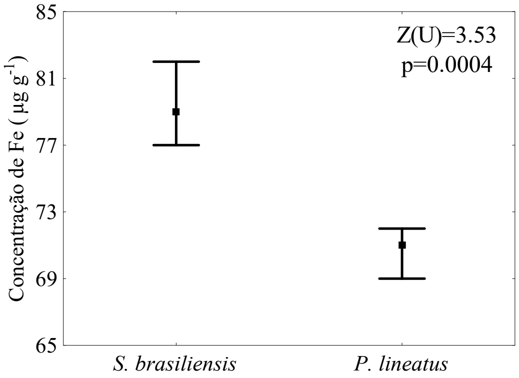

Figura 6. Concentração de Fe determinada por espectrofotometria de absorção atômica (mediana e quartis) em escamas de *S. brasiliensis* e *P. lineatus* coletadas no Rio Amambai, Alto Rio Paraná.

5.4. DISCUSSÃO

Por meio da técnica de LIBS foi possível detectar a presença de metais nas escamas de *S. brasiliensis* (Fe) e de *P. lineatus* (Fe e Pb), indicando que os poluentes ficam incorporados na matriz inorgânica das escamas e, consequentemente, tal análise pode fornecer um registro de poluição ambiental (Guambe et al., 2012). A presença de metais nas escamas dos peixes está relacionada ao fato de essa estrutura estar em maior contato com o meio, devido às suas funções de auxilio hidrodinâmico e de proteção das condições adversas da água (Brraich; Jangu, 2013; Almeida et al., 2016; Sultana et al., 2016). Neste sentido, nosso trabalho sugere que as escamas podem ser utilizadas como biomarcadoras na avaliação da bioacumulação de metais em peixes, em concordância com outros trabalhos já realizados nessa linha (Darafsh et al., 2008; Lin-Sun et al., 2009; Mahboob et al., 2015; Almeida et al., 2016).

Observamos, também, que a região das escamas na qual o laser incide não acarreta diferenças significativas nas leituras das intensidades dos metais analisados, para as duas espécies analisadas.

Na comparação interespecífica das intensidades registradas pela análise de LIBS para o Fe, observamos que *S. brasiliensis* apresenta maior concentração do metal em suas escamas. Esse resultado possivelmente esteja relacionado ao hábito alimentar carnívoro dessa espécie. Por estar no topo da cadeia alimentar, a biomagnificação do metal se revela com mais intensidade nessa espécie predadora. O Fe é considerado um elemento essencial, mas seu excesso pode ser prejudicial aos peixes (Gorur et al., 2012).

Somente para a espécie *P. lineatus* foi detectado Pb pela técnica de LIBS. Esta espécie, por ser detritívora, ingere sedimentos para, a partir destes obter a matéria orgânica que lhe serve de alimento. Os sedimentos de rios são grandes depositórios da maioria dos metais pesados, pois estes metais tendem a sofrer decantação e acumulação no fundo dos rios. Dessa forma, espécies detritívoras estão mais expostas à contaminação por metais pesados (Gupta et al., 2009; Yi et al., 2011). É importante salientar que o Pb é um elemento não essencial, considerado tóxico mesmo em pequenas concentrações, pois pode causar danos neurodegenerativos, além de ser potencialmente genotóxico para os peixes (Nadeem et al., 2008; Monteiro et al., 2011; Voigt et al., 2014).

Em função dos mais variados impactos negativos sobre nos ambientes aquáticos, há necessidade crescente de desenvolvimento de novas técnicas e estratégias para utilizar os peixes como bioindicadores ambientais. A aplicação da técnica de LIBS para avaliação da concentração de metais em escamas de peixes, quando comparada com o método de espectrofotometria de absorção atômica, revelou concordância no que se refere à maior concentração de Fe nas escamas de *S. brasiliensis*. Além disso, a técnica de LIBS apresentou maior sensibilidade na detecção do Pb, revelando a presença desse metal nas escamas de *P. lineatus*, apesar de sua concentração estar abaixo do limite de detecção através do método de espectrofotômetro de absorção atômica. A técnica de LIBS não exige tratamento da amostra em comparação ao método de espectrofotometria de absorção atômica, o que proporciona economia de tempo e gastos que seriam despendidos nesta operação. E avalia a composição química em diversos tipos de materiais, sendo que até o momento o único trabalho disponível é o de Alvira et al. (2015), que analisaram Cu e Pb em escamas de *Oreochromis niloticus* no México.

Em síntese, observamos que a técnica de LIBS foi eficiente para detectar Pb nas escamas de *P. lineatus*, demostrando ser uma importante ferramenta preditiva de monitoramento ambiental. Além disso, os resultados indicam que a utilização de escamas de peixes como bioindicadoras ambiental pode ser feita na avaliação do potencial de risco sobre a ictiofauna e, consequentemente, para os seres humanos que podem consumir esses peixes.

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CAPÍTULO 6. CONSIDERAÇÕES FINAIS

O presente estudo indicou a ocorrência de mutagenicidade em peixes do Rio Amambai e da Microbacia Tarumã. Esse fato pode estar relacionado à presença de contaminantes no ambiente aquático em decorrência, possivelmente, da redução da cobertura vegetal original e da expansão de atividades antrópicas.

Os metais analisados não ultrapassaram as concentrações máximas estipuladas pela legislação brasileira (ANVISA), na musculatura e no fígado das espécies analisadas, apesar de o Cd e o Pb serem considerados tóxicos para os organismos, em qualquer nível de concentração. Desta forma, o ambiente analisado requer atenção no que diz respeito à presença de contaminantes.

Desta maneira, os biomarcadores estudados revelaram informações importantes para a geração de políticas públicas que priorizem a recuperação da vegetação ripária em torno do Rio Amambai e na microbacia Tarumã para o monitoramento e a conservação da biota aquática. Considerando-se que a Microbacia Tarumã é um “berçário” de espécies de peixes que habitam o Rio Amambai e o Rio Paraná, recomenda-se a adoção de políticas de recuperação e preservação ambiental no sistema estudado.

Além disso, a técnica LIBS mostrou-se promissora para detectar a presença de metais em peixes, pois as escamas podem ser removidas sem a necessidade de sacrificar os peixes, que podem ser devolvidos para o ambiente. Desta forma, a pesquisa possibilitou determinar as condições que os peixes se encontram no ambiente, baseada em nova ferramenta de monitoramento ambiental, preditiva. E, assim, poder contribuir para preencher as lacunas no conhecimento do atual estado de preservação dos ambientes aquáticos.

Conclui-se, portanto, que as espécies estudadas podem ser consideradas boas indicadoras da qualidade do ambiente onde vivem e que os métodos utilizados na pesquisa se mostraram satisfatórios para responder aos objetivos propostos.