UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM
ECOLOGIA AQUÁTICA E PESCA

Caroline da Silva Montes

USO INTEGRADO DE BIOMARCADORES PARA O MONITORAMENTO DOS EFEITOS DE METAIS EM *Serrasalmus rhombeus* PROVENIENTES DE RIOS AMAZÔNICOS

Orientador(a): Prof. Dra. Rossineide Martins Rocha

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26 de fevereiro de 2016

Banca Examinadora:

Dra. Rossineide Martins Rocha
Orientadora

Dra. Lílian Lund Amado - UFPA
Membro

Dr. Leandro Juen - UFPA
Membro

Dra. Adriana Costa Guimarães - UFPA
Membro

Dr. Anderson Manoel Herculano da Silva - UFPA
Membro

Dra. Jussara Moretto Martinelli - UFPA
Suplente

DEDICATÓRIA

À minha família que em todos os momentos me apoiou muito, entendeu minha ausência, vibrou com cada vitória e me abraçou nos momentos mais difíceis.

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RESUMO GERAL

USO INTEGRADO DE BIOMARCADORES PARA O MONITORAMENTO DOS EFEITOS DE METAIS PESADOS EM *Serrasalmus rhombeus* PROVENIENTES DE RIOS AMAZÔNICOS

Diversos contaminantes tais como: metais, pesticidas e outros compostos orgânicos contribuem para a contaminação dos sistemas aquáticos. O metal lançado na água, quando não absorvido pelos organismos, se acumula no sedimento e pode ser lançado novamente na água através da ressuspensão do sedimento ou pela reação de oxidação-redução. Com objetivo de verificar os efeitos dos contaminantes no meio aquático, alterações morfológicas, ultraestruturais e bioquímicas em tecidos branquiais e hepáticos de uma espécie nativa da região amazônica, a piranha preta, *Serrasalmus rhombeus*, foram analisadas como ferramenta de avaliação da qualidade ambiental de rios Amazônicos, além de verificar a eficiência desta espécie como organismo bioindicador em monitoramento ambiental *in situ* e *ex situ*. Foram realizadas coletas em rios da região amazônica: O rio Itaciaunas, afluente da bacia do Tocantins, localizado na região do Carajás, sofre influência de efluentes da exploração de minério da região; O rio Crepori e Tropas, afluentes do rio Tapajós, localizado na região sudoeste do Pará, na região do tapajós, é uma área conhecida pela intensa atividade garimpeira e o Rio negro (AM) em uma área sem influência antrópica com elevados graus de matéria orgânica. Nos locais selecionados foram coletados simultaneamente material biológico (peixes), amostras de água superficial e de sedimento de fundo para a determinação das concentrações de metais pesados (Cd, Cr, Cu, Zn, Mn e Hg). As análises das brânquias e do fígado evidenciaram as alterações histológica, ultraestrutural e bioquímica. Essas alterações foram eficazes na avaliação da qualidade da água, pois conseguiram

distinguir os animais das diferentes áreas. A combinação de vários tipos de biomarcadores em diferentes níveis mostrou um panorama geral da saúde dos peixes e a correlação com as concentrações de metal pesado nos compartimentos ambientais. Dentre os metais, o cádmio e o cobre apresentaram valores significativamente maiores que o permitido na legislação brasileira, sendo verificado na água superficial, no sedimento e nos músculos dos peixes. Enquanto o mercúrio não foi considerado tão prejudicial à saúde dos animais do ponto de vista morfofisiológico. Desta forma, podemos inferir que a qualidade dos rios estudados está comprometida em função das atividades antrópicas adjacentes. Portanto, concluímos que o biomarcador é uma ferramenta bastante válida e de fácil aplicabilidade. Além disso, o estudo da exposição aguda dos metais selecionados mostrou que a matéria orgânica pode oferecer uma proteção natural para os rios dependendo do tipo de metal, no caso, o cádmio ainda com DOM pode trazer prejuízos severos nos animais. Ademais, a espécie escolhida *Serrasalmus rhombeus* pode ser utilizada como espécie sentinela em estudos de biomonitoramento.

Palavras chave: metais pesados, *in situ*, *ex situ*, Amazônia, peixe, sedimento.

GENERAL ABSTRACT

INTEGRATED USE OF BIOMARKERS FOR MONITORING THE EFFECTS OF HEAVY METALS IN *Serrasalmus rhombeus* FROM AMAZON RIVER

Various contaminants such as metals, pesticides and other organic compounds contribute to environmental pollution. The metal released in the water, if not absorbed by the organisms, accumulates the sediment and can be launched again in water by resuspension of the sediment or by oxidation-reduction reaction, such processes potentiate the concentration of trace metals in water solution. Therefore, it was performed several studies to evaluate the effects of contaminants in the exposed animals. Thus, the aim of this study was to analyze the morphological, ultrastructural and biochemical changes in gill and liver tissues in a species native to the Amazon region, the black piranha, *Serrasalmus rhombeus*, as an evaluation tool for environmental quality in Amazonian rivers besides verifying the efficiency of this species as bio-indicator organism for environmental monitoring *in situ* and *ex situ*. This study was conducted sampling in rivers of the Amazon region: The Itaciaunas river, a tributary of the Tocantins basin, located in the Carajas region, is influenced by effluents from ore mining in the region; The Crepori, Tropas River, tributaries of the Tapajós River, located in the southwestern region of Para, in the Tapajos region, is an area known for intense mining activity and Rio Negro (AM), an area without anthropogenic influences with high concentrations of organic material (DOM). In selected locations were simultaneously collected biological materials (fish), surface water samples and bottom sediments to determine the concentrations of heavy metals (Cd, Cr, Cu, Zn, Mn and Hg). The analysis showed that changes in ultrastructural, histological and

biochemical level were effective in assessing water quality therefore able to distinguish animals from the collection points. Furthermore, the combination of various types of biomarkers in different levels showed an overall picture of the health status of this fish correlating with the high heavy metal concentration in the environmental compartments. The metalic elements cadmium and copper was significantly greater than allowed in the Brazilian legislation for surface water, the sediment and fish tissues also presented high levels of these metals. Whilst, the mercury was not considered harmful to the health of animal from a morphological point of view. Thus, it is suggested that fish may be adaptively responding to the background Hg Amazon, since despite the large mercury concentrations in tissues few irreversible changes were observed. Thus, it concludes that the biomarker is a very valuable tool and easy to apply. We can infer that the quality of the studied rivers is committed on the basis of adjacent human activities. Moreover, the study of acute exposure of selected metals showed that the organic matter can provide a natural protection for the streams depending on the type, speciation and concentration of metal, in this case, cadmium in high even the levels of DOM can cause severe damage in animals. Besides, the *Serrasalmus rhombeus* can be utilized as sentinel species in biomonitoring studies.

Key words: heavy metals, *in situ*, *ex situ*, Amazon, fish, sediment

SUMÁRIO

INTRODUÇÃO GERAL

O desenvolvimento das atividades antropogênicas acoplado ao avanço tecnológico tem contribuído para o aumento de compostos xenobióticos no meio ambiente, fato que prejudica os diversos compartimentos ambientais (solo, água e ar) (BOTKIN E KELLER, 2000). No entanto, o ecossistema aquático é considerado o mais suscetível à contaminação por receber diretamente agentes químicos ou indiretamente por meio de águas de chuva e carreamento superficial dos solos (BERTOLETTI, 1990). A poluição aquática está comumente associada com a descarga de efluentes domésticos, industriais ou agrícolas e pode ocorrer de forma intencional ou acidental, a partir de fontes naturais ou em decorrência da atividade humana. Tal realidade pode gerar modificações ambientais drásticas, reduzindo a diversidade de espécies chaves ou aumentar a densidade de outras espécies indesejáveis (ZAGATTO E BERTOLLI, 2006).

Dentre as diversas fontes de poluição existentes a contaminação por metais pesados pode ter efeitos mais devastadores no equilíbrio ecológico dos diversos compartimentos ambientais (AYANDIRAN et al., 2009). Uma vez que eles podem diminuir a qualidade da água e sedimento afetando a saúde dos organismos que dependem destes recursos. Além disso, podem representar um grande perigo em função da sua capacidade tóxica, de persistência e de bioacumulação (SEKHAVATJOU et al., 2010). Muitos metais tem função vital para a fisiologia biológica, contudo, a extrapolação nos limites pode torna-los prejudiciais ou letais para os organismos. Enquanto o chumbo, cádmio, cromo e o mercúrio são elementos tóxicos e não essenciais, que podem bioacumular e biomagnificar em tecidos-alvos afetando

estruturas celulares com consequências, muitas vezes, irreversíveis (VIEIRA et al., 2011; ALASHEMI et al., 2012 CARDWELL et al., 2013).

Os sedimentos e partículas em suspensão desempenham um papel importante na adsorção dos metais pesados dissolvidos. Eles também podem ser um reservatório potencial de metais, liberando-os para a coluna de água quando ocorre alterações das condições físicas e químicas. Além disso, os níveis de certos elementos metais na água esta relacionada com a quantidade de descargas das indústrias (KARSSABIi et al., 2007). Vale ressaltar que a presença de um contaminante em um sistema não sugere necessariamente uma contaminação e nem que os animais irão ser prejudicados, pois a poluição esta relacionada à biodisponibilidade dos contaminantes. Isto porque ele pode estar associado à matéria orgânica dissolvida, particulada e ao sedimento. Assim, sua concentração disponível é muito variável, e afetará o organismo através da relação entre sua taxa de captação e eliminação (Al-HEASI et al., 2013). Dessa forma, é essencial que sejam aplicados estudos que avaliem não só a presença de contaminantes do ambiente mas também dos efeitos que estes podem causar. Para isso, muitas técnicas e estudos têm sido desenvolvidos e aprimorados com a finalidade de avaliar os efeitos nocivos dos compostos nos ecossistemas aquáticos e nos organismos que vivem nestes ambientes (FLORES E MALABARBA, 2007; DABROWSKA ET AL, 2012).

Uma das abordagens emergentes na ecotoxicologia é a avaliação de risco “Risk assessment”, uma poderosa ferramenta da gestão ambiental que levanta dados científicos, fazendo uma projeção dos efeitos dos estressores no ambiente a fim de evitá-la. Um dos enfoques mais promissores para a detecção preventiva dos efeitos adversos é o uso dos biomarcadores (AZEVEDO E CHASIN, 2003; MAGALHÃES E FILHO, 2008). Os biomarcadores são definidos como respostas adaptativas biológicas aos estressores, evidenciadas como alterações histológicas, ultraestruturais,

bioquímicas, fisiológicas e/ou comportamentais (HUGGET et al., 1992). Segundo DECAPRIO (1997), o biomarcador é um indicador biológico que evidencia efeito resultante de exposição a um estressor que pode ser interpretado como efeito adaptativo não patogênico ou como alteração séria de um evento funcional, dependendo da toxicocinética e do mecanismo de ação do estressor. O uso desta ferramenta como indicador de poluição ambiental é bastante válido pelo custo-benefício e pelos bons resultados, uma vez que, as respostas biológicas dos organismos aquáticos podem refletir o efeito integrado dos impactos nos corpos hídricos, além de sinalizarem possíveis danos no ecossistema aquático (van der OOST et al., 2003).

Quando peixes e outros organismos são expostos a ambientes muito degradados, o efeito dos poluentes pode gerar múltiplas alterações causando consequências em populações, comunidades ou ecossistemas, dependendo do grau de contaminação e do tempo de exposição (GOULARD e CALLISTO, 2003). Desta forma, estes organismos são considerados excelentes ferramentas em estudos de monitoramento ambiental. A utilização de peixes em programas de monitoramento, testes de toxicidade em laboratório e como indicadores biológicos da qualidade dos ambientes aquáticos tem aumentado bastante nos últimos anos (Flores-Lopes e Malabarba, 2007). O incremento do uso de peixes nesse tipo de programa ou estudos se deve principalmente ao fato desses animais possuírem a ecologia e mecanismos celulares e fisiológicos bem estudados (ARIAS et al., 2007).

Como normalmente a poluição aquática ocorre de forma crônica, com concentrações subletais de poluentes, identificam-se mais comumente alterações estruturais e funcionais nos peixes do que mortalidade em massa dos organismos (POLEKSIC E MITROVIC-TUTUNDZIC, 1994). Essas alterações morfo-funcionais têm sido muito utilizadas como biomarcadores para indicar tanto a exposição quanto os

efeitos de poluentes ambientais (MARTINEZ E SOUZA, 2002). Além disso, para diminuir os danos, os organismos utilizam defesas do sistema antioxidante, incluindo enzimas como: superóxido dismutase (SOD), Catalase (CAT), Glutationa s transferase (GST) e etc. Este sistema pode prevenir a formação de ROS, que pode reagir com as macromoléculas produzindo peroxidação lipídica (LPO) (REGOLI E GIULIANI, 2014).

Quando um contaminante é absorvido pelo organismo, este é transferido para o fígado e rim, para serem biotransformados por meio de enzimas, para se tornar hidrossolúvel e então ser excretado por diversos órgãos como pele, intestino, rim e brânquias. Portanto, esses os órgãos são considerados alvos por participarem diretamente de processos de transformação e acumulação de agentes químicos, dessa forma, podem ser utilizados em diagnósticos da saúde do animal e consequentemente do ambiente em que foi capturado (CAMARGO e MARTINEZ, 2007).

As brânquias são extremamente importantes para a respiração, osmorregulação, equilíbrio ácido-básico e excreção de nitrogênio (EVANS et al., 2005). O fígado é o órgão de grande importância para os peixes, sendo responsável pelo anabolismo das proteínas, lipídios carboidratos e catabolismo do nitrogênio, glicogenólise, desintoxicação e função de vitelogênese (BRUSLÉ E ANADON, 1996). Esses órgãos podem ser alterados por agentes do meio ambiente como os poluentes, as toxinas, os parasitas e os microrganismos (SCHWAIGER et al., 1997; BALDISSEROTTO, 2002).

Nas vias hepáticas as alterações podem ser refletidas por várias patologias como inflamação, atrofia, necrose, degeneração vacuoloar, degeneração gordurosa, estagnação de bile, hepatite, cirrose, congestão e tumores que podem levar o animal à morte (HIBIYA, 1982). Baseado neste contexto, o presente estudo tem como principal

objetivo utilizar a integração de biomarcadores como parâmetro norteador na avaliação da qualidade ambiental de três rios amazônicos.

HIPÓTESE

H0: Ambientes de minas adjacentes aos rios não exercem efeitos negativos sob a qualidade de água e nem geram risco de contaminação para a ictiofauna (*Serrassalmus spp*) residente.

H1: Ambientes de minas adjacentes aos rios exercem efeitos negativos sob a qualidade de água e geram risco de contaminação para a ictiofauna (*Serrassalmus spp*) residente.

OBJETIVOS

Objetivo geral

Avaliar a qualidade ambiental da água de rios Amazônicos em nível biológico utilizando biomarcadores de efeito e exposição em *Serrasalmus rhombeus*

Objetivos específicos

a) Determinar as variáveis físico-químicas pH, condutividade elétrica, temperatura, turbidez, Oxigênio Dissolvido (OD) e material em suspensão da água superficial diferentes pontos dos rios Amazônicos;

b) Determinar concentração de metais pesados (Cd, Cr, Mg, Mn, Cu e Hg) no músculo de *Serrasalmus spp,* na água superficial e no sedimento de fundo do rio de diferentes pontos dos rios Amazônicos;

c) Identificar as alterações histológicas nas brânquias e no fígado de *Serrasalmus spp* coletados de diferentes pontos dos rios Amazônicos, a partir dessas alterações atribuir o grau e o índice de severidade;

d) Verificar alterações ultraestruturais nas brânquias, de *Serrasalmus spp* coletados de diferentes pontos dos rios Amazônicos;

e) Determinar as atividades enzimáticas nos tecidos branquiais, bem como, peroxidação dos lipídios dos animais coletados em diferentes pontos dos rios Amazônicos;

f) Exposição aguda de cádmio e cobre em piranhas em diferentes tipos de água. Uma rica em matéria orgânica do rio Negro e outra água de laboratório;

g) Determinação dos níveis de amônia, Na^{+}, K^{+}, Na^{+}, Mg^{2+}, Ca^{2+} e Cl^{-} na água;

h) Determinação dos níveis de amônia, osmolaridade, Na^{+}, K^{+}, Na^{+}, Mg^{2+}, Ca^{2+} e Cl^{-} no plasma;

METODOLOGIA

Áreas de estudo

Para a realização deste estudo foram selecionados rios Amazônicos de águas claras e escura. As coletas foram realizadas nos rios: Itaciaúnas, afluente da bacia do Tocantins, localizado na região do Carajás, sofre influência de efluentes da exploração de minério da região; Crepori e Tropas, afluentes do rio Tapajós, localizado na região sudoeste do Pará, na região do tapajós, é uma área conhecida pela intensa atividade garimpeira, e o Rio negro (AM) em uma área sem influência antrópica com elevados níveis de matéria orgânica. Foram coletados amostras de sedimento e água de superfície no local de captura dos peixes. O sedimento foi coletado com auxílio de uma draga do tipo van-vem de aço inoxidável. Após, as amostras eram depositadas em sacos plásticos e identificadas. As amostras de água superficiais eram acondicionadas em garrafas esterilizadas e em seguida resfriadas. Os animais foram capturados com a arte de pesca linha de mão. No rio Negro não foram coletada as amostras de sedimento de fundo e água da superfície e os animais não foram pescados, porque foi realizado experimento in situ. Após a captura os peixes foram sacrificados com um corte na medula espinhal anterior a nadadeira dorsal. Em seguida os animais foram pesados (g), medidos (cm) e examinados externa e internamente para identificação de lesões macroscópicas. Logo em seguida foi retirado os órgãos (brânquia, fígado e músculo) e acondicionados de acordo com cada processamento.

Quantificação dos metais pesados (Zn, Cd, Mn, Cr e Cu)

As análises de metais traços dos materiais bióticos (pescado) e abióticos (água e sedimento) seguiram pela mesma metodologia de acordo com KRUG, 2008. Foi pesada

uma massa de aproximadamente 0,2 g de cada amostra em tubos de Teflon, em seguida foi adicionado a cada tubo: 3 mL de HNO_3 concentrado, 1 mL de HCl e 1 ml de H_2O_2 para.A digestão seguiu-se no microondas Mars, CEM. Ao final do processo o conteúdo de cada tubo foi transferido a tubos tipo falcons e aferidos com água mili-Q ao volume final de 25 mL. Posteriormente as análises foram realizadas em um Espectrômetro Óptico de Emissão com Plasma Induzido (ICP-OES).

Determinação do mercúrio total

As amostras de pescado e sedimento foram submetidas à digestão ácida, com a adição de 2 ml de HNO_3 e $HClO_4$ (1:1), 5 ml de H_2SO_4 e 1 ml de H_2O, em chapa aquecedora a 230-250°C por 20 minutos, resfriados à temperatura ambiente, aferidas em frascos volumétricos de 50 ml e homogeneizadas. O mercúrio disponível em solução iônica foi analisado por espectrofotometria de absorção atômica por geração de vapor a frio (EAA-VF/G) com um Mercury Analyzer HG-3500, de acordo com o método proposto por AKAGI et al. (1995).

Avaliação histopatológica

Os tecidos branquiais e hepáticos utilizados pelas análises histopatológicas foram fixados em solução Bouin. Para a microscopia de luz, os tecidos foram incluídos em parafina e cortados à 5 µm de espessura em micrótomo manual Leica (RM 2245). Os cortes foram corados com solução Hematoxilina e eosina (H.E.) e analisados em microscópio (Nikon Eclipse ci). A prevalência de cada tipo de mudança foi determinada de acordo com PREREIRA et al., 2013. Dez filamentos inteiros por arco foram escolhidos aleatoriamente e analisados. A escala de gravidade gradação (SGS), com seis graus (0-5) foi aplicado qualitativamente, considerando a extensão e gravidade de cada

lesão. A medida foi definida como a percentagem de filamentos com um tipo de lesão em cada peixe amostrados. Para quantificar a gravidade de cada alteração histológica, os diferentes níveis de gravidade foram atribuídos. A gravidade de cada lesão por média de filamentos afetada foi determinada como o número de espaços lamelares e interlamelares afctados por um determinado nível de gravidade, dividido pelo número de filamentos que mostra que tipo de mudança histológica. Um grau zero foi dada aos valores encontrados em peixes com poucas lesões e para definir os graus remanescentes, a extensão e níveis de gravidade foram combinados para mostrar um aumento do número de lamelas e espaço interlamelar por filamento ferido. Todos os valores obtidos, a partir da contagem de extensão e gravidade, foram, em seguida, dividido em intervalos numéricos e combinados para gerar o SGS.

Microscopia eletrônica de transmissão (MET)

As amostras de tecido branquiais foram fixadas em Karnovsky (paraformoldeído 4%, glutaraldeído 2% em tampão cacodilato de sódio 0,1M pH 7,4). Após fixação as amostras foram lavadas em tampão cacodilato de sódio 0,1M pH 7,4 e pós-fixadas em tetróxido de ósmio 2%. Em seguida desidratadas em série crescente de acetona, embebidas e incluídas em Epon 812. Após a inclusão os blocos foram cortados em ultramicrótomo e os cortes semifinos corados com azul de toluidina 1% para definição da área a ser utilizada para os cortes ultrafinos. Os cortes ultrafinos foram contrastados em acetato de uranila e citrato de chumbo, analisados e fotografados em microscópio eletrônico de transmissão (LEO 906 E).

Parâmentros bioquímicos

A GST (Glutationa – S – Transferase) foi determinada pela reação de conjugação de 1 mM de glutationa (GSH) com 1 mM de 1-cloro-2,4-dinitrobenzeno (CDNB), de acordo o com método descrito por HABIG E JAKOBY (1981). A leitura foi feita no leitor de micro placas multi moldar VITOR x3. A Catalase foi determinada segundo o método descrito por BEUTLER (1975), medindo-se pelo espectrofotômetro a taxa de decomposição enzimática do H2O2 a 240 nm. A diminuição da absorbância representa a atividade da enzima, que é expressa em unidade de CAT/mg de proteína, onde uma unidade é a quantidade de enzima necessária para hidrolisar 1µm de H_2O_2/min/mg de proteína, à 30° C e pH 8. Enquanto que as amostras para análise da LPO foram homogeneizadas em metanol, centrifugadas 1000g x por 15 minutos a 4ºC para a extração da fração S9. A atividade do LPO (Hidroperóxido Lipídico) será determinada pelo método de FOX (Ferrous Oxidation-Xylenol Orange) como descrito por JIANG et al. (1992). Os resultados foram expressos em mmolCHP/g de tecido úmido. Para determinação da concentração proteica, medição da quantidade de proteína nas amostras baseou-se no método espectofotométrico de BRANDFORD (1976), utilizando-se soro albumina bovina com padrão.

Exposição Aguda à metais pesados (Cd d Cu)

O experimento consistiu em expor as piranhas em cádmio e cobre com água de "água preta" do Rio Negro e água do laboratório. O rio Negro é rico em matéria orgânica. A água do Laboratório INPA apresentou os seguintes parâmetros físico-químico: baixa dureza (pH 6,28;. 6,40 mg O_2/L) , por um período de 1 h, 2 h e 3 h. Um total de sete repetições de peixes por tratamento foram individualmente colocados em recipiente de plástico de 3,5 L previamente limpas com água destilada. Os seguintes

tratamentos de Cu e Cd foram, A - Rio Negro: (1) Controle RN, (2) RN 5 µM Cu, (3) RN 10 µM Cu, (4) RN 0,1 µM Cd, (5) RN 10 µM Cd, B – água do INPA (6) INPA Controle, (7) INPA 5 µM Cu, (8) INPA 10 µM Cd.

Amostragem

Após a exposição, os animais foram cuidadosamente removidos dos aquários, sacrificados por meio de uma overdose de anestesia por etil-3- aminobenzoato de ácido metanossulfónico (MS-222, Acros Chemicals, Geel, Bélgica 195), em seguida foi realizada a biometria dos animais. As amostras de sangue foram retiradas a partir do veia caudal, utilizando uma seringa previamente heparinizada (heparina a partir de Sigma-Aldrich, Co, St.Louis, EUA), as amostras de sangue foram imediatamente centrifugadas durante 2 min a 13200 rpm a 4 ° C. O plasma foi cuidadosamente pipetado para frascos criogênicos e congelados em nitrogênio líquido. Parte do tecido branquial de cada peixe foi removido e armazenado em solução Bouin (75% saturada de ácido pícrico ácido PA, 20% de formaldeído, 5% de ácido acético) para análise histológica e outra parte foi acondicionada em nitrogênio líquido para a análise bioquímica e concentração de metal.

Avaliações analíticas

Amostras de água (5 mL) foram coletadas a cada hora durante o experimento. Foi determinada Amônia total (Am) por colorimetria usando o método do salicilato-hipoclorito (VERDOUW et al., 1978). Concentrações de Na + K+, Ca2+, Mg2+ e Cl - na água foram medidos usando chama espectrofotometria de absorção atômica. Os Fluxos iónicos foram calculados (em pmol kg-1 h -1), multiplicada pela massa dos peixes (em kg) Na + (NAFJ líquido), o volume (em L), e o tempo (em h). A osmolaridade do plasma foi medida na amostra de plasma fresco usando o avançado ™

Micro Osmometer (Modelo 3300, Advanced Instruments, EUA). Concentrações de íons no plasma de Na +, K +, Ca + e Mg + foram analisados utilizando um Analisador de eletrólitos 9180 (corporação AVL Scientific, 213 GA, EUA) e glicose plasmática, lactato e amônia foram determinados com kits enzimáticos comerciais (R-Biopharm AG, Darmstadt, Alemanha).

ANÁLISE ESTATÍSTICA

Os dados foram analisados utilizando a análise de variância ANOVA (oneway) e o teste não-paramétrico de Kruskal-Wallis (K-W), dependendo da validação de normalidade e homogeneidade, através dos testes Kolmogorov-Smirnov e Levene, respectivamente. Resultados estatisticamente significativos seguiram para comparação entre grupos utilizando os testes de Tukey (HSD, paramétrico) e postos de H (não paramétrico). As diferenças estatísticas serão consideradas significativas para $p < 0,05$. A análise estatística será realizada utilizando software Sigma e Prisma. Para as análises das alterações histopatológicas foi utilizado o teste de multivariada por análise dos componentes principais (PCA).

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CAPÍTULO 1

Está sob a norma da Revista: Archives of Environmental Contamination and Chemistry

Biological effects on Piranhas (*Serrasalmus Rhombeus*, Teleostei: Characidae) from Amazonian Rivers with different historical of mercury pollution

Abstract:

The aim of this study is to use biomarkers on *Serrasalmus rhombeus* to assess, from a biological perspective, the water quality in an Amazonian region with intense mining activity. Specimens of piranhas were collected from two rivers with different histories of mining exploitation. Muscle fragments were used for mercury analysis and liver samples were employed in histological and biochemical protocols. Sediment samples were collected for HgT analysis. The highest concentrations were observed in fishes from Crepori River (G2). Animals in G2 demonstrated intense oxidative stress, as indicated by the lower GST activity associated to high levels of lipoperoxidation compared to animals from G1. Morphological parameters confirmed the difference between the areas. Therefore, the damages observed in fishes collected in this environment indicate a long-term risk to the ecosystem of this region. These results emphasize the advantage of using these types of biomarkers as useful tools to diagnose the health of river environments.

Keywords: Gold mining; biomarker; GST, LPO; Histopathology

INTRODUCTION

Mercury (Hg), an important and well - known metallic element occurs naturally in the environment in three oxidation states (Hg^0, $Hg1^+$ and Hg^{2+}) (Dórea et al., 2006). However, since the soil is a natural deposit of mercury, studies clearly show that harmful anthropogenic activity resulting in soil erosion could lead to the release of Hg in the rivers and consequently make this element bioavailable in the aquatic environment. In many Amazonian regions, there are several sources that generate the release of mercury into the environment (gold mining, Hg leaching from soils following deforestation, hydroelectric damming), which ultimately may present serious toxic risks to local populations, since mercury can bioaccumulate and high concentrations may affect neurological systems (Deprew et al., 2013; Martín-Doimeadios et al.,2014).

The toxicity profile of Hg is related of several factors and the organic methylmercury, originating from the inorganic form, is more damaging due to its high capacity for bioaccumulation. In fact, more than 90 % of the mercury accumulated in fish muscle tissue is commonly in a methylated form (Berzas-Nevado et al., 2010). Brazilian legislation (Portaria nº 685/98) establishes distinct limits for total Hg concentration: for carnivorous fish species (1 mg kg-1) and for non-carnivorous species (0.5 mg kg-1).

Fish fauna have been considered suitable organisms for biomonitoring as they are very sensitive to modifications in the aquatic environment, leading to changes in their biological responses, even at low levels of pollution (Sarkar et al., 2006). Thus genetic, biochemical, morphological and behavioral responses, measured in exposed fish can be useful biomarkers for environmental biomonitoring (Monteiro et al., 2013; Colin et al., 2016).

The use of biomarkers as assessment tools of the effects of environmental pollution it is very effective and provides good results (van der Oost et al., 2003). They have been successfully used as tools for monitoring environmental pollution also as sentinels in early warning systems in ecological risk assessment (Filipak neto et al., 2008). Several biological responses are currently used as biomarkers, ranging from biochemical levels, e.g. assessing antioxidant defense enzymes and lipid peroxidation levels in the cells (as an indicator of oxidative damage), to higher levels of biological organization, assessing the effect of contaminants on morphological structures using histopathology. Both are very powerful tools in identifying adverse effects of compounds on target organs (Lushchak, 2011).

The species *Serrasalmus rhombeus* (Linnaeus, 1766), commonly known as black piranha, occurs in rivers and lakes restricted to South America, from the river Plate basin to Orinoco. This fish is an interesting model to evaluate toxic effects of mercury in the environment due to its high position in the food chain being a voracious predator, its ease to capture and its non-migratory behavior, which makes it an excellent biomonitor/sentinel organism (Barbosa et al., 2003). The objective of this study was to assess mercury effects using biomarkers at biochemical and morphological levels in piranhas from two rivers with different mining histories, Tropas and Crepori, located in the Tapajós region, in Eastern Amazonia, Brazil.

METHODS

Study area and sampling

The study was conducted in two neotropical rivers located in the State of Pará, Tapajós region, Brazil, Tropas and Crepori **(Fig. 1)**. These rivers are situated in a historical mining area, considered the largest gold mining area of the world.

Consequently, tons of mercury have already been discharged in this region (Berzas-Nevado et al., 2010). Tropas (S 6°68'36"/ W 57°42'88") has a recent history of Hg exploitation, and is defined as gradient 1 (G1), whereas the Crepori (S 6°35'389"/ W 46°76'38"), a river with a longer-term history of mining, is defined as gradient 2 (G2). The study was conducted in July 2012, a period of decreasing rainfall and low water levels of the rivers. Three collection points on both rivers were determined: TR-H (upstream region in the Tropas River), TR-M (intermediate region in the Tropas River), TR-L (downstream region in the Tropas River), CR-H (upstream region in the Crepori River), CR-M (intermediate region in the Crepori River), CR-L (downstream region in the Crepori River). 10 specimens of *Serrasalmus rhombeus* were captured by hand-lines at each sampling point. The animals were killed by transecting the spinal cord, subsequently fishes were weighed (g) and sized (cm). After biometry measurements, tissue samples (liver and muscle) were removed. For total mercury concentration (HgT) and biochemical analysis samples were cooled in ice and subsequently stored in an ultrafreezer of -80°C and for the histopathological analysis, samples were fixed in Bouin's solution.

Simultaneously with fish capture, sediment samples (10) were obtained from each point by a Van Veen grab sampler. These samples were directly transferred to an polyethylene tray which was adequately sterilized with HCl and wrapped in identified plastic bags for subsequent analytical procedures. Afterwards, they were oven dried at 60°C, passed through a 9 mm sieve and macerated to obtain a fine sediment extract.

Total Mercury Analysis

Biological and sediment samples were submitted to acid digestion by adding 2 mL HNO_3 and $HClO_4$ (1:1), 5 mL H_2SO_4 and 1 mL H_2O on a hot plate at 230-250°C for 20 minutes. The sample solution was then cooled and diluted to 50 ml with double

distilled water. A blank and standard solution digests using 25, 50 and 100 ll of 1 lg/ml standard Hg solution were subjected to the same treatment. Mercury available in ionic solution was analyzed by using cold vapor atomic absorption spectrophotometry (EAA-VF/G) with Mercury Analyzer HG-3500 (Akagi et al., 1995).

Biomarkers

Activity of the antioxidant and detoxification enzyme, glutathione–S–transferase (GST; EC 2.5.1.13)

Liver tissue samples were weighed and homogenized (1:4 – weight:volume) in a buffer containing Tris-HCl (100 mM, pH 7.75); EDTA (2 mM), and Mg^{2+} (5 mM) (Gallager et al., 1992). Next, they were centrifuged at 10,000 x *g* for 20 min at 4 ^{o}C. Supernatants were stored at -80 ^{o}C to determine glutathione-S-transferase (GST) activity (Habig et al., 1974; Habig and Jakoby,1981). The conjugation of 1mM reduced glutathione (GSH, Sigma) with 1mM of reactant 1-chlorine-2,4-dinitrobenzyne (CDNB, Sigma), a process catalyzed by GST, was assessed. The resulting conjugate has maximum absorbance at 340 mm. Readings were performed using a spectrofluorometer (Victor 2, Perkin Elmer) with microplate reader. Results of enzyme activity were expressed per total protein content, which was quantified by a commercial kit (Biureto, Doles). Enzyme activity was expressed in GST units (UGST)/mg of protein, and one enzyme unit corresponds to the amount of enzymes that conjugate 1 μmol of CDNB per min and per mg of protein present in the homogenate, at 25 ^{o}C and pH 7.

Lipoperoxidation (LPO)

Lipoperoxidation was determined in terms of lipid hydroperoxide content, following the modified FOX (ferrous orange xylenol) method (Hermes-lima et al., 1995; Jiang et al., 1991; Jiang et al., 1992; Monserrat et al., 2007). Frozen samples were

weighed and homogenized (1:9 - weight:volume) in methanol 100% cooled at 5°C. Homogenized samples were centrifuged at 1.000 *x* g per 10 min at 4° C. The supernatant was used for the analysis of lipid hydroperoxides. To perform the assay, the following reactants were sequentially added to the microplate wells: 90 μL $FeSO_4$ (1 mM); 35 μL H_2SO_4 (250 mM); 35 μL of orange xylenol (1 mM); 170 μL H_2O milli-Q, and 20 μL of sample. Samples were incubated at room temperature for 30 min, the period required for the reaction to stabilize. After this period, samples were read in a spectrofluorometer microplate reader (Victor X3, Perkin Elmer) at 550 nm. Then, 10 μL of a cumene hydroperoxide solution (CHP; 0,175 mM, Sigma) was added to each microplate well, thus obtaining a final concentration of 5 nM CHP in the assay. CHP is used as standard lipid hydroperoxide. After 15 min, the time required for the reaction to become stable, absorbance was read again. Results were expressed in terms of lipid hydroperoxide (as reactive substances to FOX) in equivalents of CHP/g of tissue. concentrations were determined by the Bio-Rad protein assay for a 96-well plate reader (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the standard (Bradford, 1976).

Histopathology

Liver portions were fixed in Bouin's solution (20% formaldehyde, 5% acetic acid and 75% picric acid saturated) for 24 h at room temperature (29°), washed in distilled water, dehydrated in a progressive series of ethanol and embedded in paraffin. Sections (5 μm thick) were stained with haematoxylin/eosin (H&E). The slides were prepared in triplicate per sample (each containing five sequential sections) and were mounted with Entellan (Merck). Photomicrographs were taken with a Carl Zeiss optical microscope (Axiostar Plus 1169–151). A semi-quantitative approach was applied based on the histopathological condition indices (Bernet et al., 1999). The estimation of the hepatic

histopathologial condition indices is based on the concepts of an importance factor (IF) for each alteration predetermined and a score value, a numerical attribute that reflects the degree of the dissemination of the alteration: (0) unchanged; (2) mild occurrence; (4) moderate occurrence; and (6) severe occurrence (diffuse lesion). Therefore, each alteration received an index that corresponded to the multiplication of the importance factor by the occurrence value. Were selected 15 sections per animal, being assessed all the animals collected in the field. Then, every specimen receive a histopathological index (IHT) based on the sum of all alterations values.

Indexes were classified: class 1 (index < 10), undisturbed tissue structure with slight modifications; class 2 (index 10-25), undisturbed tissue structure moderately altered; class 3 (index 26-35), altered tissue with noticeable lesions; and class 4 (index >35), altered tissue with severe and irreversible lesions (Zimmerli et al., 2007).

Statistical analysis

Differences of fish length and weight, mercury concentrations in fish and in sediment, and histopathological indexes (IHT) between both rivers were tested through univariate PERMANOVA tests performed at Euclidean distances. A non-restricted ordination using analysis of Principal Coordinate (PCO) was performed to view the differences in multivariate space of samples based on Gower distance matrix, created from histopathological changes. A PERMANOVA test was implemented with "river" as fixed factor (2 levels: TR (Tropas river) and CR (Crepori river) and the combination "sector and river" as fixed factor (six levels: TR-H, TR-M, TR-L, CR-H, CR-M, CR-L). For all significant tests, paired sample comparisons were used *a posteriori* to identify different treatment groups. A total of 9,999 residue permutations in a limited model were used to calculate the p values for PERMANOVA. All analyses were performed using the program PERMANOVA + software PRIMER-E 2008 (Anderson et al., 2008).

Biochemical data on glutathione-*S*-transferase (GST) activity and lipid peroxidation (LPO) were tested via Kolmogorov-Smirnov for normality, and via Levene test for homogeneity. Significant differences were tested via one–way ANOVA followed by TUKEY's test using the statistical package STATISTICA 7.0. The correlation between weight and length and concentration of total mercury in fish and in the sediment were tested by Pearson correlation (ρ).

RESULTS

Biometry and Mercury concentration (HgT)

Weight and length showed a positive correlation, (p = 0,94) and multivariate PERMANOVA tests didn't show significant differences between the weights of fish from both rivers (p = 0,115). However, regarding total length, fish from G1 had a trend to have higher values than fishes from G2 (p = 0,052). In addition, there was also a strong correlation between values of total mercury (HgT) and weight (p = 0,80) on both rivers. PERMANOVA test for the analysis of mercury concentration in the muscle and in the sediment indicated a significant difference between rivers. The Crepori River (G2) showed significantly higher values of HgT in the sediment (370 µg/kg $^{-1}$) and fish with higher concentrations of mercury in the muscle (1.128 µg/kg $^{-1}$) compared to the values from the Tropas River. However, most animals fell within acceptable standards of mercury concentration for carnivore fish (1000 µg/kg $^{-1}$, ANVISA, 2003) **(Table 1)**.

Biomarkers

Figure 2A shows the response of GST in piranhas from Tropas and Crepori rivers. The results indicate that each river exerts a different influence on the biochemical responses of the fishes. G1 presented animals with higher liver GST activity (248 ± 31

U GST/mg protein) than in livers of piranhas from G2 (158 ± 19 U GST/mg protein); $p<0,05$. On the other hand, LPO content in the liver of piranhas was inversely proportional to GST activity, as its values were much higher in individuals from G2 (52 ± 6 nM CHP/g of tissue) than in individuals from G1 (22 ± 2 nM CHP/g of tissue); $p<0,05$ (**Fig 2B**).

Most (90%) liver tissues of fish from the Tropas river (G1) exhibited normal hepatic architecture consistent for juveniles, showing regular hepatocytes without anomalies. However, other some evaluated tissues (9%) presented an altered appearance, with light brown color and some dark plates, whilst, most animals in G2 (82%) fitted this description. Although fish from both areas showed changes in their tissues, the semi-quantitative analysis of tissue changes allowed to clearly identify differences in frequencies of tissue changes in different areas. Fish from G1 showed normal liver parenchyma, comprised by the middle lobe vein surrounded by sinusoid capillaries, which were organized in double lines of *anastomized* hepatocyte *cords*. Hepatocytes were polygonal shaped with spherical and centralized nuclei and the presence of pancreatic exocrine acini within the hepatic parenchyma (**Fig. 3)**. Piranhas captured in G2 showed a high frequency of histological changes at several stages. Disorganization of hepatocyte cords, cell hypertrophy, leukocyte infiltration, which generated circulatory disorders (vascular congestion and hemorrhage), increased lipid droplets (steatosis), cellular vacuolization, and necrosis (**table 2**). In addition, the bile duct had structural and cell alterations, characterized by the increase in connective tissue and nuclear dilation (**Fig 4b**).

Histopathological indexes (IHT) in the individuals pointed out a significant difference between fish captured in G1 and G2. Fish from G2 showed, on average, 81 ± 21 of IHT; on the other hand, animals from G1 had, on average, IHT of 14 ± 13. The

data analysis of multivariate PCO also pointed out segregation between the fish collected in both sampling rivers (G1 and G2). Most fish from G1 were healthy with no hepatic tissue alterations, while piranhas from G2 showed several severe and irreversible alterations. A significant difference between the river sectors was observed regarding histopathological changes, following an order of frequency for fish tissue damages, as follows: (G1) TR-H (a) < TR-M (b) < TR-L (c) and (G2) CR-H (a) < CR-M (b) < CR-L (c) **(Fig 5)**.

DISCUSSION

Mercury occurs naturally in the Amazonian environment, however, the exact origin of this metalic element in Amazon soils and rivers is still uncertain (Malm, 1998). The association between human activities and changes in physical–chemical parameters of water may modify the degree of methylation/ demethylation of Hg and consequently its accumulation in fish tissues. It is important to highlight that in Amazon ecosystem the patterns of fish exposure to Hg, trophic level, feeding habits, age/size and fish-Hg concentration may be easily disrupted since regular annual flooding can modify the aquatic environment (Vieira et al., 2011). Then it is of the utmost importance in this region that studies evaluate the effects of mercury in fish in these adverse characteristics, once Hg contamination of aquatic systems is a concern for humans. The Tapajós River basin, one of the main tributaries of the Amazon River, has been one of the largest gold-mining regions in Amazonia for several decades (Martin-Doimeadios et al., 2014). Therefore, analyses of aquatic organisms from this region offer a unique model to study the effects of this type of exposure.

The present study revealed a significant difference among selected rivers. The samples of sediment and fishes from Crepori River (G2) showed high levels of mercury

concentration compared to G1. This difference may be related to the historical pollution of these rivers. Crepori River (G2) was one of the first river explored in the mining activities in 1959 "golden age" (Bidone et al., 1997). Moreover, according to studies conducted over the past decade in the Tapajos region in order to determine the concentration of mercury in fish piscivorous and non - piscivorous, the level of Hg has increased over this period. This problematic may be caused by the activities performed on this region but also because of mercury background of this area (Dórea et al., 2006, Sakar et al., 2006). Evaluating the data of the total metal concentration in organisms is rather valid, however, the effects on different biological levels shows a larger setting of the local situation, because is possible correlate the conditions of the environment and fish health status, therefore this analysis acts as early ecological warning.

In this study, the fishes collected in G2 show high levels of HgT concentration in tissues, but were acceptable for human consumption for a carnivore species (1mg/Kg), according to the World Health Organization. After concluded the biomarker data were also identified difference between the studied areas. Fishes from Crepori river were much damaged than those captured in Tropas river G1. The Results suggest that G2 has a larger mercury reservoir than G1, thus the health of fish has been harmed mainly in Crepori river (G2). In addition, the injuries in piranhas tissues might be related to a recent exposure to Hg in the contaminated sediment, which has accumulated mercury over the years due to gold-mining activities, once sediment works as a mercury reservoir and biological processes caused by bacteria present in the sediment trigger recycling processes of this material, making it bioavailable again (Porcela, 1994).

During an intoxication process, the liver is the organ responsible for the detoxification and biotransformation of chemical compounds. Thus, the liver is a target organ of metal contamination and that is why it responds effectively to environmental

changes (Brusle and Anandon, 1996). Mercury generate reactive oxygen species (ROS), which lead to a higher activity of biotransformation and antioxidant enzymes (GST), thereby keeping ROS levels within physiological limits. This disequilibrium between antioxidants and prooxidants might generate an oxidative stress situation causing damage in several biomolecules, induced by the excessive prooxidants (Amado et al., 2009). Glutathione-S-transferase (GST) is a phase II metabolic detoxification enzyme, and it is responsible for the reduction of toxicity of compounds, making it more hydrophilic through the reaction with reduced glutathione (GSH). Hence, xenobiotic elimination systems eliminate these conjugates to the extracellular medium (Limon-Pacheco and Gonsebatt, 2009). GST is also considered an antioxidant enzyme because it enables the elimination of lipid hydroperoxides (and other oxidation products) from the cellular medium, cutting off the chain reaction of lipoperoxidation. Thus, this enzyme is frequently used as a pollution biomarker (van der Oost et al., 2003).

In this study, GST activity was higher in the livers of fish from G1, while lipid peroxidation was higher in the livers of individuals from G2. This situation suggests that the Hg levels and exposure time that piranhas from G1 were submitted to lead to GST activation, enabling the detoxification processes, which ensures the absence of oxidative damage in lipids. Thus, although animals captured in G1 triggered defensive responses trying to avoid damage at initial stages, they could not completely counteract the negative effects of HgT exposure since they also exhibited some tissue changes, although of lower impact. On the other hand, as they are exposed to higher levels of Hg, piranhas from G2 already show inactivation of GST, which contributes to higher levels of peroxidated lipids as well as to greater tissue damage, as shown in table 2 and in the multivariate analysis.

GST is directly involved in the detoxification and excretion of toxic metals and

its activity might be inhibited due to increased exposure (Srikanth et al, 2013), situation observed in the present study. This biochemical reaction pattern was also observed in a study performed in order to assess the effect of acute exposure to inorganic mercury in *Pomatoschistus micros* using biochemical biomarkers (Vieira et al., 2009). Fish exposed to high concentrations showed negative correlation between GST enzyme and LPO, possibly caused by the decreased detoxification capacity to face chemical and oxidative stress. However, Mela et al., 2014 identified high LPO rates, GST activity, and tissue changes only in liver of trairas, *Hoplias malabaricus*, which accumulated levels of HgT at or above 1mg/kg. In the present study, however, these biochemical and tissue changes in piranhas already appeared at lower concentrations of HgT.

It is important to accentuate that environmental contamination is complex, since several toxic agents occur in mixtures in the environment (Norwood et al., 2003). Nevertheless, studies on natural populations are important because they reflect actual interactions between local contaminants and their effects on organisms. Therefore, the biochemical damages observed it is related not only to the presence of mercury in the environment, but also to its association to other factors, such as other metals and/or changes in physical and chemical properties of the water. According Souza et al., 2013 there is a strong interaction between mercury and arsenic and this was responsible for several biochemical and tissue changes in *Centropomus parallelus* collected in a Neotropical estuary.

Congestion of sinusoids and hypertrophy of hepatocytes have been suggested as an initial stage of damages to the liver and, as such, they provide an excellent example of histological biomarkers of exposure to contaminants (van Dyk et al., 2012). In this study, fish from both rivers showed these alterations; however, only fish from G2 showed a high prevalence. Other types of non-specific lesions recorded in the liver of

fish captured in G2 were nuclear alterations, which had been previously associated to necrosis, a more severe alteration (Liebel et al., 2013). Fish from G2 also showed high incidences of melano-macrophage centers (MMC). Melano-macrophage centers are different clusters of cells that contain pigments, which might be present in considerable amounts under certain conditions, such as anemia (Reddy, 2012).

Multivariate data on histological changes in fish enabled us to confirm a significant difference between the several sectors of the rivers (G1) and (G2). Such results indicate gradients of exposure to Hg along the river, with downstream sectors showing a much stronger influence on the fish than upstream sectors. Our results corroborate the results observed in *Hoplias aimara* (Outuberbet al., 2012), who noticed this pollution gradient pattern in aquatic environments in Suriname. According to these authors, there is an atmospheric gradient deposition of this easily volatilized material.

The results of the present study indicate that biochemical and morphological changes observed in liver tissue of piranhas collected in Amazonian rivers were determining factors in the structural alteration of this organ and, possibly, of its functioning. Therefore, although the concentration of mercury in the muscle tissue is below the limit for human consumption set forth by the World Health Organization, these results represent a potential risk for the aquatic community in the long term, since species on the top of the food chain, such as piranhas, show alterations in their liver structure; which challenges their chances of survival under the action of sub-lethal effects of environmental contaminants.

CONCLUSION

The use of biomarkers in the liver tissue of *Serrasalmus rhombeus* was effective in assessing damages at biochemical and cellular levels in fish collected in the Tapajós

region. In addition, this top-predator species responded accurately to different environmental conditions, mainly by showing differences between sampling points, which makes it an excellent sentinel species. This study also suggests that animals defense mechanisms are managing to adapt to the conditions on site, at least in the Tropas river (G1; region with a shorter history of contamination) while fish in the Crepori (G2; region with a long-term mining history) might be under greater threat. This type of approach might be used to evaluate effects before they occur at a higher biological organization level (such as populations, communities, and ecosystems), which is more ecologically relevant and where damages, when detected, are already more difficult to remedy. Regarding mercury contamination, and aiming at human consumption, analyses showed acceptable levels. However, periodical monitoring mus be conducted so that the action of contaminants is observed over time.

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TABLES

Table 1. Fish biometry and total mercury concentration (HgT) in the sediments collected and in muscle tissue (wet weight of fish captured in the Tropas (G1) and Crepori (G2) Rivers). (*) Indicates significant differences ($p<0.05$) of G2 compared to G1.

Area/ River	Weight (g)	Length (cm)	HgT muscle (µg/kg)	HgT sediment (µg/kg)
G1 (n=30)	488 ± 138.4*	27.5 ± 5	0.252 ± 0.10*	0.60 ± 0.25*
G2 (n=30)	274.2 ± 172.1	21 ± 5.6	0.542 ± 0.23	0.280 ± 0.80

Table 2. Histopathological changes in the livers of *S. rhombeus* collected in the Tropas (G1) and Crepori (G2) Rivers, Tapajós region – Pará – Brazil.

Tissues alterations	State	Tropas (G1)	Crepori (G2)
Hepatic disorganization	I	+	+++
Melanomacrophages	II	++	+++
Hyperaemia	I	+	+++
Absence of nuclei	II	O	++
Cytoplasmic vacuolization	I	O+	++
Cytoplasmic degeneration	III	O+	+
Hypertrophy	II	O	++
Necrosis	III	O	++
Steatosis	I	+	+++
Fatty degeneration	II	O	+
Leukocyte infiltration	I	+	+++

Score value: O = none, O^{+} = very discreet, + = mild, ++ = moderate, +++ = severe

FIGURES

Figure 1 Map of the study area located in the Tapajos region. Sampling points at the Tropas (G1) and the Crepori (G2) river are indicated by black diamonds.

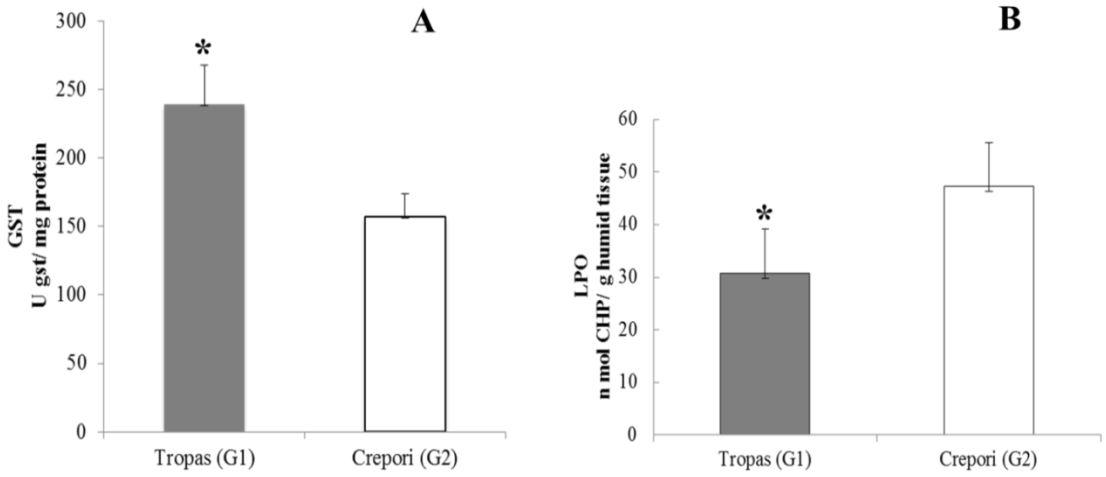

Figure 2 Oxidative stress biomarkers in liver of *Serrasalmus rhombeus* from Tropas (G1) and the Crepori (G2). **A**- glutathione-S-transferase (GST) activity, **B** – Lipid peroxidation (LPO). Values are expressed in averages ± standard deviation. (*) Indicates values with significant difference.

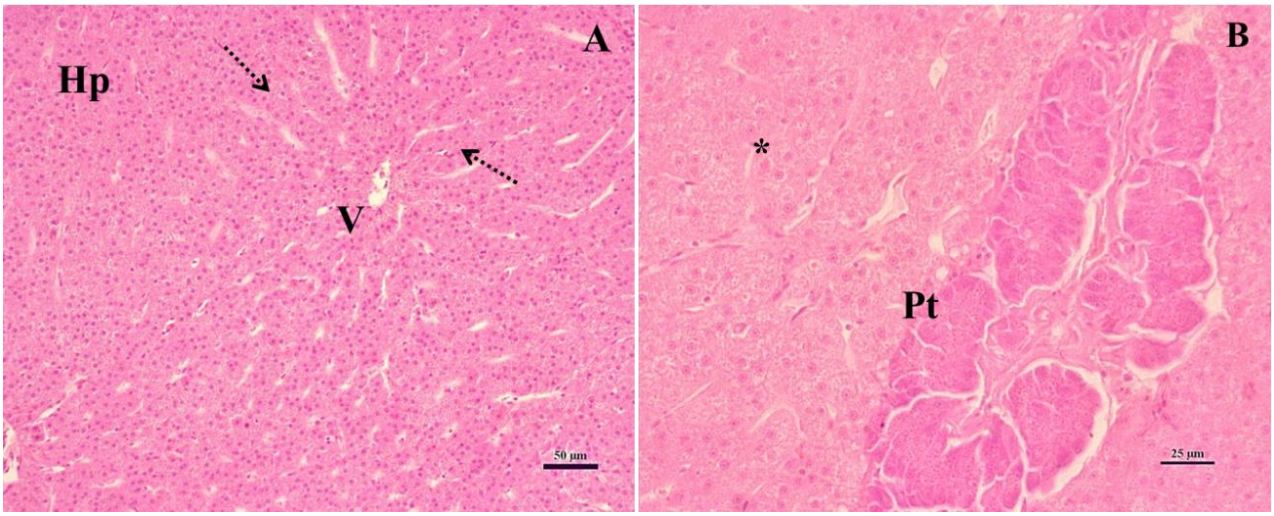

Figure 3. Liver tissue of *Serrasalmus rhombeus* collected in G1 and stained with HE. **A -** Normal structural architecture of the hepatic parenchyma (Hp), central vein (V), sinusoid capillaries organized between double lines of hepatocyte *cords* (dotted arrows)**. B –** exocrine pancreatic tissue and polygonal Hepatocytes (*).

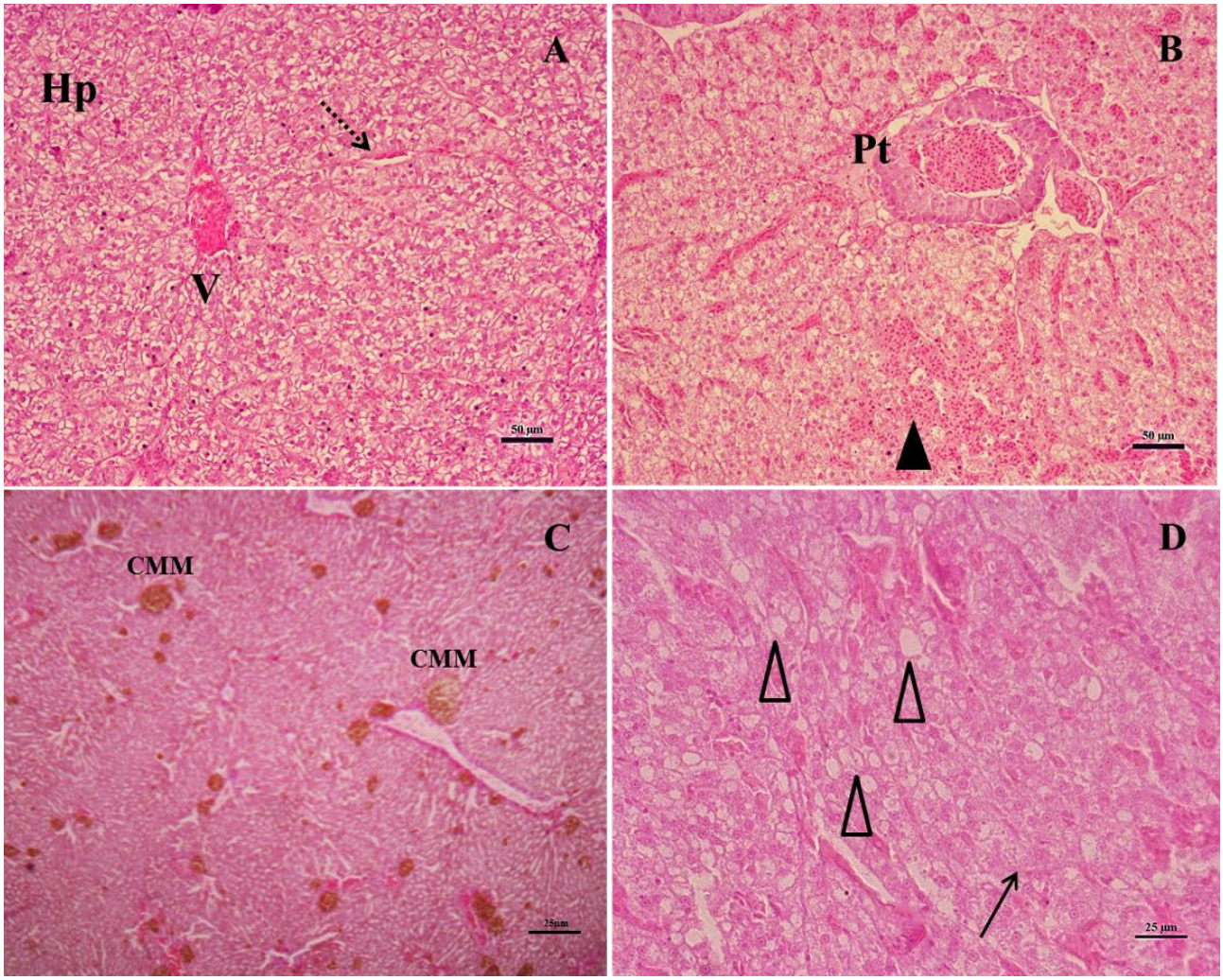

Figure 4. Liver tissue of *Serrasalmus rhombeus* collected in G2 and stained with HE. **A –** Altered structural architecture of hepatic parenchyma (Hp) disorganized, congested central vein and disorganized sinusoid capillaries between the hepatocyte *cords* (dotted arrow)**. B –** Exocrine pancreatic tissue (Pt) with leukocyte infiltrates (full-headed arrow). **C –** Tissue with strong presence of melano-macrophage centers (MMC). **D** – Vacuolization of hepatocytes (hollow-headed arrow) and necrotic extensions of the tissue (thin arrow).

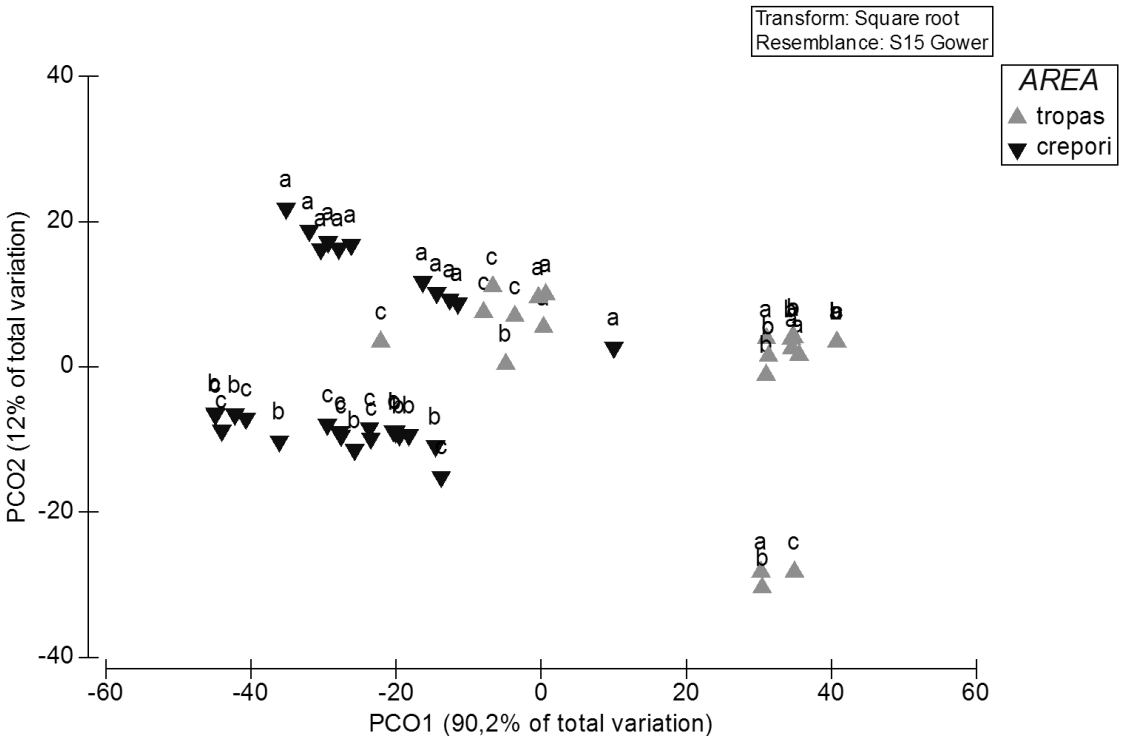

Figure 5. PCO analysis with Gower distance coefficient, resulting from a histopathological abundance matrix for fish from the Tropas (G1) and Crepori (G2) Rivers in the Tapajós region – Pará – Brasil. **a** (upstream region of the river), **b** (intermediate region of the river) and **c** (downstream region in the Tropas River).

CAPÍTULO 2

Está sob a norma da Revista: Journal applied of Ichthyology

Biomarkers in piranhas for assessing toxicological effects of heavy metal pollution in amazon environmental

SUMMARY:

The exploitation of metals in the Amazon is quite old and has generated income for many countries. From the 60's this activity has intensified and continues until nowadays and in some regions this activity is carried out illegally. For decades the Amazon region has been receiving waste loads contaminating several areas even pristine. Thus, this study aimed to, using biomarkers of exposure and effect in carnivorous fish, piranhas, abundant in the Amazon. In order to know if there is difference between a pristine area and one completely influenced by mining activity, from a biological point of view were performed a relation between the quality of the environmental compartments (water and sediment) with the biological responses of the animals exposed to these areas. Also, verify the most significant role in the segregation of environmental factors, if there are differences.

Introduction

Mining activities are one of the major sources of metals released into the environment. These activities can contaminate the surrounding environment and are often quite detrimental to the human population and ecosystems located in the proximity of the mining site (Cassela et al., 2007). This activity causes damages from the beginning of the process and even after the mining operations end, because large mining tailing ponds remain in the area due to the waste accumulated in pyramidal structures, causing environmental impacts (Krishna et al., 2013). In the Southeastern region of the Amazon, Brazil, the Carajás Mineral Province (CMP) has many oxide–copper–gold deposits, such as Igarapé Bahia, Salobo, Sossego and Cristalino. These deposits have become prime targets for mineral exploration worldwide because they commonly show high-grade and high-tonnage ore (Moreto et al., 2014). Despite environmental and social concerns about this economic activity, the mining extraction frontier continues to expand (Bebbington et al., 2008).

Environmental contamination is a global concern, moreover, the methods used to evaluate the ecosystems are often insufficient or lead to uncertainty as pollution is usually composed of a complex mixture of agents (Beyer et al., 2014). Chemical analyse alone are not sufficient to properly assess the adverse effects of the complex mixture of water contaminants, therefore, a biomarker-based biomonitoring is a promising approach to provide early-warning signs of exposure (van der Oost et al., 2003; Martinez-haro et al., 2014; Bae and Park, 2014). The biomarkers that show the effects caused by the action of pollutants are determined and evaluated in order to have an insight of the environmental situation and aquatic organisms, such as fish, and have been often used in this study over the years (Colin et al., 2016). Histological-ultrastructural analysis, and biochemical changes in different fish tissues, such as gill, is

widely used in biomonitoring and aquatic toxicology (Montes et al., 2015; Tabassum et al., 2016). Thus, the morphological and physiological changes in the gills of fish can provide an assessment method to evaluate how environmental stressors can affect fish populations (van der Oost et al., 2003). Alterations in gill epithelium are a consequence of a range of contaminant exposures, with the severity of changes depending on the pollutant concentration and exposure period (Mallat, 1985; Alazemi et al., 1996; Evans et al., 2005). Many studies also showed that metals can produce free radicals and reactive oxygen species, which result in oxidative stress. However, organisms have antioxidant defense systems to protect them from oxidative stress. Since the discovery of the important role of free radical damage in the mechanisms of toxicity of many environmental pollutants (xenobiotics), the application of biomarkers of oxidative stress in aquatic organisms has increased (Srikanth et al., 201. In this study the objective is to evaluate if the ultrastructural, histopathological and biochemical responses of gills, as biomarkers of exposure, are able to differentiate different areas in the same river, however at different points that undergo different influences. This was achieved using gills from native species, the black piranha (*Serrasalmus rhombeus*), collected in two different points in the Itacaiunas River in the Amazon region. Histological, ultrastructural changes and oxidative stress responses of gills (CAT, GST activity and lipid peroxidation) were determined.

Material and Methods

Sampling

The study points were located upstream and downstream of the Itacaiunas River located in the Carajás Mosaic, Pará, Brazil. This region has the largest deposit of Fe-Cu-Au ore and other minerals exploited by several mining activities, however, most of this

mosaic is protect by Brazilian Federal Laws. The reference point was specified because it is located in the protected part and upstream of the Itaciunas River, defined as **POINT 1** - P1 (5°59'68 " - 50°43'36 "). **POINT 2** - P2 (5°51'40 " - 50°27'25 "), downstream of the river and affected by several gold, manganese and copper exploration mines.

Sediment, surface water and fish (*Serrasalmus rhombeus*) samples were collected simultaneously in the selected points in two different seasons, the dry season (October 2011) and rainy season (April 2012). In addition, the water parameters such as: pH, temperature (°C), Electrical conductivity (μS.cm-1), total dissolved solids (ppm) and dissolved oxygen (ppm) were measured using a previously calibrated HI 9828 HANNA® multiparameter probe. A total of 80 fishes were caught in the Itacaiunas River using hook and line. The animals were sacrificed by a spinal puncture, and the biometric measures were taken: length (cm) and weight (g). Next, tissue samples (muscle, liver and gill) were removed and stored according to the analysis (Bouin Solution and Liquid nitrogen).

Water and bottom sediment collection

10 replicates of bottom sediment were collected using a Van-Veen stainless steel drag in areas of deposition and low water flow. After collection, the sediment samples were then transferred to a polyethylene container previously sterilized with HCl, placed in labeled plastic bags and placed in coolers. The samples were then dried at 60 °C, sieved using a 9mnm sieve and then macerated to obtain a fine sediment extract and kept until the early analytical procedures. Simultaneously, 15 replicates of surface water samples were collected at both sampling points and stored in 500 ml polyethylene bottles, previously sterilized with HCl, and then cooled on ice and stored at -20 ° C until the analytical procedures.

Trace metals analysis (Zn, Cd, Mn, Cr, Cu)

The trace metals in the biotic (fish) and abiotic (water and sediment) materials were determined according to Krug, 2008. The Samples were weighed on Teflon tubes (0.2 g), and added to each tube: 3 ml of HNO_3, 1 ml of HCl and H_2O_2. The acid digestion was followed by the microwaves Mars, CEM procedure. Next, the contents were transferred to falcon tubes and milli-Q water was added to a final volume of 25 ml. The analyses were performed on an Optical Emission Spectrometer with Induced Plasma (ICP-OES). The water samples were filtered under vacuum using cellulose acetate filter of 0.45 uM pore size and 22 mm diameter. Next, the samples were acidified and analyzed by ICP-OES. The potential to cause adverse biological effects was assessed through the estimation of sediment quality guideline quotients (SQG-Qs) (Long and MacDonald, 1998). These quotients were calculated as the ratio between the concentration of individual chemicals and their respective PEL value. A score was then obtained according to their toxicological risk: SQG-Q < 0.1 as non-impacted sediments; 0.1-1 as moderately impacted and >1 as strongly impacted.

Biomarker measurements

Histopathology

The gill tissues were fixed in Bouin's Solution (25 % Picric acid, 75% Paraformaldehyde, 1 % Acetic acid). After regular histology procedures, the samples were embedded and included in paraffin blocks to obtain 5 mm thick cuts using a rotary microtome (Leica RM 2245). The sections were stained with hematoxylin and eosin (HE) and examined under a light microscope (Nikon Eclipse ci). One gill arch was randomly chosen and analyzed. The prevalence of each type of change was determined according to Pereira et al., 2013. Ten entire filaments per arch were chosen randomly and analyzed. A severity gradation scale (SGS) with six degrees (0–5) was applied

qualitatively, considering the extent and severity of each lesion. The extent was defined as the percentage of filaments with a type of lesion in each fish sampled. To quantify the severity of each histological change, the different levels of severity were attributed. The severity of each lesion per average of affected filament was determined as the number of lamellar and interlamellar spaces affected by a given level of severity, divided by the number of filaments showing that type of histological change. A zero degree was given to values found in fish with less histopathology, and to define the remaining degrees, the extent and severity levels were combined to show an increasing numbers of lamellae and interlamellar spaces per injured filament. All values obtained, from the counting of extent and severity, were then divided into numerical intervals and combined to generate the SGS.

Transmission Electron Microscopy (TEM)

The samples were fixed in Karnovsky (4% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4). After fixation, the tissues were washed in 0.1M sodium cacodylate buffer pH 7.4 and post-fixed in 2% osmium tetroxide. They were then dehydrated in series of acetone, embedded in Epon 812 and included in blocks. Next, they were cut in ultramicrotome and thin sections were stained with 1% toluidine blue to define the area to be used for ultrathin sections. The ultrathin sections were contrasted with uranyl acetate and lead citrate, analyzed and photographed in a transmission electron microscope (LEO 906 E).

Estimation of catalase (CAT) (EC 1.11.1.6):

The catalase (CAT) activities were determined according to the method described by Beutler (1975), measuring by spectrophotometer enzymatic decomposition rate of H_2O_2 at 240 nm. The absorbance decrease by the enzyme activity was expressed

in CAT unit / mg of protein, where one unit is the amount of enzyme required to hydrolyze 1μm H_2O_2 / min / mg protein at 30 ° C and pH 8.

Estimation of Glutathione – *S* – Transferase (GST) (EC2.5.1.18):

The tissue samples were weighed, homogenized in GCL buffer and then centrifuged to extract the S9 fraction. The dosage was based on the methodologies proposed by Habig et al. (1974) and Habig and Jakoby (1981). It evaluates the combination of 1 mM GSH (Sigma) in 1mM reagent 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) by GST-catalyzed process. The formed complex conjugate has a maximum absorbance at 340 nm. The reaction medium used is 0.1M phosphate buffer, pH 7.00. Readings were performed in a spectrofluorometer (Victor2, Perkin Elmer) using a microplate reader.

Lipid peroxidation (LPO)

Tissue samples were homogenized in methanol, centrifuged to extract the S9 fraction. The LPO (lipid hydroperoxide) activity was determined by the Fox method (FerrousOxidation-Xylenol Orange) as described by Jianget al. (1992). The FOX method is based on the oxidation of Fe 2+ (ferrous ammonium sulfate) to Fe 3+ by hydroperoxide in an acid medium in the presence of a pigment conjugate Fe (III), xylenol orange. 20 uL of the sample was incubated for 30 minutes at room temperature with 270μL reactive mixture containing 1 mL xylenol orange, FeSO4 250μM, 25mM and 4mM H2SO4 butilatohidroxitolueno 90% methanol. Readings were taken in a multi microplate reader shape VITOR x3. The results were expressed in mmolCHP / g wet tissue. The protein concentration was determined by measuring the amount of protein in the samples based on the Bradford method (1976) using bovine serum albumin as the standard.

Statistical analysis

All data are reported as mean ± standard error. Kolmogorov-Smirnov was applied to evaluate normality, while Levene's test was used to test the homogeneity of variance. The one-way analysis of variance (ANOVA) was used for comparing the statistical data among the sampling points, followed by multiple comparison test (Tukey). While the Kruskal–Wallis test was applied for the data with no normal distribution or variance heterogeneity. The statistical packages SigmaStat 3.5 were used for statistical analysis. In addition, principal component analysis (PCA) was performed in order to get a comprehensive view of biomarkers responses between seasons and points.

Results

Water quality Parameters

The data of the water quality indices was not significantly different between points and seasons. The interval values of temperature (29 - 31), pH (4 - 6), electrical conductivity (32 - 77), total dissolved solids (2 - 5) and dissolved oxygen (6 – 7) were according to the Brazilian regulations of CONAMA 357/2005.

Biometric parameters

Table 1 shows the values corresponding to the weight and length of the piranhas. There were no significant differences in the biometric values of the fishes captured, however, the condition factor calculations (K) were significantly higher in fishes from P2 - rainy season (Fig 1).

Trace metals in water and sediment samples

Tables 2 and 3 show the data corresponding to the total metal concentration and toxicological significance of sediments and water samples. With regard to the metal trace values analyzed in the surface waters of the Itacaiúnas River, it was verified that

Cd and Cu in P2 in both seasons were significantly higher compared to P1 and also exceeded the determined limit. The sediment analysis, P2 - Dry season presented samples with high levels of Zn, Mn and Cu. It is important to highlight that copper sediment were high in P2 in the dry and rainy season and also in P1 – in the rainy season. In fact, SQG-Qs determined in sediments collected along the Itacaiunas River indicated that most of the metals were classified as moderately impacted, while Cu was classified as strongly impacted, indicating adverse biological effects.

Trace metals in muscle tissue

The analysis reported that fishes sampled in P2 (dry and rainy season) and P1 (rainy season) presented high levels of cadmium (Cd) in the tissues. Moreover, the copper (Cu) values are higher in piranhas in P2 – Dry season. However the others values are in accordance with the normal patterns as stated in the Brazilian laws and regulations for human consumption (Table 4).

Gill morphology

The architecture of the gills of fish collected in P1 showed primary filaments and secondary lamellae, slender appearance and well defined cell types, where: pillars, epithelial cells without stratification and mucus cells (Fig. 2A and B). The fish gills collected in P2 in the Itacaiunas River showed various types of damages (moderate and severe) in the gill tissues such as: aneurism and lifting of the epithelial layer, causing swelling of the lamellas and reducing the superficial respiration area. The lamellae fused together and necrosed with mucoid depositions along the surface. (Fig. 2C and D). Transmission electron microscopy confirmed the results obtained by light microscopy. The ultra-thin section of the control fish gills exhibited normal pavement cells varied in form from squamous to polygonal with elongated nucleus, electro-dense cytoplasmic matrix with well-developed Golgi apparatus, rough endoplasmatic

reticulum, ribosomes, and a small number of mitochondrias in comparison with the chloride cells. While the piranhas in P2 showed a completely different lamellar structure, with thicker lamella and irregular shape, the tissue in the filament region showed cell proliferation, as shown in (Figure 3) where the intense proliferation of mitochondria in mucus cell can characterize tissue damage.

Biochemical analysis

The results obtained in the analysis of enzymatic activities (CAT and GST) and lipid peroxidation were also determinant for the segregation of fishes studied. The catalase activity evaluated in piranhas in P2 (rainy season) was lower when compared to the control (P1) and the other points, which results in compliance with lipid peroxidation (LPO) also higher in the gill tissue of piranhas in P2 in the dry and rainy season. However, the GST activity was not significantly different between points, which showed an average around (156.4 ± 36.9) (figure 4).

Multivariate analysis

To examine the discriminating power of the set of biomarkers studied, we carried out PCA. The data matrix was constructed based on 10 biomarkers (8 – histological and 2 – biochemical) as depended variables and 40 sampled individuals as grouping variables. The result showed that two main groups were formed with 73.8% of total accumulated variance (figure 5).

Discussion

Industrial-scale mining is an activity that enhances the economy and development of countries, however it also impacts the environment. The mining activity and its waste disposal have been long considered as major sources of environmental degradation due to metal contamination (Wittman, 2012; Su et al., 2014). In the present

study, we assessed the concentrations of heavy metals and its impact on histological, ultrastructural and biochemical changes in *S. rhombeus* inhabiting an Amazonian river, at different sampling points along the Itacaiunas River (Upstream and Downstream), in an area next to the mining operations. Heavy metal contaminations can have devastating effects on the ecological balance of the environment and diversity of aquatic organisms.

They can decline water and sediments quality and can adversely affect fish health and other biological attributes like taxonomic richness, trophic structure, and health of individual organisms (Boening, 2000). In fact, a full understanding of heavy metal kinetics in fish is important for natural resource management and for human consumption of fish. The presence of such elements in the environmental can induce changes in gill functions and structural and biochemical alterations have been suggested as useful biomarkers of environmental contamination (Costa et al., 2009; Souza et al., 2013; Sabullah et al., 2015).

Fish gills have a basic structural design with a single epithelium surrounded by different cell types but with a complex mechanism to perform important functions such as gas exchange, ammonia excretion, maintenance of the blood acid–base balance and ionic regulation (Evans, et al., 2005). In the present study, the morphological analysis of gills evidenced changes in fishes from the different sampling points, especially in piranhas in P2. The changes observed in the animals from this area were either filament or lamellar epithelium proliferation, which in many cases resulted in a complete lamellar fusion. This can reflect the high concentration of metals (Cd and Cu) determined in the water and sediments collected along the Itacaiunas River. Most of these histological changes are interpreted as a non-specific response to stress and are described in fish exposed to a wide spectrum of pollutants, such as heavy metals and organic contamination. However, Alterations in the surface ultrastructure can reflect the

health and physiological state of the fish. On the other hand, several studies that assessed the effects of specific metals in the structure of the gills have been performed. Monteiro et al., 2009 and Brunelli et al., 2011 evaluated the effect of different concentrations of Cu and Cd in *Oreochromis niloticus* and *Thalassoma pavo* respectively. They observed several types of damages caused by the exposure to metal. These results corroborate with the histopathology analysis reported in piranhas in this study. The proliferation of mucus cells and detachment of the respiratory epithelium in gills were largely detected in both studies. This result can be interpreted as an initial adaptation of certain pollutants, once mucous cells can be efficient in seizing the toxic agents and thereby assist in preventing the entrance of these agents into the gills, and the detachment can reduce the entrance of toxicants into the circulatory system (Perry and Laurent, 2012). In this case, the organism can reduce certain permanent damage, such as bioaccumulation. This situation was observed in the fishes in P2, as they exhibited high cadmium and copper concentrations in the tissues analyzed and severe damages in the gill tissues, but no mortality was reported.

The study of the prevalence of lesions demonstrated that most fishes had some degree of gill injury even in the preserved area. This information highlights the fact that in natural environments complex mixtures affect the organisms and many factors can interfere in the health status of the fishes. However, the multivariate data obtained allowed establishing correlations between biomarker responses and trace metal analysis and also the ecological status of each sampling point, demonstrating the existence of a pollution gradient. This approach can be sufficient to define an overall picture of the environmental conditions. This also enabled to relate the histology data with the ultrastructure analysis. The ultrastructural features of the chloride cells in freshwater fish have a great abundance of mitochondria in their cytoplasm. The profile of these

mitochondria varied from ovoid to elongated and sinuous, with electro-dense matrix, a result also observed by Samanta et al., 2015 and Paruruckumani et al., 2015. In contaminated places these cells proliferate and the amount of mitochondria increases considerably, and in this study this situation was reported for the fish collected in P2. The results are in accordance with the water and sediment analysis, because of the high metal concentrations verified at this sampling point.

Copper is an essential element involved in many biological activities, namely Cu/Zn superoxide dismutase, cytochrome c oxidase or transcription factors, but it becomes toxic when in excess in the cells, unlike cadmium, which is a non essential element and very toxicant for the organism (Saad et al., 2014). The metal contamination can increase the intracellular formation of ROS (Rigoli and Giuliani, 2014). Since induction of antioxidants represents a cellular defense mechanism to counteract toxicity of ROS, they have been extensively used in several field studies to assess the extent of pollution in rivers, lakes and coastal waters (Zanette et al., 2015; Benedetti et al., 2015; Franco et al., 2016). This study verified the CAT and GST activity and lipid peroxidation, the results also show differences between fish from different areas. Only the fishes in P2 showed modifications in the CAT activity and higher lipid peroxidation in the gill tissue. Lipid peroxidation is a free radical-induced oxidative degeneration of lipids, therefore an increase in the levels of LPO in the tissues could be attributed to the accumulation of heavy metals. Similar results were also verified by Nunes et al., 2015, which reported that high levels of cadmium and copper decrease the catalase activity and increases the levels of LPO.

Conclusions

This study was successful, because we verified in a natural environment the quality of health status of the fishes using efficient tools. Therefore, we confirm that there is a pollution gradient in the Itacaiunas River due to different influences and the health status of fish clearly reflects these different influences, furthermore, the species chosen in this study was considered an excellent bioindicator.

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Tables

Table 1. Mean±Standard of the biometric values (Weight and length) from *Serrasalmus rhombeus* captured in different points (P1 and P2) along Itacaiúnas River.

Season	Location	Weight (g)	Total length (cm)
Dry	P1	503.8± 298.09	28.056± 5.53
	P2	432.7±213.46	25.271± 4.32
Rainy	P1	385±311.84	23.55± 6.33
	P2	582.5±399.17	26.771± 5.95

Table 2. Mean±Standard of heavy metals from Itacaiúnas River water (mg/L^{-1}) sampled in points 1 and 2 (dry and rainy season). (*) denote statistically significant differences among points and (°) denote statistically significant differences among seasons.

Season	Location	Zn	Cd	Mn	Cr	Cu
Dry	P1	0.78±0.004	0.0012±0.0005°	0.04±0.007	0.002±0.004	1.5±0.67
	P2	0.12±0.009	0.0044±0.0001**	0.0102±0.0003	0.0014±0.0031	2.6±0.97*
Rainy	P1	0.014±0.0002	0.0009±0.0002	0.012±0.0044	0.005±0.02°	0.3±1,1
	P2	0.9±0.006	0.0026±0.003*	0.014±0.005	0.0027±0.007*	2,1±0,88*
WHO (guidelines mg L^{-1})		3	0.001	0.5	0.05	2

Table 3. The concentration of heavy metals in sediment from Itacaiúnas River (ppm) Median±standard deviations of metal (mg/Kg^{-1}) and toxicological significance (SQG-Qs) of sediments collected P1 and P2 along the Itacaiuna river. (*) denote statistically significant differences among points and (°) denote statistically significant differences among seasons. M: moderately impacted and S: strongly impacted.

Season	Location	Zn	Cd	Mn	Cr	Cu
Dry	P1	258.7± 88.5(M)	0.0089± 0.0002(M)	146.9±° 71.8(M)	57.8± 26.7(M)	65.4± 29(M)
	P2	560±** 56.645(M)	0.0088± 0.0034(M)	595±** 109.2(M)	58.5± 41.8(M)	463±** 230.1(S)
Wet	P1	307.4± 181.52(M)	0.0034±° 0.0009(M)	333.9± 10.9(M)	70.8± 16.3(M)	405.4±°° 125(S)
	P2	457± 159.73(M)	0.006± 0.0088(M)	501.6±* 149(M)	63.5± 176.4(M)	430.8± 113(S)
Reference (AC)		560	0.005	781.3	346.2	233.9

Table 4. Bioaccumulation (mg/Kg $^{-1}$) in the muscles of piranhas from Itacaiúnas River on both Points and seasons. (*) denote statistically significant differences among points and (°) denote statistically significant differences among seasons. M: moderately impacted; S: strongly impacted.

Season	Location	Zn	Cd	Mn	Cr	Cu
Dry	P1	3.34±1.2	0.014±0.006	0.22±0.19	0.14±0.05	1.05 ±0.005
	P2	4.1±0.6	0.046±0.017*	0.31±0.2	0.27±0.09	2.090±0.008°
Wet	P1	4.9±0.04	0.047±0.02	0.49±0.07	0.094±0.01	0.7±0.02
	P2	4.67±0.13	0.0509±0.02	0.24±0.051	0.05±0.03	0.6±0.01

Table 5. Prevalence (%) of gill histopathological changes observed in *S. rhombeus* captured in different points (P1 and P2) on both season of Itacaiúnas river.

Season	Location	Prevalence (%)							
		LF	**FEP**	**LEP**	**Va**	**An**	**Ed**	**Lif**	**N**
Dry	P1	5	20	25	20	0	0	20	0
	P2	70	60	85	45	12	2	75	0
Wet	P1	10	15	35	10	3	0	30	2
	P2	95	40	90	60	75	0	65	3

Note: LF (lamellar fusion); FEP (filament epithelium proliferation); LEP (lamellar epithelium proliferation); Va (vasodilatation); An (aneurism); Ed (edema); Lif (lifting); N (necrosis).

Figures

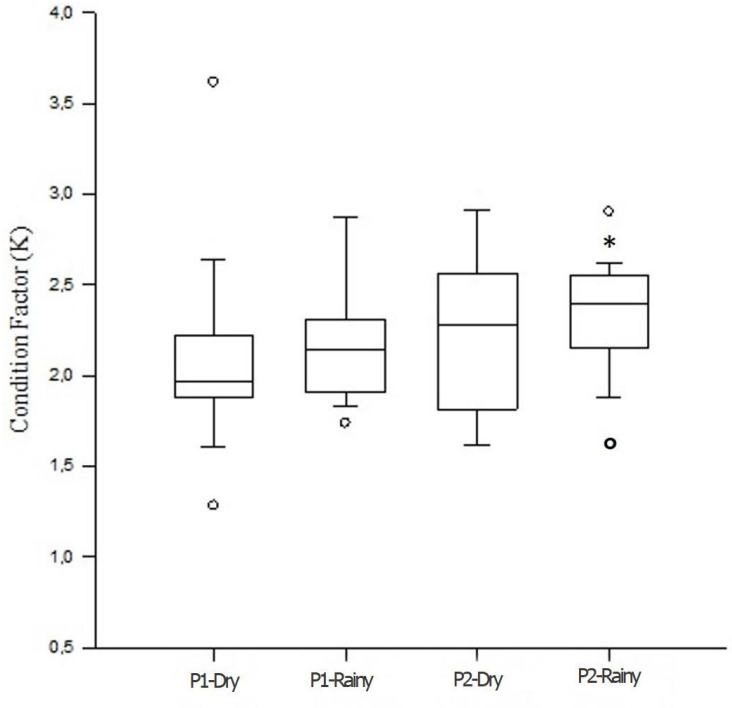

Figure 1. Mean ± Standard of the Condition factor (K) values from the piranhas collected during the study. P1 (Upstream) and P2 (Downstream) of Itacaiunas river on both seasons.

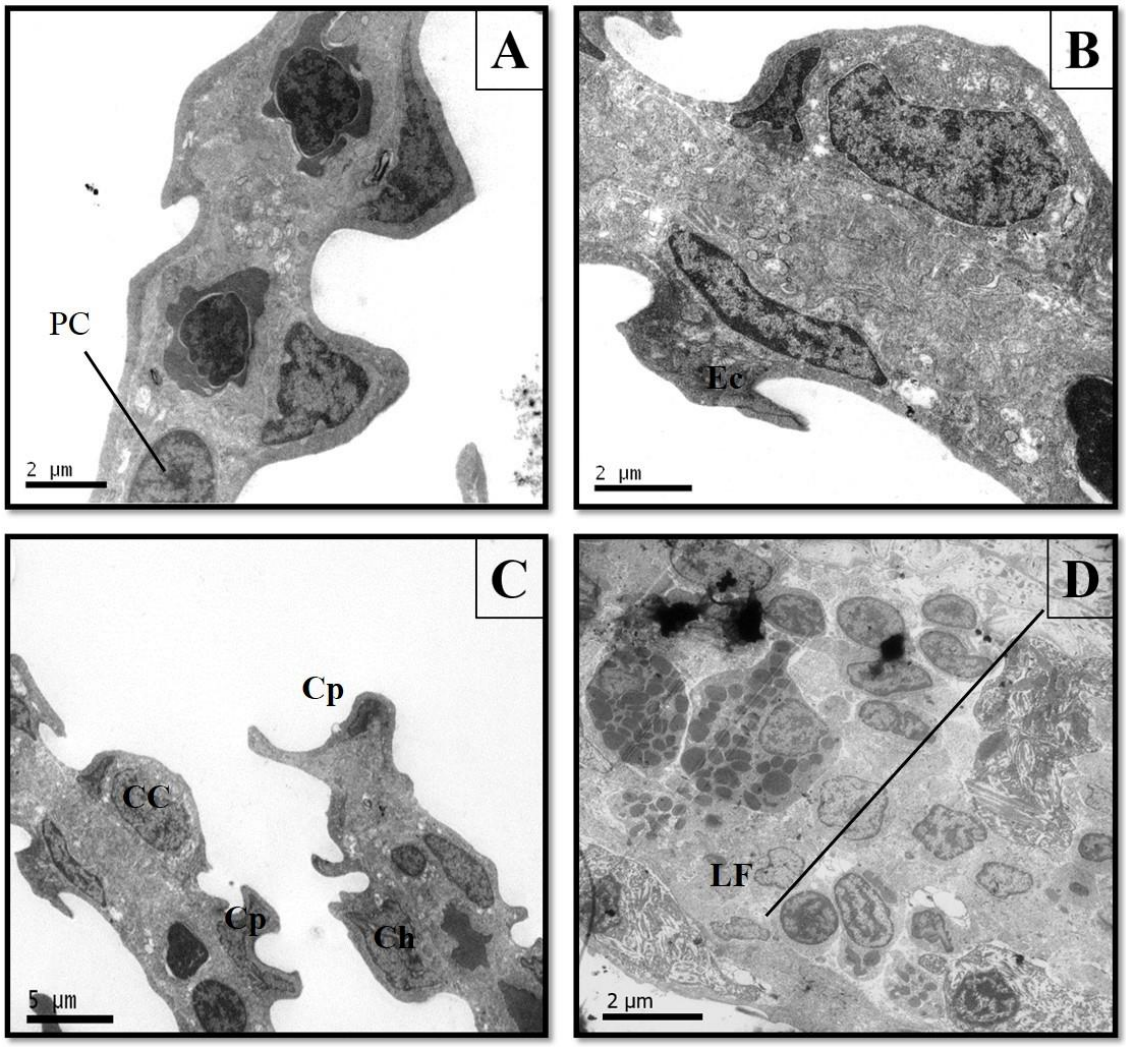

Figure 3. Transmission Eletromicrography (TEM) (A) Normal morphology of a gill tissue. Pillar cell (PC), Chloridric cell (CC) and Epithelial cell (Ec); (B) Detail of a normal lamella. Célula clorídrica (CC); (C) Branchial tissue alteraded with edema (Ed), Cellular hypertrophy (Ch) and disarrangement in the architecture in the tissue (*); (D) Detail of the second. Moderate alteration with Hyperplasia causing lamelar fusion (LF) and cell proliferation (Cp).

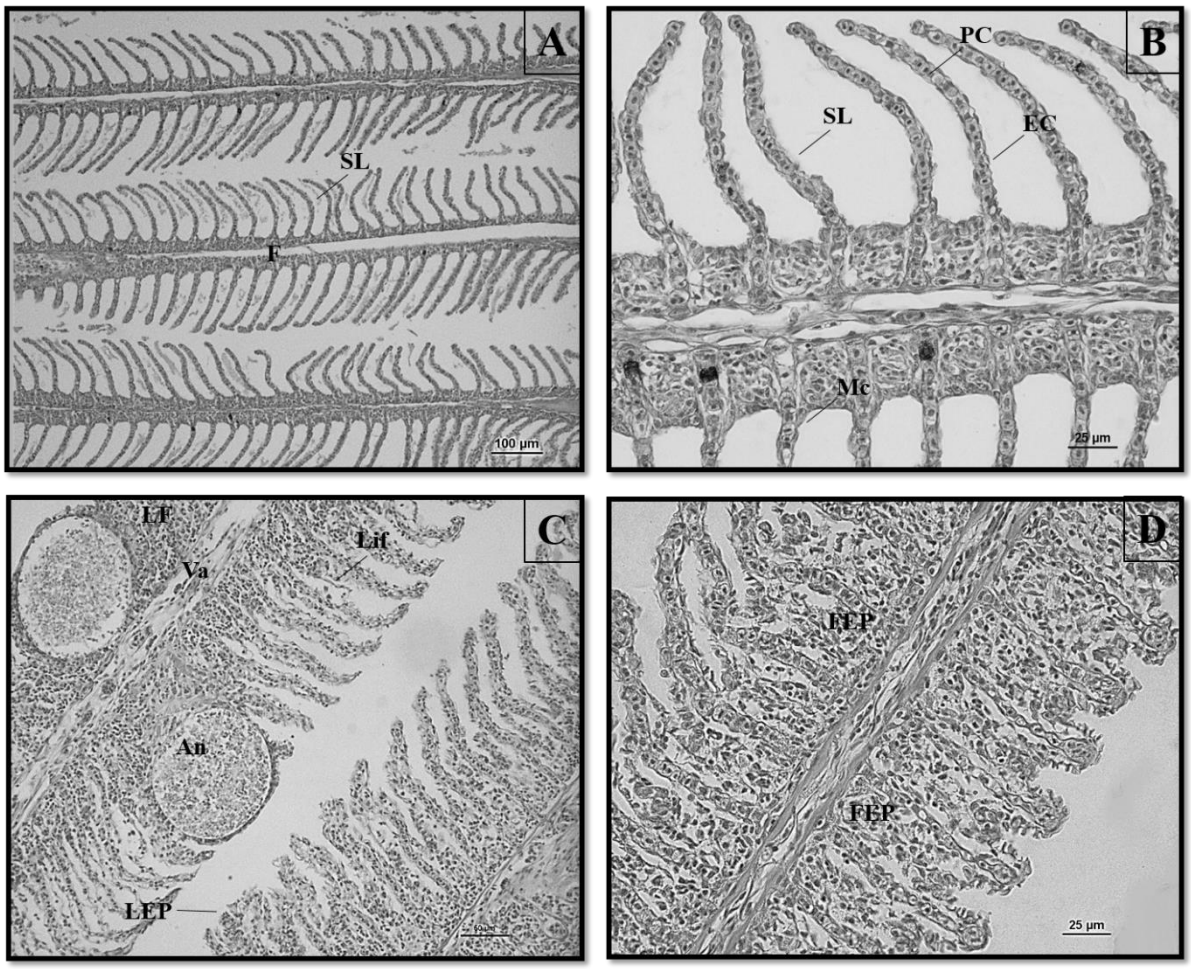

Figure 2. Gill tissues photomicrography of *S. rhombeus*. **(A)** Fishes captured in P1 with normal structure for teleost Branchial: filament (F) and second lamella (SL); **(B)** Detail of the second lamella with a slender appearance and without damages in the cells: PC – Pillar cell, EC – Epithelial cell, Mc – Mucous cell; **(C)** Fishes captured in P2 presented branchial tissue with lesions: Aneurism (An), lamellar lifting (Lif), lamellar fusion (LEP); **(D)** Filament with severe alterations: Necrosis (Nc) and intense cell proliferation causing completely fusion of the filament (FEP).

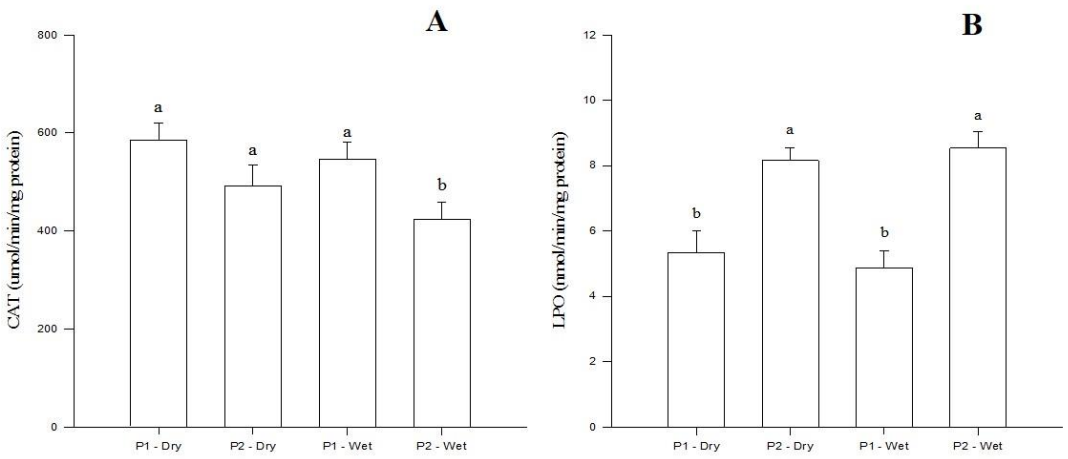

Figure 4. (A) Gill Catalase activity (µmol/min/mg protein) and (B) lipid peroxidation (µmol/min/mg protein) in piranhas. Data are expressed as mean± Standard; letters indicate significant differences among sampling locations ($p < 0.05$).

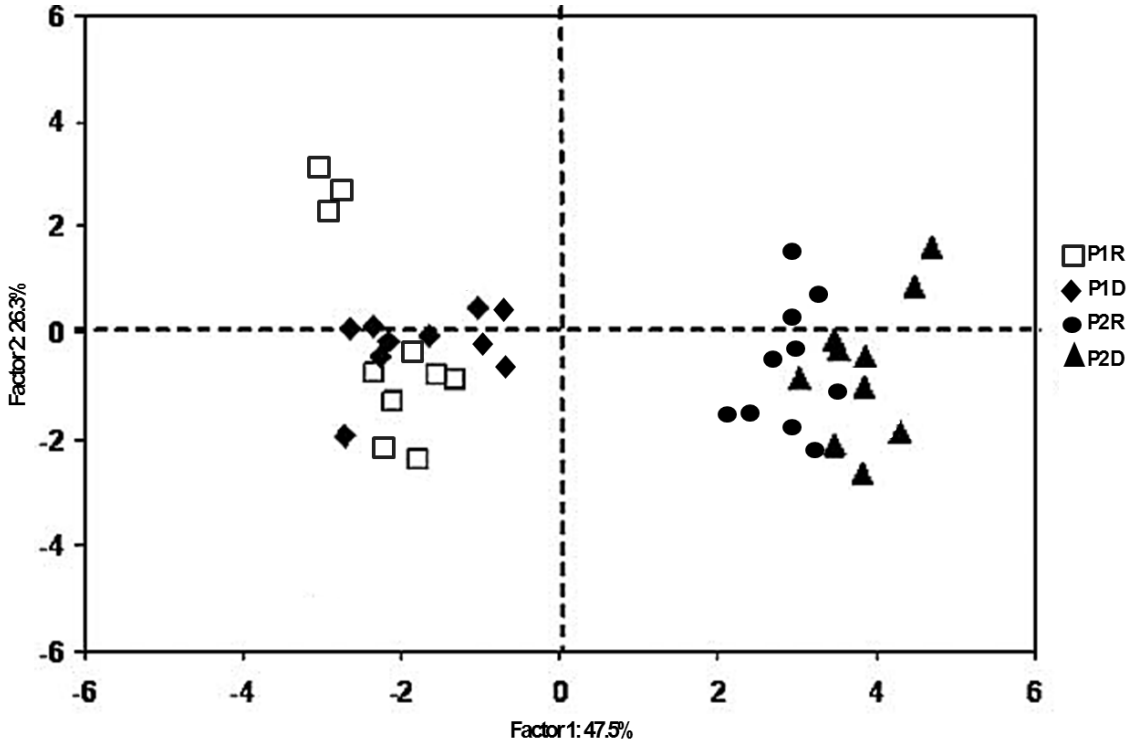

Figure 5. PCA plots of the variances of biomarkers in piranhas among points. P1R – point 1 rainy season; P1D – point 1 dry season; P2R – point 2 rainy season; P2D – point 2 dry season.

CAPÍTULO 3

PROTECTIVE EFFECTS OF DISSOLVED ORGANIC MATTER (DOM) IN SHORT-TERM EXPOSURE OF BLACK PIRANHA, *SERRASALMUS RHOMBEUS*, TO METALS IN RIO NEGRO RIVER WATER

Está sob a norma da Revita: Aquatic toxicology

Abstract: The goal of the present study was to assess the degree of protection conferred by NOM against Cu and Cd accumulation in the gills of piranhas (*Serrasalmus rhombeus*) on Rio Negro River, Amazon compared with a soft water from laboratory. In this study, animals exposed to copper and cadmium in the waters of the Rio Negro showed higher concentrations of metals (Cd and Cu) in the gills tissues compared to animals exposed in laboratory water. Cadmium is know to perturb ion balance, cause biochemical and morphological damages, whilst copper is a essential metal to organisms. In this study we observed that the cadmium even in the presence of DOM still harmful to the gill tissues in high concentrations, because, many anomalies in the fish gills were detected and quantified also biochemical alterations, as CAT and SOD, especially in fishes exposure to cadmium. This study conclude that the DOM provide a natural protection, depending the metal and the concentration.

Key Words: Cadmium, copper, BLM, toxicity, water quality, teleost

1. INTRODUCTION

The environment has been constantly impaired, due to several anthropogenic activities coupled with economic development. However, the aquatic ecosystem is considered more susceptible to pollution and contamination because it is the final recipient of all discharge produced (Botkin and keller, 2000). So the increase in range of pollutants and the multiple mechanisms of toxicity are the current issues we face today. It is well know that among the different types of pollutants, heavy metals can cause adverse effects on the aquatic individuals, also, can generate an imbalance in the trophic structure, because they can form a major hazard because of their toxicity, persistence, and bioaccumulation in the food chains (Sakar et al., 2006).

Most metals are essential for the physiological function processes in fish. However, tolerable limits and environmental changes may in turn affect the metals biokinetics of the fish leading to mortality, while sublethal concentrations may lead to behavioral, biochemical, and histological changes in organisms (van der Oost et al., 2003). The heavy metals can enter the bodies to a small extent via food, drinking water and air. In fish, gills are the first sites of direct contact with any external pollutant along with the skin and so gills are also the first sites to elicit structural and functional responses (ba-Omar et al., 2011). The gills are multifunctional organs directly involved in gas exchange, osmoregulation, acid-base balance and elimination of nitrogenous excreta (Poleksic and Mitrovic-Tutundzic, 1994; Evans, 2005). This organ owned an extensive surface area in contact with water, thus ends up being the primary target after exposure to contaminants, becoming an excellent water quality indicator (Mallat, 1985; Tophon et al., 2003).

It is important elucidate that the presence of a metal compound in the environment does not necessarily mean a risk to the organisms, it is important verify issues such as bioavailability. The role of organic ligands in metal complexing in natural waters has received little attention because of uncertainties regarding both the abundance and nature of dissolved organic carbon compounds. Recent data show that the bulk of dissolved organic matter (DOM) in natural waters consists of highly oxidized and chemically and biologically stable polymeric compounds closely resembling soil humic substances (Al-Heasi et al., 2013). Some studies suggest that the natural organic matter is readily adsorbed after complexation and can reduce the toxicity of some metals. Depending the type of the water, the metallic elements will have a certain kind of behavior.

The Amazon River ranks first among the World rivers due to its drainage area. It represents about 20% of the world fresh water flux from land to ocean. Tributaries of the Amazon River exhibit a range of chemical characteristics and classified into three types on the basis of their appearance as ‘‘white waters’’, ‘‘clear waters’’ and ‘‘black waters’’. The ‘‘black waters’’ from the Rio Negro are relatively acid, low in total cations and rich in dissolved organic carbon (Aucour et al., 2003). In the Negro River, kaolinite dominates the clay fraction of the suspension. The marked difference in water chemistry between the two rivers and inputs of other sources such as varzea lakes (Moreira-Turcq et al., 2003).

Based on this context, the present study was performed to describe, the comparative response of a large set of biomarkers in piranhas, *Serrasalmus rhombeus*, exposed to environmentally relevant concentrations of Cd and Cu in two different types of water, a natural water from Rio Negro and other from the laboratory (Deionized water), in order to evaluated the effect on the natural organic matter of the river on the

toxicity of the selected metals. The responses of these parameters were evaluated considering their suitability to detect biological effects associated with exposure to low and high environmental concentrations of copper and cadmium.

2. MATERIAL AND METHODS:

All experiments were performed in December 2014 on board a research vessel (the Ana Clara, from Manaus) during an expedition to the Anavilhanas Archipelago of the Rio Negro, approximately 110 km upstream from Manaus. All procedures were in compliance with Brazilian national and INPA animal care regulations. Fish used for this study were the carnivorous black Piranha, *Serrasalmus rhombeus*, (mean weight 143 ± 58 g and body length 19 ± 2.4 cm). The fishes were caught in the local area by INPA fishermen, after they were held on board in large tanks served with the water pumped directly from the Rio Negro (temperature = 29–32 °C, Dissolved Oxygen Content = 7-9). The fishes were not fed during the experiment.

2.1.Chemicals

The metals (cadmium and copper) to which fish were exposed were dosed as stable salts, cadmium chloride ($CdCl_2 \cdot 2H_2O$; MW. 228.34, BDH Analar) and copper sulfate ($CuSO_4 \cdot 5H_2O$; MW 249.7, Sigma) in degrees of purity of 99.5% and 99.0%, respectively. Stock solutions of the tested compounds were prepared in ultrapure water immediately before dilution into test solutions.

2.2.Experimental Design

The experiment set up consisted of exposing the piranhas to cadmium and copper with water of the “black water” of Rio Negro rich with dissolved organic matter (DOM) and other with the water of the INPA Laboratory (soft water: pH 6.28, 6.40 mg

O_2/L), for a period of 1h, 2h and 3h. A Total of seven replicates of fishes per treatment were individually exposed in plastic bottles of 3,5 L previously clean with distilled water. The following treatments of Cu and Cd were, A – Rio negro water: (1) RN Control, (2) RN 5 µM Cu, (3) RN 10 µM Cu, (4) RN 0,1 µM Cd, (5) RN 10 µM Cd, B – INPA water (6) INPA Control, (7) INPA 5 µM Cu, (8) INPA 10 µM Cd. In addition, water samples were collected every hour in order to verify some variation of ammonia, chloride and sodium in the water during the experimental period and for metal analysis.

2.3.Sampling

After the exposure the animals were carefully removed from the aquaria, euthanized by means of an overdose of anesthetic by ethyl-3- aminobenzoate methanesulfonic acid (MS-222, Acros Chemicals, Geel, 195 Belgium) and the biometrics were performed. Blood samples was taken from the caudal blood vessel using a heparinised syringe (heparin from Sigma-Aldrich, co, St.Louis, USA), then, the blood samples were immediately centrifuged for 2 min at 13,200 rpm at 4 °C. Plasma was carefully pipetted into cryogenic vials and frozen in liquid Nitrogen. The gills were removed, cleaned and stored in Bouin Solution (75% Saturated picric acid PA, 20% Formaldehyde, 5% Acetic acid) for histological analysis and other pieces in liquid nitrogen for biochemical and metal content analysis.

2.4.Analytical techiniques and calculations

Water samples (5 mL) were collected every hour during the experiment. Water total ammonia (Am) was determined colorimetrically by using the salicylate–hypochlorite method (Verdouw et al., 1978). Chloride levels and Na^+ concentrations in water were measured using flame atomic absorption spectrophotometry. Net ion fluxes were calculated (in µmol $kg^{-1}h^{-1}$) of Na^+ (J^{Na}_{net}), ammonia , K^+, Mg^{2+},Ca^{2+} chloride

were calculated from changes in concentration (in μmol l^{-1}), factored by the known fish mass (in kg), volume (in l), and time (in h). Plasma osmolality was measured on fresh plasma sample using the Advanced™ Micro Osmometer (Model 3300, Advanced Instruments, USA). Plasma ions concentrations of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} were analyzed using an Electrolyte Analyzer 9180 (AVL Scientific corporation, 213 GA, USA) and plasma glucose, lactate and ammonia were determined with commercial Enzymatic Kits (R-Biopharm AG, Darmstadt, Germany)

2.5.Histopathology

The gill tissues were fixed in Bouin's Solution (25 % Picric acid, 75% Paraformaldehyde, 1 % Acetic acid). After regular histology procedures, the samples were embedded and included in paraffin blocks to obtain 5 mm thick cuts using a rotary microtome (Leica RM 2245). The sections were stained with hematoxylin and eosin (HE) and examined under a light microscope (Nikon Eclipse ci). One gill arch was randomly chosen and analyzed. The prevalence of each type of change was determined according to Pereira et al., 2013. Ten entire filaments per arch were chosen randomly and analyzed. A severity gradation scale (SGS) with six degrees (0–5) was applied qualitatively, considering the extent and severity of each lesion. The extent was defined as the percentage of filaments with a type of lesion in each fish sampled. To quantify the severity of each histological change, the different levels of severity were attributed. The severity of each lesion per average of affected filament was determined as the number of lamellar and interlamellar spaces affected by a given level of severity, divided by the number of filaments showing that type of histological change. A zero degree was given to values found in fish with less histopathology, and to define the

remaining degrees, the extent and severity levels were combined to show an increasing numbers of lamellae and interlamellar spaces per injured filament. All values obtained, from the counting of extent and severity, were then divided into numerical intervals and combined to generate the SGS.

2.6.Oxidative stress

Gills were weighed, homogenized (1:2, w/v for LPO and CAT; and 1:4, w/v for GST and SOD) in buffer homogenization (20mM tris-base; 1mM EDTA; 1mM dithiothreitol; 500mM saccharose and 150 mM KCl), centrifuged (9000 g, 30 min, 4ºC), and the supernatant removed for analysis of the biochemical parameters. GST activity was measured according Keen et al. (1976). The supernatant was mixture in potassium phosphate buffer 0.1 M pH 7.0, 50 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 25mM of reduced glutathione (GST). The method is based in the complexation of the GST with 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance values were measured at 340 nm, and the activity was expressed as nmol of conjugated CDNB min^{-1} mg of $protein^{-1}$. Lipid peroxidation was estimated following the methodology by Jiang et al., 1992. Supernatant were precipitate (1:1 v/v with 12% TCA), and centrifuged (5000 g, 10 min, 4ºC). The samples (30µl) were added in reaction buffer (methanol 90%; 100 mM xylenol orange; 25 mM H_2SO_4; 4 mM BHT and 250 mM ferrous ammonium sulfate). LPO levels were expressed as (µmol cumene hydroperoxide.mg $protein^{-1}$). Total antioxidant competence against peroxyl radicals was evaluated according to Amado et al., 2009. The gills samples were diluted (1:20, w/v) in cold buffer (Tris–HCl 100 mM; EDTA 2 mM; $MgCl^{2+}$ 5 mM; pH 7.75) and centrifuged (10000 g, 20 min, 4ºC). The proteins were previously standardized for the same concentration values. Subsequently, microplate half was filled with 10 µL of the supernatant was mixture with reaction buffer (HEPES 30 mM; KCl 200 mM; $MgCl_2$ 1 mM; pH 7.2) and the

same fulfilled for another microplate half (the same samples). Superoxide dismutase (SOD) assay was determined according to the protocol of McCord and Fridovich (1969). The samples were diluted in reativic solution (phosphate buffer 50mM; xantina 1 mM; NaOH 1 mM; citocromo-c 1.9 mM). Inhibition of reduction rate of cytochrome c by the superoxide radical at 550 nm and 25 °C is the basis of this method. SOD activity is expressed in U SOD per mg of protein, assuming one U of SOD as the quantity of enzyme that promotes the inhibition of 50% of reduction rate of cytochrome c. CAT activity was determined by the method described by Beutler (1975). The samples were mixture with reaction buffer (Tris 1M com EDTA 5mM). The rate of inhibition of H_2O_2 decomposition absorbance was measured at 240 nm in a spectrophotometer. CAT activity was expressed as H_2O_2. min^{-1}.mg $protein^{-1}$.

2.7.Heavy metals analysis

Gills samples were acidified (1:30, w/v) with HNO_3 1N (70°C; by 48 h) and filtered. Cd and Cu in measured by an atomic absorption spectrophotometer Perkin Elmer brand - AAnalyst 800. The results were expressed in µg L^{-1} of metal (cadmium or copper).

2.8.Statistical analysis

All data are reported as mean ± standard error. Kolmogorov-Smirnov was applied to evaluate normality, while Levene's test was used to test the homogeneity of variance. The one-way analysis of variance (ANOVA) was used for comparing the statistical data among the sampling points, followed by multiple comparison test (Tukey). While the Kruskal–Wallis test was applied for the data with no normal distribution or variance

heterogeneity. The statistical packages SigmaStat 3.5 and GraphpadPrism were used for statistical analysis.

3. RESULTS

3.1.Fluxes

Based on the evaluated data, it was noted that treatment with 0,1 µM cadmium with Rio Negro water didn't presented significant difference in the parameters. Whilst, the values of Cl^- and Na + increased significantly in both black and INPA water during the three hours. Ca^+ increased only in the first time (Fig. 1).

In the exposure treatment with Cu, the ammonia flux presented no significant difference between the other concentrations, excepted for RN 5µM, the values was high in the hour two. The Na + was high in all treatments except control. With respect to Ca^+ in the treatment RN 10 µM was high only in the first hour. The Cl^- and Mg^{2+} levels were highest at 5 µM with water INPA (Fig. 2).

3.2.Osmolality and Plasma ions

Fig. 3 shows the results of osmolality and the concentrations of the plasma ions. The values of osmolality didn't differ much between treatments, only the fishes exposed to water of the Rio Negro in treatments 5 uM Cu and 1 uM Cd presented significantly lower than control. With respect to plasma ions from fishes exposed to cadmium, K+ was higher in the INPA 1 uM treatment and Mg^{2+} in the treatment RN 1 uM. With respect to copper exposure, such K^+ and Ca^{2+} were significantly higher on treatment INPA 5 uM. In addition, also INPA 5 uM treatment presented high levels of glucose and lactate (Fig. 3).

Metal accumulation

No fish died in the control groups. However, the exposure with RN 1mM of Cadmium only one fish died. Accumulation of heavy metals in the gill of fish *S. rhombeus* is shown in fig. 4. The accumulated metal were significantly elevated on fish gills exposed in Cu 10mM and Cd 1mM with Black water of the Rio Negro River.

3.3.Measurement of biochemical parameters

SOD activity decreased ($P<0.05$) in fishes from treatment INPA 1µM Cd. GST activity was significantly lower in animals from treatments 10 µM Cu and 1µM Cd both with Rio Negro water (Fig. 5). Whereas CAT (Fig. 5E-F) and LPO (Fig. 6), didn't present a significant difference between treatments.

3.4.Histopathological analysis

The results of analysis of gill lesions prevalence and histopathological assessment are presented in Table 1. Gill epithelium of *S. rhombeus* showed several histopathologic alterations; 90% of the fish presented three or four lesions. The fishes from control group with Rio Negro and INPA water presented tissues with no damages, normal architecture and well defined cells (Fig. 6). However, the fishes exposed to Cd and Cu presented gills with alterations and the grade was related with the concentration. The main lesions observed were vasodilatation and epithelial hyperplasia, occasionally resulting in lamellar fusion (Fig. 7). These histological alterations were observed at varying degrees of extension and severity. Hyperplasia and vasodilatation were scored as the maximum severity (grade 3), whereas the maximum extent (grade 4) was scored for hyperplasia and lifting. The highest mean assessment value of lesion was found for

hyperplasia, followed by lifting, while the lowest was found for aneurisms and necrosis (Fig 8).

4. DISCUSSION

Natural waters contain organic matter (DOM), which is a complex mixture of organic components derived from the decomposition of plants, animals and microorganisms from either terrestrial materials external to the aquatic system, and generated within the water column (McKnight and Aiken, 1998, Richards et al., 2001). DOM is mainly comprised of the humic substances, humic acid and fulvic acid, together with carbohydrates, proteins and lipids. The humic material is able to complex metals and therefore plays an important role in protection against metal toxicity in aquatic animals, some studies affirm that 50% of DOM are dissolved organic carbon (DOC) (De Schamphelaere et al., 2004, Kramer et al., 2004). Pandey et al. (1999) reported the role of humic acid in the sequestration of metals by formation of soluble complexes, an event extremely important in affecting the retention and mobility of metal contaminants in the environmental (Arnold et al., 1998).

In this work we evaluated the effects of the Rio Negro water, an Amazon River rich in DOM, on the accumulation of selected metals in fish gills and the biological responses of the organisms. Bioaccumulation patterns of metals in tissues of fishes can be utilized an indicator of environmental metal contamination. Copper exerts a wide range of physiological and histopathological effects on fish, whilst cadmium is a non-essential metal (Arellano et al., 1999). The results pointed that the gill tissues from the animals threatened with Rio Negro water had more concentration of metal compared to those from INPA water. However, the main alterations in gills (histological and biochemical) were observed in fishes exposed to metals in INPA water.

The anomalies as necrosis and lamellar fusion are alterations of severe character,

the lammelar fusion is caused by a intense proliferation of mucous or pavement cells, acting as s barrier to the tissue not accumulate more pollutants, so, a defense mechanism against more damages. Monteiro et al., 2008 exposed *O. niloticus* to different concentrations of copper, they observed that there was no relation between the high concentration and the severe gill damages. They believed that the acute exposure can affect immediately the gills and as a defense an intense proliferation of cells can reduce more damages. In our work, the INPA water is very soft water with no organic matter, so the metals can't bind with cations in the water and can attaching in the gill tissues quickly and generating the injuries. However we didn't observed difference in the experiment with high concentration of cadmium with water of Rio Negro and INPA. Accordingly Balistieri and Mebane et al., 2014 cadmium is a metal that have some specificity to complex with other metals and with cations and anions. In this research the influence of cadmium in the gill tissues was very strong even in the presence of the organic matter.

Many studies show that metals, essential and non-essential, can induce oxidative stress by generating free radicals and reactive oxygen species (Souid et al., 2013). The toxic effects of metals in animals, via oxidative stress, are well known. Organisms utilize enzymatic and non-enzymatic defense against oxidative stress due to metal exposure in an attempt to keep the injury as small as possible by metabolizing ROS and by repairing the occurring damage, although this is not always possible (Basha and Rani, 2003). In fact, some metallic compounds can trigger specific responses in which distinct pathways are involved and the decrease of antioxidant enzymes (as observed in our study) can be consequent to the inactivation of a specific signaling pathway (Regoli, 2003). In this study the enzymes showed significant changes in its activities, except GST had no significant difference between treatments. In addition, the LPO analysis

also revealed that there was no damage in tissue lipids in the gills of piranhas from RN 1mM cd. SOD - CAT is considered as the first line of antioxidant defense as it catalyzes the dismutation of O_2 into molecular oxygen and H_2O_2 (Alscher et al., 2002). The enhancement of ROS generation, caused probably by the accumulation of metals in gills, may stimulate the biosynthesis of SOD and induced enzyme activity on fishes from Rio Negro river treatments, in which, cadmium exposure was significantly higher than the copper treatment. Sensitivity of SOD and CAT activities to metal exposures were also supported with our previous results (Atli and Canli, 2010). Decreased SOD activity might be an indicator of damage in the antioxidant mechanisms caused by metal exposure and water hardness, similar result was observed in this study, because only INPA water treatments were significant lower than treatments with Rio Negro water. The GSTs enzymes can catalyze the reaction of organic peroxides with GSH, thus preventing lipid peroxidation, the treatment with cadmium in Rio Negro water presented fishes with peroxidation in lipid and high levels with GST but not significantly. The reasons for the difference could be explained by the inactivation of the pathways for the rapid absorption of the metals for the gills. Since, in spite of antioxidant defenses not present significant activities, damage to tissue as regards apoptosis indicates tissue damage. According Dutton and fisher, 2013 metals accumulation first in the intestine than to other tissues and the the bioaccumulation route happens in prolonged exposure with approximately 12 hours of exposure. In this study, treatment was carried out 3 hours of exposure. However, we could observed differences between treatments. In the treatments with laboratory soft water, the metals can be accumulated and transferred rapidly to the blood pathways, while in the treatments with Rio Negro water, this process has not been fully established. However, very little is known about the direct effects of DOM on the physiology of fishes, thus it

is important more research is needed in this field. Electrolytes loss has been described in fish exposed to several heavy metals, including Cd (Camargo et al., 2009). Freshwater fish take up most of the ions necessary for homeostasis from the water via their gills, the drop of plasma electrolytes are apparently caused by an increased efflux of ions across these organs and an impairment of active ion uptake by the Chloride cells of the gill (Garcias-Santos et al., 2005), so an imbalance in this mechanism could injury the fish stability. In this research the results of plasma ions didn't show robust responses. Monteiro et al., 2005 affirm that this mechanism of action is most affected by long-term exposure (days) or acute exposure to high concentrations can lead to death.

5. CONCLUSION

This work allows us to have a simple view of the interaction of DOM with the toxicity of some metallic compounds, in this case, copper and cadmium. The natural features can provide some protection for the organisms reducing the toxicity the metals. We believe this protection maintaining only a certain time and depend of the concentration of the exposure. Maybe, in the long run, it may not have the same effect. Therefore, it is important to have more detailed studies regarding the time of exposure.

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1. TABLE

Table 1. Prevalence (%) of gill histopathological changes observed in *S. rhombeus*

Treatment	**Prevalence (%)**							
	LF	**FEP**	**LEP**	**Va**	**An**	**Oed**	**Lif**	**N**
RN Control	5	0	0	10	0	2	10	0
RN 5µM Cu	54	30	35	30	20	35	55	2
RN 10 µM Cu	48	35	25	10	6	15	30	0
RN 0,1 µM Cd	65	25	80	20	52	0	45	5
RN 1 µM Cd	72	42	68	40	48	38	70	16
INPA Control	5	5	5	10	0	5	20	0
INPA 5 µM Cu	75	57	62	52	50	36	66	0
INPA 1 µM Cd	78	65	88	46	45	22	74	15

Note: LF (lamellar fusion); FEP (filament epithelium proliferation); LEP (lamellar epithelium proliferation); Va (vasodilatation); An (aneurism); Oed (Oedema); Lif (lifting); N (necrosis).

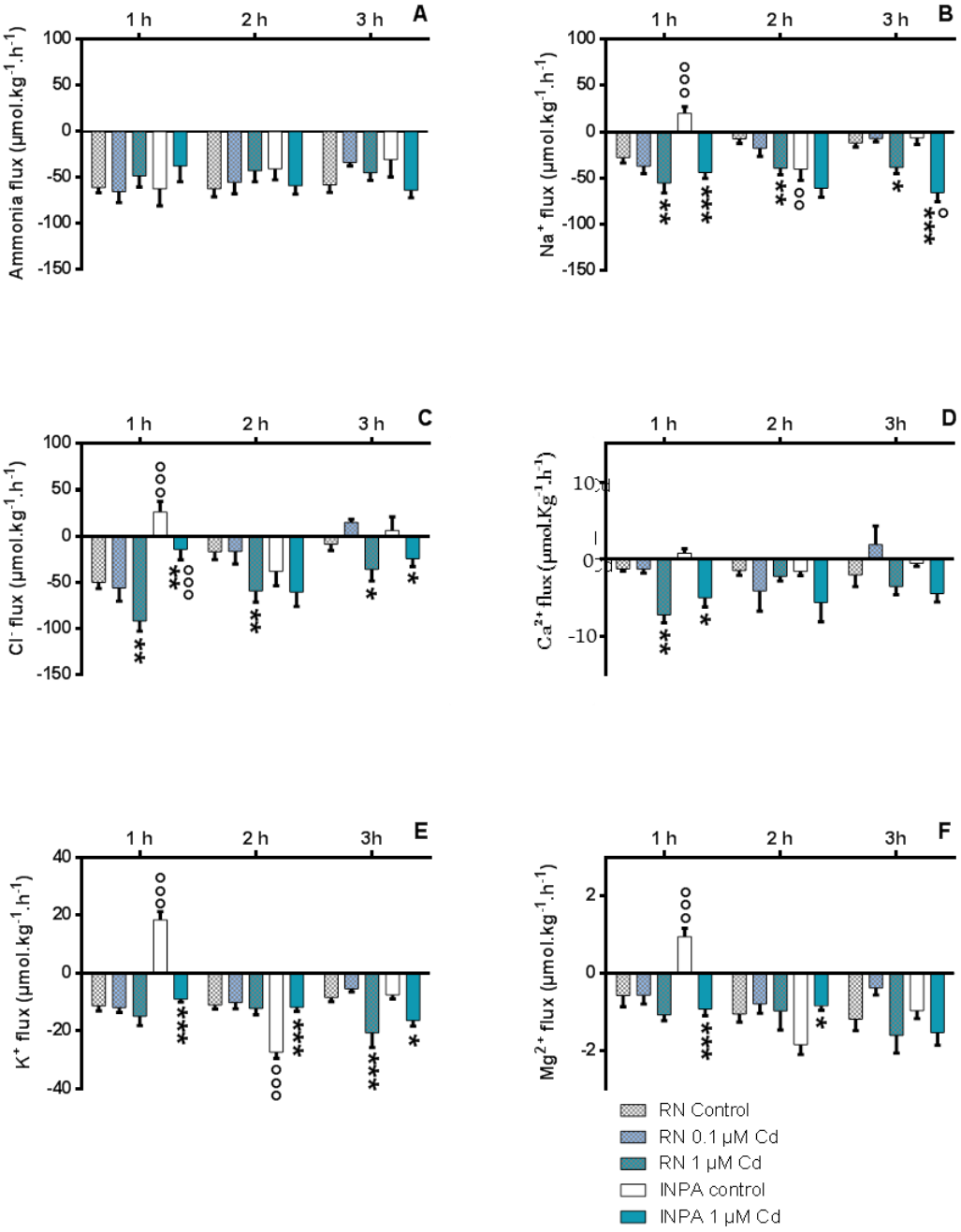


Figure 1. Conconcentrations (in µM) of ammonia, sodium, potassium, chloride, calcium and magnesium in water fluxes exposed to cadmium. Results are represented as means ± SE. * indicate significant differences between the groups against the control and ° indicate significant differences between water.

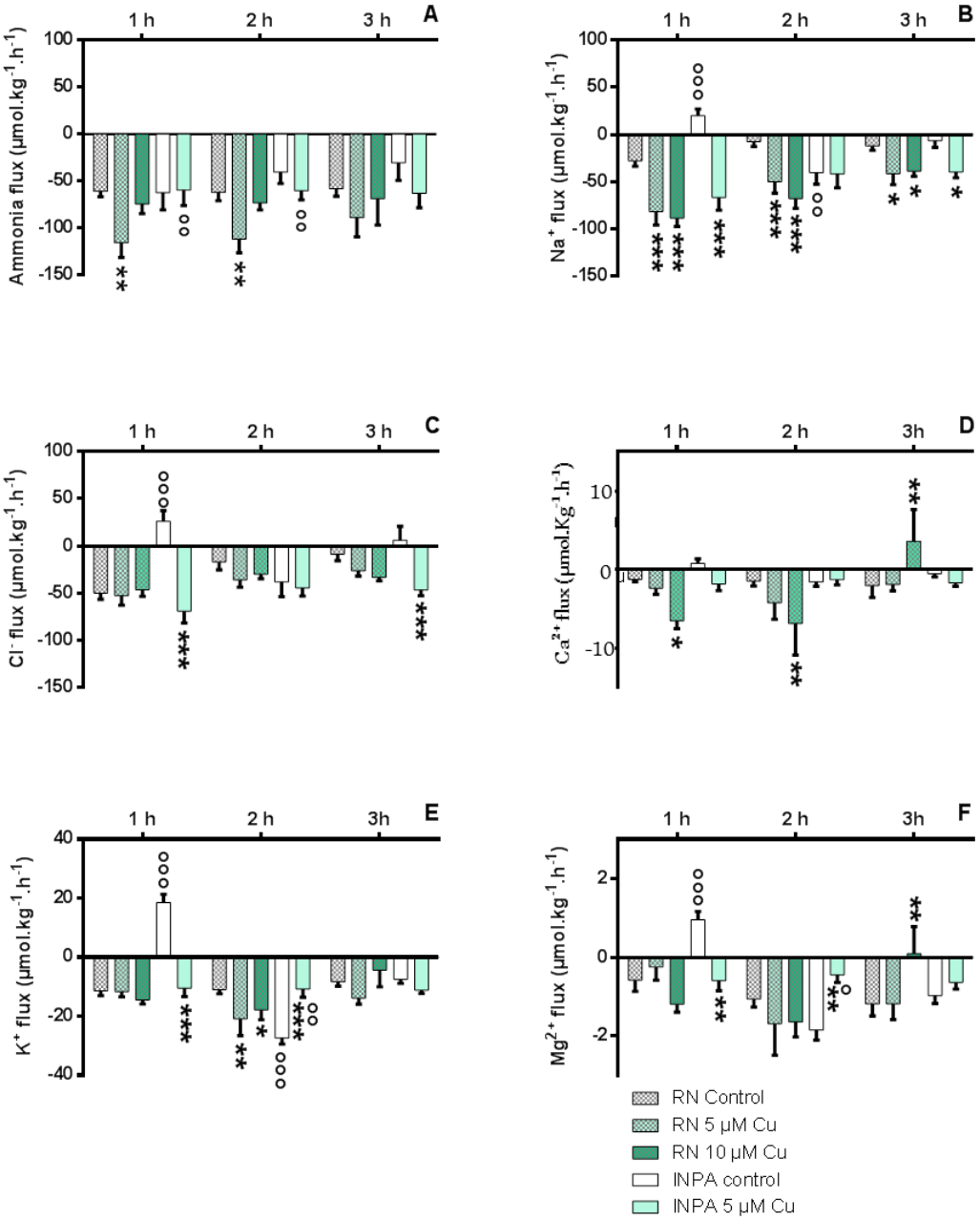
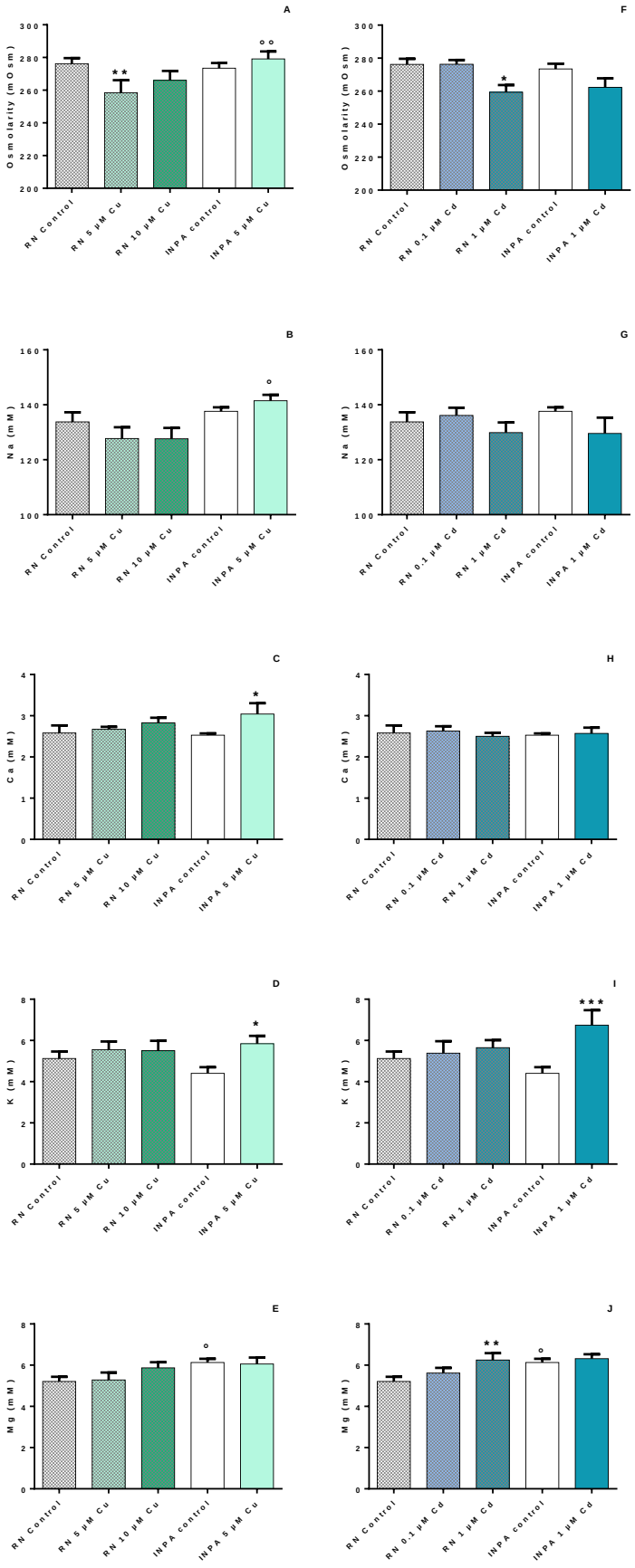


Figure 2. Conconcentrations (in µM) of ammonia, sodium, potassium, chloride, calcium and magnesium in water fluxes exposed to copper. Results are represented as means ± SE. * indicate significant differences between the groups against the control and ° indicate significant differences between water.


A
Osmolarity (mOsm)
300
280
260
240
220
200
**
°°
RN Control
RN 5 µM Cu
RN 10 µM Cu
INPA control
INPA 5 µM Cu
F
Osmolarity (mOsm)
300
280
260
240
220
200
*
RN Control
RN 0.1 µM Cd
RN 1 µM Cd
INPA control
INPA 1 µM Cd
B
Na (mM)
160
140
120
100
°
RN Control
RN 5 µM Cu
RN 10 µM Cu
INPA control
INPA 5 µM Cu
G
Na (mM)
160
140
120
100
RN Control
RN 0.1 µM Cd
RN 1 µM Cd
INPA control
INPA 1 µM Cd
C
Ca (mM)
4
3
2
1
0
*
RN Control
RN 5 µM Cu
RN 10 µM Cu
INPA control
INPA 5 µM Cu
H
Ca (mM)
4
3
2
1
0
RN Control
RN 0.1 µM Cd
RN 1 µM Cd
INPA control
INPA 1 µM Cd
D
K (mM)
8
6
4
2
0
*
RN Control
RN 5 µM Cu
RN 10 µM Cu
INPA control
INPA 5 µM Cu
I
K (mM)
8
6
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2
0
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RN Control
RN 0.1 µM Cd
RN 1 µM Cd
INPA control
INPA 1 µM Cd
E
Mg (mM)
8
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RN Control
RN 5 µM Cu
RN 10 µM Cu
INPA control
INPA 5 µM Cu
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Mg (mM)
8
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RN Control
RN 0.1 µM Cd
RN 1 µM Cd
INPA control
INPA 1 µM Cd

Figure 3. Plasma conconcentrations (in µM) of sodium, potassium, chloride, calcium and magnesium in plasma osmolality (in mOsm. Kg H^2O^{-1}) in piranhas exposed to cadmium and copper at different concentrations and type of water (Rio Negro and INPA). Results are represented as means ± SE. * indicate significant differences between the groups against the control and ° indicate significant differences between water.

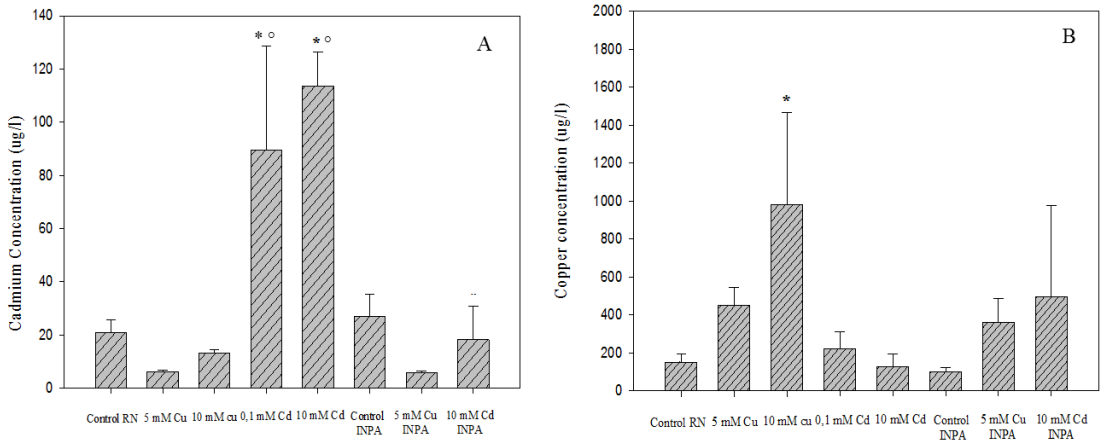

Figure 4. Total Cadmium (Cd) and Copper (Cu) concentration (Ug/l) in the gill tissues of piranhas exposed to metals with water from Rio Negro and INPA Laboratory. Results are represented as means ± SE. * indicate significant differences between the groups against the control and ° indicate significant differences between water.

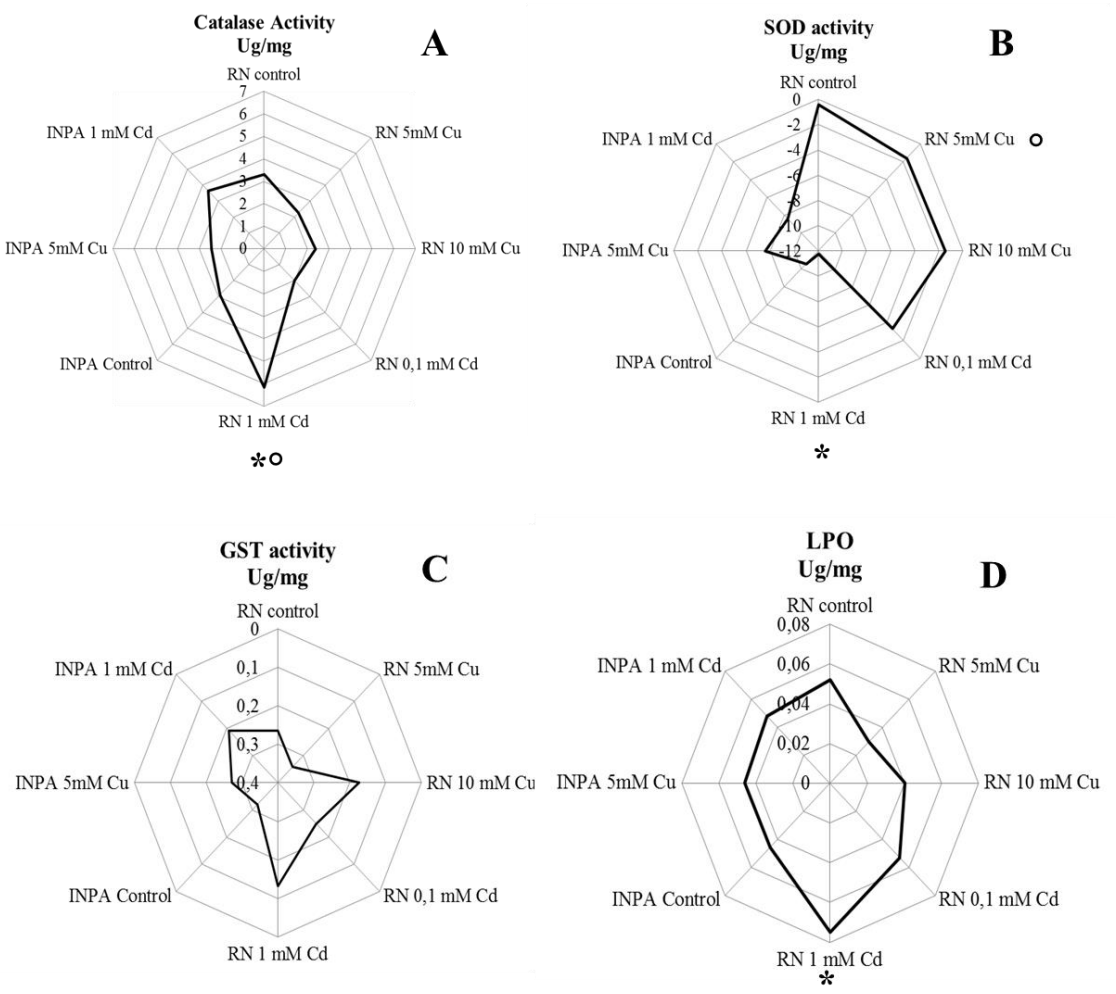

Figure 5. Catalase activity (A); Superoxide dismutase (SOD) (B); Glutationa –s-transferase (GST) (C) and Lipid peroxidation (LPO) (D) in gills of *S. rhombeus* kept under control and exposed to different concentrations of Copper and Cadmium with Rio Negro Water and INPA water. Results are represented as means ± SE. * indicate significant differences between the groups against the control and ° indicate significant differences between water ($p<0,05$).

Figure 6. Photomicrographs of *Serrasalmus rhombeus* tissues gills stained with HE from control group using Rio Negro (A-B) and INPA water (C-D). Normal arrangement of primary lamellae (PL) and secondary lamellae (SL) with all cell types, 1 – Pavement cells, 2 – Pillar cell and 3 – Chloride cell.

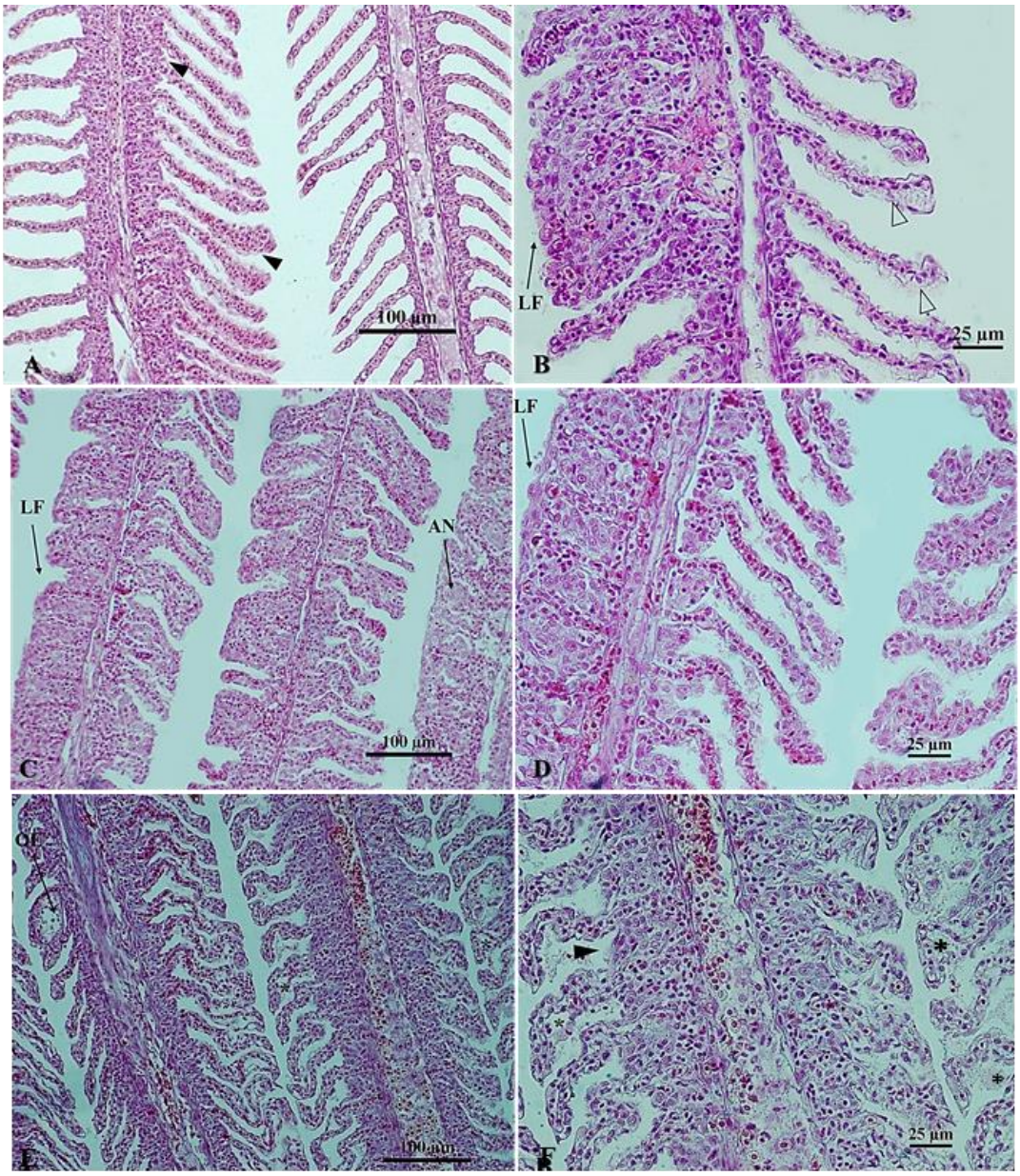

Figure 7. Photomicrographs of *Serrasalmus rhombeus* tissues gills stained with HE exposed to copper with different concentrations, RN 5 µM (A-B), RN 10 µM (C-D) and INPA 5 µM (E-F). Cellular proliferation on initial stage (full arrow head), lamellar fusion (LF), Desquamation (empty arrow head), necrosis (*) and aneurism (AN).

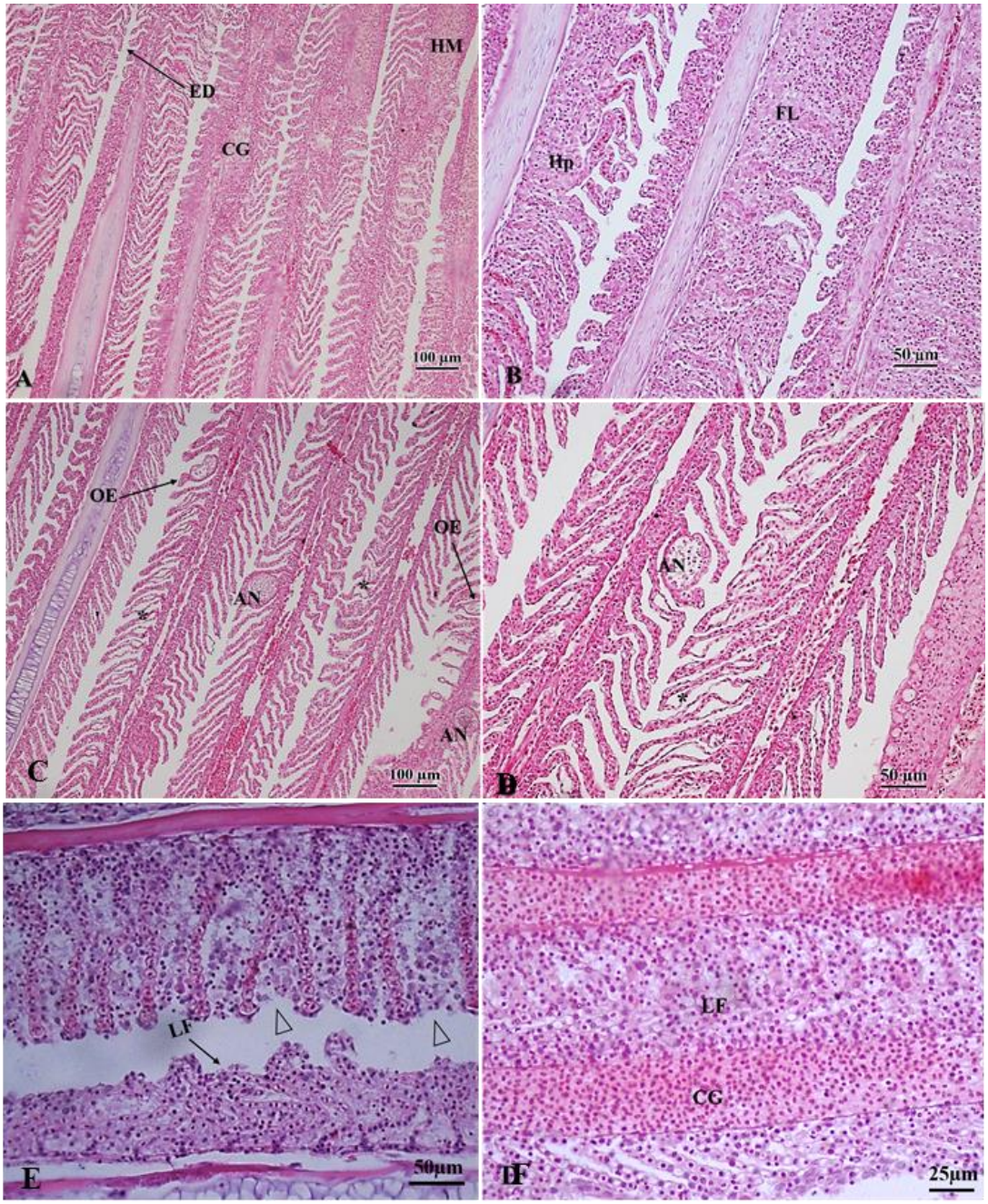

Figure 8. Photomicrographs of *Serrasalmus rhombeus* tissues gills stained with HE exposed to cadmium with different concentrations, RN 0,1 µM (A-B), RN 1 µM (C-D) and INPA 1 µM (E-F). Intense cellular proliferation (full arrow head), Oedema (OE), Detachment of the respiratory epithelium, lamellar fusion (LF), Congestion of the filaments (CG) and aneurism (AN).

CONSIDERAÇÕES FINAIS

A proposta do trabalho foi avaliar a qualidade ambiental de rios Amazônicos em nível biológico. De acordo com os dados bioquímicos e morfológicos foi possível diferenciar as diferentes áreas estudas. Sendo assim, os biomarcadores utilizados foram eficazes na avaliação dos rios Itacaiunas, Tropas e Crepori. A alta incidência de alterações histopatológicas e bioquímicas nas piranhas coletados indicam claramente o comprometimento da saúde desses peixes e da qualidade da água deste local. O nível das respostas biológicas observadas mostra que os peixes estão sofrendo os efeitos dos poluentes presentes na água e sedimento. Os dados de exposição aguda apontam que o cádmio é um metal altamente tóxico ainda com a proteção natural por matéria orgânica dos rios.