UNIVERSIDADE FEDERAL DO PARANÁ

ANA CAROLINA DE DEUS BUENO KRAWCZYK

BIOMONITORAMENTO DA BACIA HIDROGRÁFICA DO MÉDIO RIO IGUAÇU EM UNIÃO DA VITÓRIA, PR, UTILIZANDO BIOMARCADORES DE CONTAMINAÇÃO AMBIENTAL

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ecologia e Conservação, Área de Concentração em Conservação do Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial para obtenção do grau de Doutor em Ecologia e Conservação.

Orientadora: Dra. Helena Cristina da Silva de Assis.

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UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **ANA CAROLINA DE DEUS BUENO**, intitulada: "**Biomonitoramento da bacia hidrográfica do médio Rio Iguaçu em União da Vitória, PR, utilizando biomarcadores de contaminação ambiental.**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

Curitiba, 29 de Abril de 2016.

Prof HELENA CRISTINA DA SILVA DE ASSIS (UFPR)
(Presidente da Banca Examinadora)

Prof ANA TEREZA BITENCOURT GUIMARÃES (UNIOESTE)

Prof CESAR APARECIDO DA SILVA (UFPR)

Prof IZONETE CRISTINA GUILOSKI (UFPR)

Prof RAFAEL BUENO NOLETO (UNESPAR)

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"Porque eu bem sei os pensamentos que tenho a vosso respeito, diz o Senhor, pensamentos de paz, e não de mal, para vos dar o fim que esperais."
Jeremias 29:11

RESUMO

A maioria dos corpos hídricos brasileiros recebe despejo inadequado de esgoto e resíduos agrícolas e industriais. Na região do Médio Iguaçu, a atenção voltada para o Rio Iguaçu é de cunho econômico e ambiental, principalmente pelo fato de a água ser utilizada no abastecimento público. Assim, avaliações da integridade ambiental neste rio são essenciais para entendimento de possíveis fontes poluidoras que possam prejudicar a qualidade da água que é utilizada e os xenobióticos que se destacam no comprometimento da biota. O primeiro objetivo deste trabalho foi monitorar a resposta da espécie *Astyanax bifasciatus* por meio de um conjunto de biomarcadores bioquímicos no fígado, no cérebro e na musculatura axial e biomarcador genético nos eritrócitos, aliados a análises físicas e químicas da água, incluindo contaminantes emergentes, no Rio Iguaçu, na região do Médio Iguaçu. Além disso, foram utilizados testes ecotoxicológicos com *Daphnia magna* e *Desmodesmus subspicatus* para a compreensão de efeitos de toxicidade da água sobre outros organismos. Desta forma, a qualidade da água desta região foi avaliada durante três estações sazonais para compreender as alterações na biota entre estações sazonais e a ação antrópica. O segundo objetivo foi avaliar efeitos subletais em peixes saudáveis expostos à água do Rio Iguaçu. Neste contexto, foi feita análise de metal na água e no músculo dos animais. Os resultados confirmaram a importância da utilização dos biomarcadores para avaliar efeitos de contaminação ambiental na biota aquática, e, também, demonstraram que a água do rio apresenta xenobióticos que desencadeiam efeitos de neurotoxicidade e estresse oxidativo nos peixes. As atividades das enzimas acetilcolinesterase, glutationa, glutationa S-transferase e glutationa peroxidase foram as que responderam às alterações entre estações sazonais em adição aos agentes estressores na água. A cafeína foi o contaminante emergente identificado durante o biomonitoramento, caracterizando poluição aquática por efluente doméstico. No tecido dos animais analisados no bioensaio, verificou-se acúmulo de alumínio, metal sem consideração em pescado na legislação brasileira. Os peixes expostos apresentaram resultados semelhantes em relação às repostas de neurotoxicidade, estresse oxidativo e danos de membrana, o que foi atribuído à qualidade da água e à possível mistura de contaminantes disponível à biota. O estudo mostra a necessidade de se discutir as fontes poluidoras nos limites jurisdicionais, bem como maneiras de mitigar a poluição aquática nesta região.

Palavras-chave: bioensaio, ecotoxicidade, contaminantes emergentes, metais

ABSTRACT

Most Brazilian water bodies receive inappropriate disposal of sewage and agricultural and industrial waste. In the Middle Iguaçu region, the focus is of economic and environmental nature, mainly because of the fact that the water is used for the public supply. Therefore, evaluation of the environmental integrity in this river are essential to understand the possible pollution sources that might affect the quality of the water that is used and the xenobiotics that stand out in the commitment of the biota. The first goal of this study was to monitor the response of *Astyanax bifasciatus* specie through a set of biochemical biomarkers in liver, brain and axial muscle and genetic biomarker in erythrocytes, together with physical and chemical analysis of the water including emerging contaminants in the Iguaçu River, in the Middle Iguaçu region. Furthermore, ecotoxicological tests with *Daphnia magna* and *Desmodesmus subspicatus* were used to understand toxic effects of the water in other organisms. Thus, the water quality was evaluated during three seasonal seasons to understand the changing in the biota among seasonal seasons and anthropic action. The second goal was to evaluate sublethal effects in healthy fish exposed to the Iguaçu River's water. In this context, metal in the water and in the animals muscle was analyzed. The result confirmed the importance of the biomarkers use to evaluate environmental pollution effects on aquatic biota, and also showed that the river's water has xenobiotics that causes neurotoxicity effects and oxidative stress in fish. The activities of acetylcholinesterase, glutathione, glutathione S-transferase and glutathione peroxidase responded to seasonal changes in addition to stressor agents in water. Caffeine was the identified emergent contaminant during the biomonitoring, characterizing aquatic pollution by domestic wastewater. In analyzed animal tissues was verified aluminum concentration, a metal without considerations to fish in Brazilian legislation. The exposed fish demonstrated similar responses to neurotoxicity, oxidative stress and membrane damage, which was attributed to water quality and the possible mixture of contaminants available to the biota. The study shows the necessity of discussing the pollution sources in the jurisdictional limits, as well as the ways to mitigate water pollution in the region.

Keywords: bioassay, ecotoxicity, emerging contaminants, metals.

LISTA DE SIGLAS

AChE - Acetylcholinesterase

AMN – Morphological abnormalities and micronucleous frequency

CAS – Chemical Abstracts Service

CAT – Catalase

CEMA – Conselho Estadual do Meio Ambiente (State Council on the Environment)

CONAMA - Conselho Nacional do Meio Ambiente (National Environmental Council)

ECA – Comet assay

EROS – Espécies reativas de oxigênio (Reactive oxygen species)

GPx – Glutathione peroxidase

GSH - Glutathione

GST- Glutathione S-transferase

IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis (Brazilian Institute of Environment and Renewable Resources)

IBGE – Instituto Brasileiro de Geografia e Estatística (Brazilian Institute of Geography and Statistics)

IQA - Índice de Qualidade da Água (Water Quality Index)

LPO – Lipoperoxidation (Lipoperoxidação)

ROS – Reactive oxygen species

SOD – Superoxide dismutase

SUMÁRIO

CAPÍTULO II

1 INTRODUÇÃO GERAL

1.1 Poluição dos ecossistemas dulcícolas e a Ecotoxicologia

O desenvolvimento industrial intenso e a urbanização muito próxima aos corpos hídricos permitem que várias substâncias químicas sejam incorporadas ao ciclo dos rios (VAN der OOST et al., 2003). As origens dos descartes destes compostos e resíduos químicos envolvem fontes industriais, domésticas e da agricultura, mais comumente citadas, além de um complexo montante de produtos farmacêuticos e de higiene pessoal (TERNES et al., 1999; KOLPIN et al., 2002; BOXALL et al., 2012), que têm demonstrado alterar significativamente os organismos e populações a longo prazo, em especial pelo potencial em alterar o processo reprodutivo (SILVA de ASSIS et al., 2013).

A estimativa de disponibilidade de substâncias químicas nos ecossistemas hídricos aumenta a cada dia, e do montante final, uma parcela muito reduzida tem os efeitos tóxicos conhecidos e/ou divulgados (ZAGATTO e BERTOLETTI, 2006). A grande questão envolvida nisso é que a poluição no ambiente aquático ocorre potencialmente pelos compostos antropogênicos, de forma pontual e/ou difusa, no entanto, os efeitos ecotoxicológicos são pouco compreendidos, pois falta linearidade entre a poluição ambiental e a resposta biológica dos organismos que são constantemente expostos a ela (BUCHELI e FENT, 1995).

O Ministério da Saúde (MS) e o Conselho Nacional do Meio Ambiente (CONAMA) estabeleceram procedimentos e responsabilidades em relação ao controle e vigilância da qualidade da água para o uso humano, a partir da portaria federal 518 (de 25 de março de 2004) e das resoluções 357 (de 17 de março de 2005) e 430 (de 13 de maio de 2011). Além de garantir a potabilidade da água, a portaria e as resoluções visam assegurar o padrão de qualidade no lançamento de efluentes, de forma que haja garantia e preservação da vida aquática.

Mesmo com a padronização da legislação, estudos que envolvem a compreensão da toxicidade de substâncias e elementos químicos são essenciais para a detecção de respostas biológicas frente a um quadro de contaminação, permitindo o entendimento do impacto ambiental sobre células, tecidos, órgãos e

perturbações metabólicas diversas (PANDRANGI et al., 1995). Isto porque há várias interações e efeitos que os xenobióticos podem causar, pois dependerá da duração e persistência do composto, sua amplitude e potencial em alterar os sistemas biológicos. Por isso, os estudos em Ecotoxicologia são uma busca na interpretação da interação contaminante – ecologia dos ambientes aquáticos – biota. A definição de Plaa (1982) para Ecotoxicologia é "*ciência que estuda os efeitos das substâncias naturais ou sintéticas sobre os organismos vivos, populações e comunidades, animais ou vegetais, terrestres ou aquáticas, que constituem a biosfera, incluindo assim a interação das substâncias com o meio nos quais os organismos vivem num contexto integrado*". Estudos nesta área foram impulsionado após a década de 70, com a criação de leis estaduais e federais nos Estados Unidos (EUA), período em que foi estabelecida a Agência de Proteção Ambiental *(Environmental Protection Agency* – EPA), e desde então, várias regulamentações, contribuições e estudos na área têm ocorrido visando compreender a interação entre os organismos e os xenobióticos disponíveis nos ambientes.

Como os ecossistemas dulcícolas são o depósito final de uma miríade de poluentes, a biota fica suscetível às diferentes formas de contaminação, com comprometimentos que poderão ser deletérios sobre as populações após longos períodos. Este entendimento dos efeitos em exposições simultâneas é um dos desafios atuais em ecologia de ambientes aquáticos, e embora o monitoramento ambiental tradicional envolva várias ferramentas de análise de integridade ambiental que identificam a contaminação e, muitas vezes, sua fonte, dificilmente estabelece uma conexão entre a saúde da biota e os poluentes disponíveis (ZHOU et al., 2008). Desta forma, a necessidade de avaliar os impactos sob a ótica da condição dos organismos, os biomarcadores servem como uma resposta dos organismos ao agente tóxico, pois representam variações bioquímicas, celulares, fisiológicas ou comportamentais que podem ser avaliadas nos tecidos ou nos fluídos dos organismos (DEPLEDGE, 1993), demonstrando diferentes respostas a diferentes estressores ambientais (HUGGET et al., 1992).

1.2 Contaminantes nos corpos hídricos

Os metais pesados representam uma forma de risco ao meio ambiente e à saúde humana, por causa da sua toxicidade, persistência, degradação abiótica e efeitos de bioacumulação (BONANNO e GIUDICE 2010; FRANCO-URÍA et al., 2010). Além da preocupação em relação à presença dos metais nas águas superficiais, o acúmulo destes compostos no sedimento e sua disponibilidade para os organismos bentônicos, destacam a necessidade de estudos que demonstrem os efeitos dos diferentes metais sobre os organismos expostos cronicamente a eles (ISLMAM et al., 2015; SIMONATO et al., 2016).

A expansão das diferentes atividades domésticas, industriais e de agricultura caracterizam bem os efeitos da superpopulação e os resíduos de produção que vão para os rios, os reservatórios finais destes processos (ISLAM et al., 2014). Os rios em áreas urbanas têm sido constantemente associados a problemas na integridade ambiental por causa da falta de tratamento dos efluentes industriais e tratamento inadequado do esgoto que favorecem a entrada de metais na água (VENUGOPAL et al., 2009).

A permanência e biodisponibilidade dos metais na água dos rios ocorre por conta dos seus múltiplos mecanismos de toxicidade, que envolve a alta afinidade dos íons metálicos por moléculas com átomos de nitrogênio e enxofre. Assim, podem agir bloqueando ou modificando a conformação ativa das biomoléculas, interferindo nas reações enzimáticas, além do tamanho reduzido, que faz com que os metais potencialmente alterem o metabolismo do organismo contaminado (CONNELL e MILLER, 1984). Além disso, os metais podem formar os organometálicos, por metilação, que são ligações covalentes com o grupo metil (-CH_3). Estes compostos apresentam tendência à lipossolubilidade, facilitando o movimento pelas membranas, o que garante a integridade da conformação do organometálico bem como sua distribuição nos diferentes compartimentos.

Vários estudos visam demonstrar o efeito das diferentes concentrações traço dos metais na água dos rios, tentando estabelecer conexões entre as concentrações verificadas e os efeitos sobre a biota (DE JESUS et al., 2014; BO et al., 2015; ISLAM et al., 2015). Cada vez mais estes estudos são imprescindíveis para o alerta referente aos riscos ambientais que os metais podem causar, bem como o risco do consumo de pescados.

Outro aspecto importante em relação à poluição aquática são os chamados micropoluentes ou contaminantes emergentes. Vários países têm gerenciado programas de monitoramento e tratamento da qualidade da água visando à detecção de fármacos e produtos de higiene pessoal nos corpos hídricos, principalmente os que são explorados para o abastecimento público, mas esta forma de tratamento no Brasil é inexistente.

Estes compostos são chamados de micropoluentes por conta da detecção em concentrações que variam de $ng.L^{-1}$ a $\mu g.L^{-1}$, e, também, são conhecidos como contaminantes emergentes porque até há pouco tempo não eram considerados ou detectados no ambiente aquático (devido às baixas concentrações). A partir de estudos e metodologias específicas para a detecção destes micropoluentes em ecossistemas aquáticos, no solo e no ar, tem-se associado a presença deles a vários efeitos adversos à biota e à saúde humana (STUART et al., 2012; JIANG et al., 2013; RIBAS et al., 2015; GUILOSKI et al., 2015; GHELFI et al., 2016). A falta de remoção destes compostos nos sistemas de tratamentos de efluente, e a consequente eliminação dos efluentes nos corpos hídricos subsidia sua ocorrência em águas superficiais (RAHMAN et al., 2009; GUILOSKI et al., 2013), o que é preocupante no tocante à biota e à saúde humana.

Além disso, a disponibilidade destes químicos tem aumentado em ordem exponencial, pois há aproximadamente 81 milhões de substâncias químicas conhecidas e registradas no *Chemical Abstracts Service* (CAS). Destas, a maioria é usada pela população em higiene pessoal, produtos de limpeza, medicamentos e alimentos, o que dificulta a restrição de uso e, principalmente, o controle do descarte do resíduo (IDE, 2014).

Geralmente as estações de tratamento de esgoto utilizam somente a remoção de matéria orgânica e nutrientes, mas isto não é suficiente porque estes contaminantes têm baixa biodegradabilidade (LE-MINH et al., 2010; AL AUKIDY et al., 2012).

Estudos prévios realizados na bacia do Rio Iguaçu demonstraram que há a presença de cafeína, produtos de higiene pessoal e estradiol (MACHADO, 2010; KRAMER, 2012; IDE et al., 2013; OSAWA, 2013). Isto intensifica a importância de estudos que permitam quantificar as concentrações destes contaminantes presentes na água utilizada para abastecimento público bem como a compreensão dos efeitos dos contaminantes para a biota presente no ecossistema aquático.

1.3 Modelo biológico – *Astyanax bifasciatus*

Dos grupos animais aquáticos, os peixes são importantes modelos biológicos para estudos ecotoxicológicos, pois são diversos e abundantes, ocupam diferentes nichos, são utilizados na dieta humana e, como estão nos corpos hídricos representam, junto a estes, os receptores finais das variadas demandas de poluição. Embora vários estudos sejam desenvolvidos com espécies tropicais e endêmicas, os efeitos da mistura de contaminantes disponível nos corpos hídricos brasileiros sobre as espécies nativas é incipiente, por isso o desenvolvimento de trabalhos com a utilização de modelos biológicos com esta característica é essencial.

Os lambaris, *Astyanax bifasciatus* (Teleostei:Characidae), representam um grupo de espécie endêmica da bacia do Rio Iguaçu. Como são peixes pequenos, não agregam valor comercial, entretanto são bastante utilizados no consumo humano e na prática de pesca. Estes animais são estritamente ocorrentes em água doce, possuem escama, são relativamente pequenos (em média apresentam até 10 cm de comprimento total) e sua coloração é variada, mas, em geral, estes animais apresentam similaridades morfológicas que dificultam sua separação em grupos específicos (MELO, 2001) (Figura 01). São forrageadores de todos os níveis tróficos e mudam de hábito alimentar conforme a disponibilidade do meio e em resposta às alterações ambientais do ecossistema (LOBÓN-CERVIÁ e BENNEMANN, 2000).

A característica do forrageamento é essencial no equilíbrio dos ecossistemas aquáticos, pois estes organismos participam da mineralização para os níveis tróficos superiores. Já a plasticidade ecológica em relação aos hábitos alimentares é dominante sobre a utilização deste grupo como bioindicadores, pois geram resposta às alterações ambientais (BARBIERI et al., 1992; BETTIM et al., 2016).

Figura 1: Exemplar de *Astyanax bifasciatus* utilizado como modelo biológico tanto no biomonitoramento quanto no bioensaio.

1.4 Biomarcadores

Nos últimos anos, a utilização de biomarcadores tem sido reconhecida, aplicada e difundida nos estudos ambientais, e representam uma ferramenta de prevenção na relação poluição-organismo, permitindo que, quando observadas, algumas alterações verificadas nos organismos possam ser reversíveis aos demais níveis ecológicos (BONNINEAU et al., 2012). Neste caso, quanto maior for o número de biomarcadores utilizados, melhor será a interpretação do comprometimento gerado pelos poluentes aos organismos, populações, comunidades e ecossistemas (HUGGET et al., 1992).

Pela teoria dos biomarcadores os efeitos em níveis superiores são sempre iniciados em modificações em processos biológicos de níveis mais baixos, como alterações morfológicas em níveis celulares, teciduais e molecular, ou expressão de proteína e alguma atividade enzimática (BAYNE et al., 1985; WALKER et al., 1996). Assim, a utilização destas ferramentas nas avaliações de integridade ambiental demonstram as respostas subletais que preconizam os danos em maior escala, quando o risco já é iminente por conta da poluição.

Vários estudos têm sido desenvolvidos na análise de integridade ambiental de ambientes dulcícolas utilizando biomarcadores de contaminação ambiental (ARELLANO et al., 1999; ABDEL-MONEIM et al., 2012; AZEVEDO et al. 2013). A

utilização dos peixes se dá porque eles apresentam uma resposta semelhante aos demais vertebrados, quando expostos a estresse ambiental, além de serem veículo de transferência de contaminantes, via cadeia trófica, aos seres humanos (AL-SABTI e METCALFE, 1995).

Mesmo em rios com contaminação pouco evidentes, uma exposição a longo prazo pode prejudicar a saúde da biota (NIPPER et al., 1998), e tais efeitos subletais precisam ser mais conhecidos, porque poderão gerar efeitos de mortandade nos ecossistemas aquáticos. Isto porque naturalmente os ecossistemas lóticos são representados por hábitats sujeitos a mudanças ao longo de seu curso como condições físicas e químicas, sazonais ou não, e a comunidade biótica está sempre sujeita a tais mudanças (VANNOTE et al., 1980).

Há vários tipos de biomarcadores que podem ser empregados para avaliar efeitos de exposição dos organismos aos diferentes tipos de poluentes específicos e às misturas que ocorrem no ambiente como os bioquímicos, os genéticos, morfológicos e hematológicos que têm sido usados (GUILOSKI et al., 2013; SILVA de ASSIS et al., 2013; ROSSI et al., 2014; BUENO-KRAWCZYK et al., 2015, SERIANI et al., 2015).

1.4.1 Biomarcadores bioquímicos

Os biomarcadores bioquímicos podem ser considerados indicadores precoces de alterações ambientais, visto que refletem a condição saudável dos organismos estudados nos mais baixos níveis de organização, além de refletirem uma resposta rápida ao estresse ambiental ao qual os organismos estejam submetidos (HUGGET et al., 1992). Nesta perspectiva, o Brasil, assim como outros países europeus e norte-americanos, tem desenvolvido pesquisas no sentido de padronizar o estudo destes biomarcadores em espécies nativas que possam ser utilizadas no biomonitoramento de corpos hídricos (ROSSI et al., 2014; BETTIM et al., 2016).

A transformação metabólica (biotransformação) é um dos processos considerados resposta dos organismos aos compostos químicos, e envolve a neutralização do composto químico em contato com o metabolismo animal, ou seja, este processo visa neutralizar a interação entre os elementos químicos e as células.

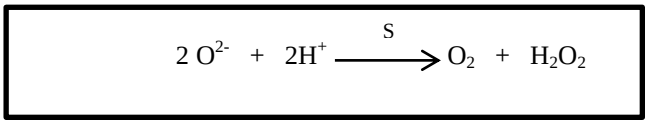
Há vários sistemas enzimáticos envolvidos para que esta neutralização ocorra, que incluem as enzimas das reações iniciais (fase I), que são as monooxigenases flavoproteínas e heme proteínas. As enzimas do CYP450 são heme proteínas que metabolizam os compostos da forma lipofílica a hidrofílica, e envolvem as reações da fase inicial de desintoxicação e excreção, porém podem liberar metabólitos reativos ou tóxicos (STEGEMAN e HAHN, 1994).

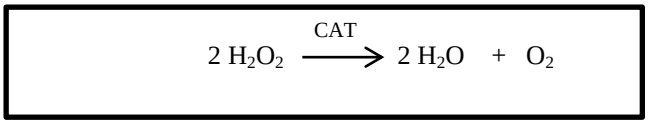
As enzimas de fase II podem ser chamadas de biomarcadores de exposição e/ou de efeito, porque podem ser alteradas por vários xenobióticos diferentes. Neste caso, tem-se a GST (Glutationa S-transferase), essencial neste mecanismo, pois conjuga com a GSH (Glutationa) os compostos lipofílicos, tornando-os hidrofílicos, para facilitar a eliminação (KIM et al., 2010). Além disso, a GSH atua na eliminação das espécies reativas de oxigênio (EROS) (RIOL et al., 2001).

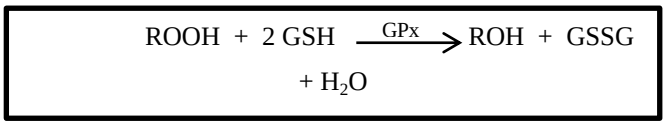
As EROS são citotóxicas e o produto do oxigênio molecular pode reagir com macromoléculas gerando, como consequência, a inativação enzimática, a peroxidação lipídica, danos à molécula de DNA e morte celular. Assim, as enzimas que são antioxidantes, são essenciais na preservação do metabolismo celular quando o organismo é exposto a um xenobiótico (VAN der OOST et al., 2003). Embora se saiba que alguns xenobióticos aumentam a liberação do ânion superóxido no organismo, e a forma como ele é citotóxico, não se pode desconsiderar que o radical hidroxila (OH^-) é mais reativo e pode formar-se pela fissão homóloga da ligação O-O da molécula de peróxido de hidrogênio (H_2O_2), pela simples mistura de H_2O_2 com sal de ferro (Reação de Fenton), pela interação do H_2O_2 com o O^{2-}, ou quando uma forma reduzida do cobre entra em contato com o H_2O_2 (HALLIWELL e GUTTERIDGE, 1999).

A enzima superóxido dismutase (SOD) é uma metaloenzima que catalisa a dismutação do radical superóxido (O^{2-}) formando como produto o peróxido de hidrogênio (H_2O_2) (1) que é, então, degradado pela ação da catalase (CAT) (2) ou pela glutationa peroxidase (GPx). A GPx degrada outros tipos de peróxidos também, e é importante na degradação de peróxidos lipídicos, evitando a lipoperoxidação lipídica, tendo como cofator para sua atividade a GSH (3) (HAYES et al., 1997; VAN der OOST et al., 2003).

(1)



(2) 

(3) 

O radical (R) pode ser um hidrogênio, um lipídio ou um éster.

Há três formas conhecidas de SOD: 1) a Cu, Zn-SOD, que é encontrada no citosol e no meio extracelular dos eucariotos; 2) a Mn-SOD, encontrada em bactérias e mitocôndrias, e 3) a Fe-SOD, presente exclusivamente em bactérias (HALLIWELL e GUTTERRIDGE, 2007).

Como citado anteriormente, a GPx age degradando o H_2O_2, mas também degrada outros peróxidos, incluindo hidroperóxidos, utilizando a GSH como como cofator, e tendo como produto a glutationa oxidada (GSSG) (HAYES et al., 1997). Justamente a característica de degradar outros peróxidos é que tem estabelecido esta enzima como um bom indicador de estresse oxidativo, pois amplia a avaliação do estresse celular (VAN der OOST et al., 2003).

A GSH atua como sequestradora de radicais, agindo na proteção celular contra danos oxidativos (CHANG et al., 2009). Este tripeptídeo (γ-glutamil-cisteinil-glicina) faz parte dos sistemas antioxidantes não enzimáticos, e é utilizada por enzimas como a GPx e a GST.

A peroxidação lipídica ou lipoperoxidação (LPO) é a perda da integridade da membrana celular e é um dos principais danos causados pelo estresse oxidativo. O que ocorre é que os grupos hidroperóxidos se ligam aos sítios hidrofóbicos dos ácidos graxos insaturados da membrana celular, alterando as membranas e lipoproteínas, além de formar radicais livres, que podem causar outras alterações. Este processo altera a permeabilidade da membrana, podem causar uma ruptura total seguida de morte celular.

1.4.2 Biomarcadores genéticos

Assim como os biomarcadores bioquímicos geram respostas que refletem a saúde dos organismos expostos a estresse ambiental, os biomarcadores genéticos

são de interesse por conta do potencial em demonstrar os danos associados com genotoxicidade. Este interesse se dá porque a consequência da exposição aos xenobióticos disponíveis nos corpos hídricos são defeitos de hereditariedade por causa de mutação, efeitos teratogênicos nas células gaméticas, redução drástica da densidade populacional, e carcinogênese (MITCHELMORE e CHIPMAN, 1998).

Vários biomarcadores têm sido usados para a avaliação da exposição dos organismos aos xenobióticos, dos quais destaca-se a frequência de micronúcleos e anormalidades morfológicas nucleares, e quebras do DNA.

A frequência de micronúcleos e anormalidades nucleares é avaliada pelo Teste do Micronúcleo, proposto originalmente por Schmid (1975) para testes com camundongos e adaptado por Hooftman e Raat (1982) para células sanguíneas de peixes utilizados em testes em laboratório.

Os micronúcleos em peixes podem ser visualizados nos eritrócitos, nas células branquiais, nas renais e nas hepáticas. Comumente, usa-se eritrócitos como ferramenta. São cromossomos inteiros ou parciais que não se incorporaram à célula filha no momento da divisão celular, e aparecem como uma estrutura pequena e arredondada, com aparência do núcleo celular, por isso o nome. As anormalidades morfológicas ocorrem pelo mesmo modo, porque como parte do material fica atrasada na mitose, faz com que o núcleo não seja oval e tenha alteração de formato (BOMBAIL et al., 2001).

O Ensaio Cometa ou Eletroforese em Gel (*Single Cell Gel Electrophoresis*) é uma das técnicas mais utilizadas em genotoxicidade por conta da sua capacidade na detecção de lesões mutagênicas (BELPAEME et al., 1998). Esta técnica investiga os impactos na integridade do DNA, reparo e recuperação nas diferentes espécies.

Diferentemente do Teste do Micronúcleo que precisa de células em proliferação para a realização do ensaio, o Ensaio Cometa pode ser realizado com qualquer célula e em qualquer condição, desde que ela seja uma célula nucleada (PANDRANGI et al., 1995). Mesmo assim, o ensaio é mais comumente realizado com células sanguíneas, embora possa ser realizado com outros tecidos, principalmente quando a intenção é detectar se há efeito genotóxico tecido-específico (MITCHELMORE e CHIPMAN, 1998). Há necessidade de cuidados com o material sanguíneo para que danos adicionais não ocorram ao DNA durante a manipulação do material, uma sugestão é o armazenamento do sangue em soro

bovino fetal, por 48 horas após a amostragem, em ambiente escuro sob refrigeração constante (RAMSDORF et al., 2009).

A investigação sobre poluição em ambientes aquáticos utilizando biomarcadores é essencial por permitir a verificação dos efeitos adversos à biota ainda nos primeiros estágios de contaminação. Em especial na região do Médio Iguaçu, onde ocorre a captação da água do rio para abastecimento público, os resíduos do tratamento de esgoto, resíduos da indústria de papel e celulose e tratamento insuficiente do esgoto para a demanda local constituem aspectos potenciais de poluição aquática.

1.5 Bacia do Médio Iguaçu

A bacia hidrográfica do Rio Iguaçu é o maior complexo hídrico do Estado do Paraná (72.000 Km^2), com cerca de 1.200 Km^2 em território argentino e 70.800 Km^2 em território brasileiro. Há ocupação de 53.330 Km^2 no estado do Paraná e 13.470 Km^2 no estado de Santa Catarina. Com direção geral leste-oeste, percorre 1.060 Km desde suas nascentes na vertente ocidental da Serra do Mar, próximo a Curitiba, até a foz no Rio Paraná. Seus principais afluentes são: o Rio Negro, Rio Potinga, Rio Claro, Rio Timbó, Rio da Areia, Rio Iratim, Rio Jordão, Rio Chopim e Rio Capanema. O Rio Iguaçu é um afluente do Rio Paraná e é formado pelo encontro do Rio Irai e Rio Atuba, na parte leste de Curitiba, na divisa com os municípios de Pinhais e São José dos Pinhais (SUDERHSA, 2000).

A região conhecida como Médio Vale do Iguaçu ou região do Médio Iguaçu comporta os municípios de União da Vitória (PR) e Porto União (SC), e é marcada por atividades econômicas como extração de areia, usina hidrelétrica, indústrias de papel e turismo. O abastecimento de água é feito por uma única empresa que faz a captação diretamente do leito normal do Rio Iguaçu.

O Rio Iguaçu apresenta o segundo pior Índice de Qualidade da Água (IQA) do país, perdendo somente para o Rio Tietê em São Paulo (IBGE, 2010). O IQA reflete a contaminação por esgoto sanitário e outros materiais orgânicos, além dos nutrientes e sólidos. Esta poluição inicia na região do Alto Iguaçu e permanece na região do Médio Iguaçu, e há indícios da sobrecarga orgânica no rio e do possível resquício dos efluentes industriais ainda nesse trecho (FREIRE et al., 2015).

2 HIPÓTESES

A utilização de espécies nativas para o entendimento dos efeitos dos possíveis poluentes dos corpos hídricos é essencial, principalmente no que diz respeito a rios que são utilizados para o abastecimento público. Desta forma, o presente estudo foi norteado por duas hipóteses que ficaram evidentes nos dois capítulos abordados, conforme destacadas a seguir:

Hipótese 1

As alterações sazonais ocorrentes no Rio Iguaçu, na região do Médio Iguaçu, juntamente com os efeitos da urbanização, agricultura e atividades industriais contribuem para a redução da integridade ambiental da água do rio, sendo tais efeitos perceptíveis na análise integrada de biomarcadores em *Astyanax bifasciatus* e análises físicas e químicas tradicionais da água mais análise de contaminantes emergentes e metais.

Hipótese 2

A exposição aguda de peixes saudáveis à água do Rio Iguaçu, na região do Médio Iguaçu, causa efeitos de estresse oxidativo e de neurotoxicidade correspondentes à poluição da água; e os peixes, pelas condições da água, apresentam acúmulo de metais no tecido muscular, o que pode subsidiar discussões sobre a qualidade de água captada para o abastecimento público da região do Médio Iguaçu.

3 OBJETIVOS

Esta tese foi dividida em dois capítulos em forma de artigo científico.

O **primeiro artigo** investigou um conjunto de biomarcadores bioquímicos no fígado (respostas antioxidantes e peroxidação lipídica), no cérebro e na musculatura axial (neurotoxicidade), e biomarcador genético nos eritrócitos (danos de DNA), integrados a análises físicas e químicas da água que incluíram as análises tradicionais mais a de contaminantes emergentes. O objetivo foi verificar a qualidade da água do Rio Iguaçu, na região do Médio Iguaçu, que é área de captação de água para abastecimento público.

No **segundo artigo** visou-se submeter peixes saudáveis à água dos diferentes pontos amostrais previamente estudados do Rio Iguaçu, na região do Médio Iguaçu, a fim de verificar os efeitos antioxidantes, de neurotoxicidade, de peroxidação lipídica e genotóxicos sobre os peixes, além do nível de metais nos tecidos musculares dos animais expostos.

CAPÍTULO I

Artigo submetido para a revista Chemosphere.

MULTIBIOMARKER IN FISH TO EVALUATE A RIVER USED TO WATER PUBLIC SUPPLY

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ABSTRACT

We aimed to evaluate the ecological integrity of a large river, which receives agricultural and urban effluents and is used to water public supply. The fish species *Astyanax bifasciatus* was used as bioindicator during winter and spring 2012, and summer 2013 at the Middle Iguaçu River basin in Paraná state, Brazil. Water chemical and physical measures and ecotoxicological tests were carried out as well biochemical and genetic biomarkers in sampled fish in each period. The studied area was divided in three sample points: SP1, located where the water is collected to public supply; SP2, located in an urbanized area, and SP3, located at an urbanized area with the discharge of the sewage treatment. Although water chemical and physical analyzes were range of the Brazilian law to hydric bodies, anticholinesterasic effects were found in winter, oxidative stress in summer and spring. The higher genotoxic effect was in winter to all sample points. The temporal variation in biomarkers and the detection of caffeine in the water call attention to the water quality in this river, mainly be used to public supply.

Keywords: ecotoxicology, biomarker, acetylcholinesterase, oxidative stress, biomonitoring

1 INTRODUCTION

The study of pollutants' contribution to the aquatic ecosystems, especially in those explored to water public supply is relevant, mainly because human activities can limit these natural resources (Osório et al., 2013) and compromise its use. Among the activities that impair water resources, industrial development and intense urbanization favors the introduction of a large volume of organic and inorganic xenobiotics (Van der Oost et al., 2003), whose discharge can affect both human population and aquatic biodiversity.

The presence of a xenobiotic in a segment of aquatic ecosystem does not suggest, by itself, injurious effect (Van der Oost et al., 2003), so in order to establish connections between the level of contamination and adverse effects on the organisms is necessary to understand the different adverse responses, which can be

measured by biomarkers of environmental contamination (Bucheli and Fent, 1995). The studies involving biomarkers present some advantages on the traditional monitoring, that considers just analytical methods to quantify some chemical and physical variables in the environment. The use of biomarkers can detect organism sublethal effects, demonstrates an integrative response to the mixture of water xenobiotics and favors the detection of risk assessments (Walker et al., 1996; Zhang et al., 2008). Following this perspective, an environmental risk assessment is a prediction of anthropogenic stressors impacts on population and communities, because of the multiple xenobiotics availability, beyond the concentrations that vary in time and space, characterizing a river as a fluctuate condition to organisms (Jager et al., 2014).

This variability justifies the use of biomarkers as predictive tool in field studies, and their importance of data regarding the health of aquatic biota and the effects to human population (Wapener et al., 2005; Ghisi et al., 2014). It is also important to use several biomarkers, which allows a suitable evaluation of xenobiotic responses in organisms (Cazenave et al., 2009; Guiloski et al., 2013), and the integrated analysis has been used to assess the ecological risk in rivers and bays (Tejeda-Vera et al., 2007; Cazenave et al., 2014).

For this study a freshwater endemic fish was used as bioindicator, *Astyanax bifasciatus* (Teleostei:Characidae). This fish genus has elevated richness in neotropical region, with distribution from the United States to Argentina (Silva et al., 2010). Besides this successful geographic distribution, this group is dominant in some watersheds, and in Iguaçu bay, because of its reproductive potential and trophic opportunism. Some characteristics as convenient size to experiments in lab, easy capture in river and omnivorism permit that these fish be used as bioindicators both in biomonitoring and in bioassays (Carrasco-Letelier et al., 2006; Rossi et al., 2011).

This study aimed to evaluate the ecological integrity of Iguaçu River in a segment that is used to water public supply during winter, spring and summer through water physical and chemical analysis, ecotoxicological test, besides the biochemical and genetics biomarkers.

2 MATERIAL AND METHODS

2.1 Study area

The study area was a longitudinal segment located in Middle Iguaçu Basin region, which is a part of Iguaçu River from South of Brazil. This region is very important to water public supply for nearly 100 thousand people, and this river also plays a key role in supplying water for industry and irrigation. The industrial activities include pulp and paper industries, that are distributed upstream and downstream the Middle Iguaçu Basin region, while the agricultural practices are developed massively upstream. This region also receives the residue from the sewage treatment downstream.

Three sample points were chosen to assess the different effects of xenobiotics. The sample point 1 (SP1) (26º 9’ 32.08” S and 51º 4’ 47.83” W) is the region where the water is collected to public supply, to be treated for drinking water. The sample point 2 (SP2) (26º 14’ 27.61” and 51º 2’ 53.99”) is a region with a massive urban occupation. The sample point 3 (SP3) (26º 14’ 58.25” S and 51º 6’ 31.80”) is characterized by a massive urban occupation with the sewage treatment discharge (Figure 1).

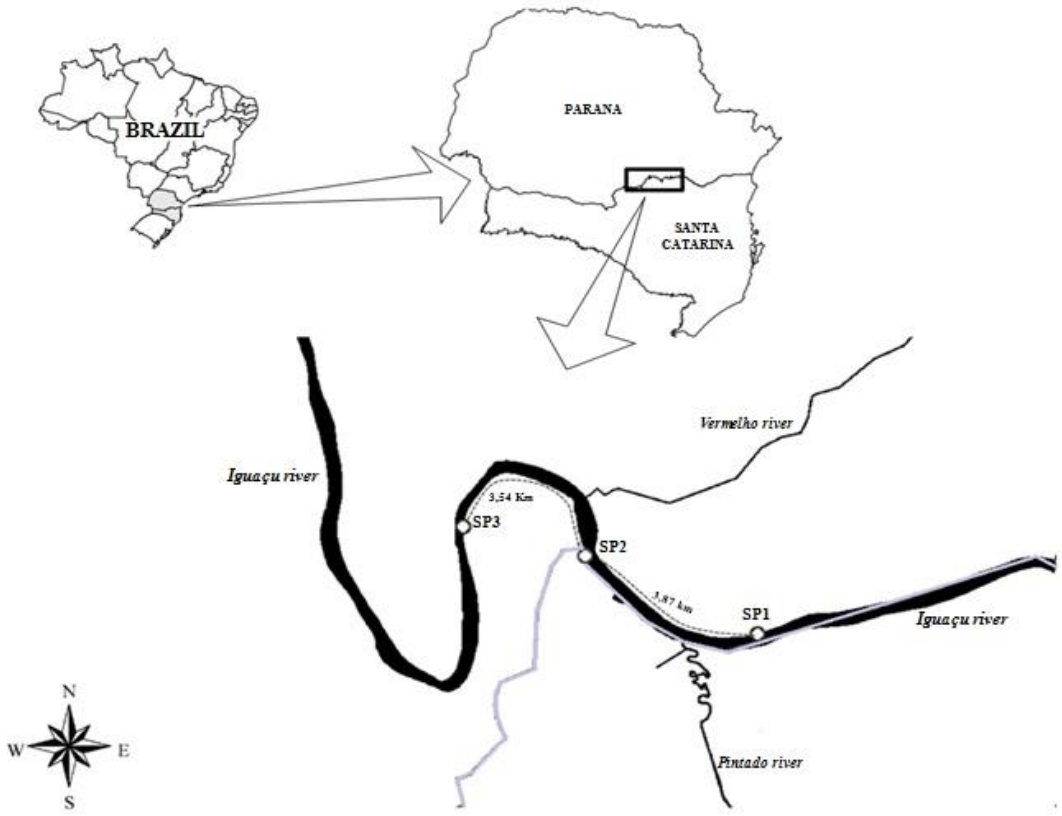

Figure 1. Study area in Middle Iguaçu Basin region in South Paraná state, Brazil. SP1: sample point 1, at region where the water is collected to public supply; SP2: sample point 2, urbanized area; SP3: sample point 3, urbanized area with sewage treatment discharge.

2.2 Water chemical and physical analyses

The water pH, dissolved oxygen (DO) ($mg.L^{-1}$), electrical conductivity ($\mu S.cm^{-1}$), redox potential(mV) and total dissolved solids (TDS)($mg.L^{-1}$) were measured by using a multiparameter probe Hanna 9898. The turbidity (NTU) ($mg.L^{-1}$), salinity (PSU) and resistivity (Ωm) were determined by a digital turbidimeter Hanna.

The water for chemical analyses was sampled from sub superficial water and stored in decontaminated (with 5% hydrochloric acid, rinsed with deionized water) polyethylene terephthalate 1Lbottle (ABNT, 1987). All the samples were preserved at 4ºC. The ammoniacal nitrogen ($N\text{-}NH_3$) (Fenato Method), nitrite ($N\text{-}NO_2$-) (Colorimetric method), nitrate ($N\text{-}NO_3$-) (Cadmium reduction method), N-total (Digestion persulfate), orthophosphate (ORP) (Ascorbic acid method) and phosphate

($P\text{-}PO_4^{3-}$) (Acid digestion) were carried out by APHA (2005). The dissolved organic carbon (DOC) was determined by HiperToc Thermo Scientific equipment.

The analytical method to identify and quantify 13 emergent contaminants to aquatic environment was developed by solid phase extraction (SPE) followed by Gas phase Chromatography Coupled to Mass Spectrometry (GC-MS/MS) or High Performance Liquid Chromatography (HPLC). It was 13 composts of different contaminant classes, in the same sample with only one extraction procedure. For the extraction of the emergent contaminants from water, 1 L of each sample was filtered throught 0.45 µm celulose acetate filters, the pH was ajusted to 3.0 and then pre-concentration was done using a method based on solid phase extraction (C18 cartridges). After the extraction, cartridges were eluted with acetonitrile and the final extract had a volume of 1 mL.

The emergent contaminants studied and analyzed by HPLC were: caffeine (CAF) (2.54 min; 273 nm), acetylsalicylic acid (AA) (3.68 min; 230 nm), salicylic acid (SA) (4.24 min, 230 nm), estradiol (EST) (6.99 min; 280 nm), ketoprofen (KET) (8.01 min; 254 nm), naproxen (NAP) (8.39 min; 230 nm), ethinyl estradiol (EES) (8.94 min; 280 nm) and estrone (EST) (10.4 min; 280 nm). HPLC analysis were carried out in an Agilent 1200 series LC chromatographic system equipped with a quaternary pump, a C18 column (250 mm, 4.6 mm, 5 µm) and a diode array detector. The mobile phase constituted of acidified water (pH 3.5) and acetonitrile in a proportion of 50% in a flow rate of 1.0 mL min^{-1}. The volume of injection was 5 µL.

The separation by GC-MS/MS was performed on a silica capillary column HP-5msi (30 m, 0.25 mm, 0.25 µm) on a model 7890A gas chromatograph (Agilent Technologies) coupled to a mass spectrometer triple quadrupole model 7000 autosampler (PAL sampler) to the contaminants: gemfibrozil (GEM), fenofibrate (FEN), 4-methylbenzylidene camphor (4-MBC), octylmethoxycinnamate (OC) and octocrylene (OCT). The temperature ramp was: 80 °C for 2 min increased to 280 °C at 15 °C min^{-1} and held for 3 min. The injector temperature was 280 °C, the transfer line, 280 °C and the ion source, 230 °C. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. A volume of 1 µL of the samples was injected in splitless mode. GC–MS/MS analysis was conducted using the electron impact ionization mode at 70 eV. The optimum conditions for each compound were applied in the selected reaction monitoring (SRM) mode (Supplementary material to access

the information about molar mass, retention time, precursor and product ion and colision energy of the selected compounds).

2.3 Toxicity factor

Daphnia magna (Crustacea:Cladocera) and *Desmodesmus subspicatus* (*Sphaeropleales:Scenedesmaceae*) ecotoxicological test were used to measure the toxicity of the river water from the three sample points. The tests were based on ABNT (2009, 2011). The toxicity factor (TF) was calculated according to the legislation CEMA (2010).

2.4 Fish sampling

The neotropical native fish *Astyanax bifasciatus* (Teleostei:Characidae) was sampled, in each sample point, during three seasonal periods along 2012 and 2013. These samples occurred in field during July/2012 (winter), November/2012 (spring) and February/2013 (summer). A total of 165 fish were sampled according to SISBIO license 35071-1. Fishing was carried out between 7:00am and 11:00am. After the fishing in each sample point, the fish were kept in a 20L buckets and immediately transported to the laboratory (10-15 minutes).

In the laboratory, fish were anesthetized; the total weight (TW) and standard length (SL) were measured and the blood was taken from caudal vein using a heparinized syringe for the Comet assay, and euthanized by cervical dislocation. Immediately, whole brain, liver, and a sample of axial muscle were removed and weighted, placed in cryotubes and stored in liquid nitrogen.

2.5 Condition factor

In order to assess the fish health in the studied river, a condition factor (CF) was measured. The CF was based on the regression curve from the data of mass (grams) and standard length (centimeter), and was calculated by the formula $CF = M*TL^b$, b is the slope of the generated curve.

2.6 Biomarkers

Brain and axial muscle were homogenized in phosphate buffer (0.1M) at pH 7.5 in a proportion of 1:10 (mass/volume) and centrifuged at 10.000x g during 20 minutes, at 4ºC. Acetylcholinesterase activity (AChE) was measured spectrophotometrically at 405nm, as proposed in Ellman et al. (1961), modified to microplate by Silva de Assis (1998). Each enzymatic assay was performed in triplicate.

Liver samples were homogenized in phosphate buffer (0.1M) at pH 7.0, and centrifuged at 15.000xg during 30 minutes, at 4ºC. Catalase (CAT) activity was measured at 240nm based on Aebi (1984). Superoxide dismutase (SOD) activity was measured at 440 nm according to Gao et al. (1998). Glutathione peroxidase (GPx) activity was measured at 340nm according to Paglia and Valentine (1967). GST activity was measured at 340nm (Keen et al., 1976). GSH concentration was obtained according to Sedlak e Lindsay (1968) and was measured at 415 nm. Lipoperoxidation analysis (LPO) was carried out using the ferrous oxidation xylenol assay and measured at 570nm (Jiang et al., 1992).

The tissue homogenate's protein concentration in liver, brain and axial muscle were carried out according to Bradford's method (Bradford, 1976), using bovine serum albumin as standard.

The Comet assay with peripheral blood (erythrocytes; ECA) was performed according to Speit and Hartmann (1999), modified by Cestari et al. (2004) and Ferraro et al. (2004).One hundred nucleoids were analyzed for each fish according to the visual classification based on the migration of DNA fragments from the nucleus. The results were categorized into classes according to Ramsdorf et al. (2009).

2.7 Statistical analysis

The data for the water physical and chemical analyses (temperature, pH, dissolved oxygen, conductivity, turbidity, orthophosphate, total dissolved solids, resistivity, salinity, ammoniacal nitrogen, nitrite nitrogen, nitrate nitrogen, phosphate, dissolved organic carbon, caffeine) was done according to the distribution pattern of the residue by the Shapiro-Wilktest and the homogeneity of variances by Bartlett test.

Once the data followed the statistical assumptions, the comparison was performed by ANOVA – one way just among sample points (SP1, SP2 and SP3).

The same procedure to the residue distribution pattern and homogeneity of variances were performed to biomarkers data (LPO, SOD, GSH, GST, GPx, CAT, AChE and ECA).These data comparison was performed by ANOVA– two way in sample points analyzes (SP1, SP2, SP3) and seasonal periods (Winter, Spring and Summer), followed by Tukey's test to unequal samples.

The somatic indexes were first tested for assumptions of normality and homoscedasticity by the Shapiro-Wilk test. Once these assumptions were satisfied; thus, ANOVA was used. Comparisons between sample points along the seasonal periods were performed using Tukey's test.

The matrices of biomarker data and the water physical and chemical data were analyzed separately by Principal Components Analysis (PCA) to verify association of it with the sample points. The correlation was evaluated by the sphericity Bartlett's test, and the inclusion of the variables in the model was considered by the Kaiser-Meyer-Olkin (KMO) criteria, which provides variables with KMO value greater than 0.5. The resultant factor loadings matrices of PCA's were associated among themselves by a Canonical Correlation Analysis to assess the association between biomarker data and water chemical and physical data.

Similarity matrices from the two original data, biomarkers and the water chemical and physical analyzes, were calculated by Euclidian distance. These matrices were associated by Mantel Test in order to verify a significant relation of biomarkers, water chemical and physical data after 5000 permutations and definition of the correlation coefficient (r). All tests were analyzed using 0.05 significance level.

3 RESULTS

3.1 Water chemical and physical analysis

The means and standard error of the water chemical and physical data corresponding to the three seasonal periods are shown in Table 1. It was not observed significant variation in data among sampling seasonal periods and sample

points. Caffeine was the only pharmaceutical detected at SP2 in spring, and the other pharmaceuticals were not detected in any of the water samples.

Table 1.Water chemical and physical data of sampling points. WT = water temperature (ºC); DO = dissolved oxygen ($mg.L^{-1}$); C = conductivity (cm^{-1}); Turb = Turbidity (NTU); ORP = organophosphate ($mg.L^{-1}$); TDS = total solid dissolved ($mg.L^{-1}$); Res = resistivity(Ωm); Sal = salinity (PSU); $N\text{-}NH_3$ = ammoniacal nitrogen ($mg.L^{-1}$); $N\text{-}NO_2^-$ = nitrite nitrogen ($mg.L_{-1}$); $N\text{-}NO_3^-$ = nitrate ($mg.L^{-1}$); $P\text{-}PO_4^{3-}$ = phosphate ($mg.L^{-1}$); DOC = dissolved organic carbon ($mg.L^{-1}$); CAF = caffeine ($ng.L^{-1}$).

Parameters	**SP1**	**SP2**	**SP3**	***p***
WT	19.87 ± 4.10	19.73 ± 4.13	20.17 ± 4.28	0.99
pH	7.87 ± 0.40	7.72 ± 0.29	7.84 ± 0.79	0.97
DO	6.03 ± 1.28	5.74 ± 0.61	6.48 ± 1.01	0.87
C	29.00 ± 9.85	27.33 ± 9.39	30.33 ± 10.81	0.97
Turb	67.27 ± 14.82	58.00 ± 18.18	64.43 ± 15.02	0.91
ORP	30.30 ± 62.54	32.87 ± 69.74	-2.23 ± 44.96	0.901
TDS	0.02 ± 0.00	33.68 ± 33.66	0.02 ± 0.01	0.422
Res	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.991
Sal	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	1.000
$N\text{-}NH_3$	0.15 ± 0.05	0.16 ± 0.06	0.10 ± 0.03	0.652
$N\text{-}NO_2^-$	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.992
$N\text{-}NO_3^-$	1.28 ± 0.37	1.31 ± 0.34	1.28 ± 0.33	0.998
$P\text{-}PO_4^{3-}$	0.05 ± 0.03	0.05 ± 0.03	0.06 ± 0.03	0.981
DOC	4.59 ± 1.53	13.99 ± 6.15	5.93 ± 1.57	0.326
CAF	0.00 ± 0.00	0.29 ± 0.29	0.00 ± 0.00	0.422

The values of abiotic variables are demonstrated in mean and mean standard error (*X* ± *MSE*).

3.2 Toxicity factor

The acute bioassay with *D. magna* and chronic bioassay with *D. subspicatus* demonstrated no pattern of toxicity to the three sample points at the analyzed periods, and they had the same toxicity factor result (TF=1).

3.3 Somatic indexes

Biological parameters of sampled fish are listed in Table 2. The CF of *A. bifasciatus* differed among sample points in spring (p<0.00) and summer (p<0.00). The sample points SP1 (p<0.00), SP2 (p<0.00) and SP3 (p<0.00) also showed significant statistical differences in CF values among the seasonal periods.

Table 2. Biological data of *Astyanax bifasciatus* sampled at the three sample points in winter, spring and summer.

Sampling Point	n	Weight (g)	Standard Length (cm)	CF
Winter				
SP1	20	12.2 ± 3.5	7.9 ± 0.3	7.5 ± 2.0
SP2	20	11.7 ± 2.1	8.1 ± 0.4	7.1 ± 1.2 **
SP3	20	12.2 ± 1.7	8.1 ± 0.4	7.4 ± 1.0
Spring				
SP1	15	9.9 ± 2.0	7.3 ± 0.7	6.2 ± 1.1 a *
SP2	15	11.6 ± 3.4	7.2 ± 0.7	7.3 ± 2.0 a
SP3	15	14.1 ± 2.1	7.7 ± 0.3	8.7 ± 1.2 b ***
Summer				
SP1	20	14.0 ± 4.6	8.6 ± 0.7	8.4 ± 2.5 a *
SP2	20	10.9 ± 1.5	8.0 ± 0.3	6.6 ± 0.9 b **
SP3	20	14.7 ± 3.1	8.6 ± 0.6	8.8 ± 1.7 a ***

The values of condition factor (CF) and biotic parameters are demonstrated in mean and mean standard deviation (X ± MSD).

Different letters indicate significant statistical difference among the sample points in each seasonal period.

* indicate significant statistical difference to each sample points along the seasonal periods.

3.4 Biomarkers

In general, there was a seasonal variation in muscle (AChE M) and brain (AChE B) AChE activities ($F_{4.70}$=7.928, p<0.00; $F_{4.72}$=7.702, p<0.00, respectively), and a higher activity was observed in summer to the three sampling points (p<0.05), and the lowest activities occurred in winter. In summer, these enzymes were spatially different (p<0.05), mainly because the fish in SP2 exhibited the lowest values of activity (Table 3).

The biomarkers LPO, GSH, GPx and GST presented a pattern in summer, which was not repeated in other seasonal periods. The GST activity showed the highest values in this season, while GSH, GPx and LPO showed the lowest ones. It demonstrates that the higher activity of the enzyme and the use of the cofactor avoided the oxidative damage in cellular membrane. The LPO was the one which presented the most significant statistical temporal variation ($F_{4.71}$=3.11; p=0.02), mainly in summer. It also demonstrated a spatial variation in spring, because SP3 had the lowest values registered to this season (p<0.05). In spring, the fish presented the highest values to GSH, although this biomarker ($F_{4.70}$=0.397; p=0.810), while GST had the lowest activities registered during this season. Both seasonal and spatial variations of these biomarkers demonstrated oxidative stress, which reduced the GST activity and elevated the GSH, being a potential cause of injury to the metabolism.

SOD did not show a significant temporal variation ($F_{4.69}$=1.444, p=0.229), while CAT activity was not spatially different in the seasonal periods, although a trend was observed ($F_{4.53}$=2.058; p=0.099). The activity increased in summer, especially in sample points SP2 and SP3.

There was no significant spatial variation to ECA during the seasonal periods ($F_{4.71}$=1.0962; p=0.36519), but the fish presented the lowest mean scores to genotoxic effects in spring and the highest ones in winter and summer (p<0.05).

Table 3. Biomarkers in *Astyanax bifasciatus* at the three sample points in winter, spring and summer.

Sample point	**SP1**			**SP2**			**SP3**		
Seasonal periods	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
n	10	7	10	9	7	10	10	7	10
LPO	29,00± 4,3^{ab}	30,78± 5,6 ^{a}	2,34± 0,2 ^{c}	22,88± 2,2^{ab}	24,96± 3,6^{ab}	3,48± 0,5 ^{c}	29,03± 4,7^{ab}	13,10± 1,6^{bc}	2,40± 0,1 ^{c}
SOD	137,43± 15,9 ^{abc}	111,57± 18,6 ^{abc}	72,86± 5,5 ^{c}	186,06± 29,7 ^{a}	151,24± 14,1 ^{abc}	165,18± 23,3 ^{ab}	172,71± 26,4 ^{ab}	169,28± 25,8 ^{abc}	91,01± 7,6 ^{bc}
GSH	10,19± 2,9 ^{ab}	12,36± 2,8 ^{ab}	5,54± 1,1 ^{ab}	19,79± 6,9 ^{a}	18,54± 2,0 ^{ab}	7,72± 1,4 ^{ab}	9,15± 1,9 ^{ab}	12,41± 4,0 ^{ab}	2,47± 0,7 ^{b}
GST	16,20± 2,3 ^{cd}	12,40± 1,5 ^{cd}	54,02± 7,7 ^{ab}	23,36± 5,2 ^{cd}	17,31± 2,9 ^{cd}	62,38± 9,4 ^{a}	12,51± 3,1 ^{d}	8,90± 0,7 ^{d}	37,81± 4,6 ^{bc}
GPx	16,41 ± 4,0 ^{ab}	12,28 ± 1,6 ^{abc}	1,87 ± 1,2 ^{c}	13,12 ± 3,1 ^{abc}	18,73 ± 5,0 ^{a}	1,04 ± 0,3 ^{bc}	13,74 ± 3,7 ^{abc}	11,45 ± 2,9 ^{abc}	1,49 ± 0,1 ^{bc}
CAT	32,15 ± 3,3 ^{a}	28,47 ± 1,3 ^{a}	30,34 ± 6,0 ^{a}	20,72 ± 6,6^{a}	21,59 ± 1,9 ^{a}	44,84 ± 5,5 ^{a}	25,83 ± 4,3 ^{a}	22,36 ± 3,5 ^{a}	43,82 ± 5,6 ^{a}
AChE M	91,32±7,43^{ab}	160,92±18,18 ^{b}	104,40±13,04 ^{ab}	86,66±7,31^{ab}	148,44± 14,62 ^{ab}	161,69±33,31 ^{b}	101,97±13,58 ^{ab}	132,36±15,01 ^{ab}	66,39±11,99 ^{a}
AChE B	83,52± 9,2^{b}	163,01± 12,1 ^{b}	473,64± 76,0^{a}	77,76± 8,5 ^{b}	143,95± 14,6 ^{b}	186,54± 10,5 ^{b}	94,00± 5,7 ^{b}	188,49± 29,7 ^{b}	435,64± 18,6^{a}
ECA	284,85±8,2^{ab}	214,21±8,0^{bc}	265,40±10,9^{a} ^{b}	305,80±10,5 ^{a}	180,14±10,8^{c}	267,22±21,9^{a} ^{b}	251,95±16,8^{a} ^{bc}	184,36±9,1^{c}	225,45±23,9 ^{bc}

The values of abiotic variables are demonstrated in mean and mean standard error (*X* ± *SE*).

Different letters (a,b,c) indicate significant differences over the seasonal periods at $p<0.05$. M=muscle, B= brain.

Lipid peroxidation (LPO) = nmol.mg protein^{-1}; superoxide dismutase (SOD) = U.mg protein^{-1}; reduced glutathione (GSH) = µg.mg protein^{-1}; glutathione S-transferases (GST) and glutathione peroxidase (GPx) = nmol.min^{-1}.mg protein^{-1}; Catalase (CAT) = µmol.min^{-1}.mg protein^{-1}; acetylcholinesterase (AChE) = nmol.min^{-1}.ml^{-1}.mg protein^{-1}and Comet assay (ECA) = score of DNA damage.

The association of the water physical and chemical data demonstrated that at least one measure of correlation was statistically significant (Bartlett test; x^2=129.91; *p*<0.05), the KMO criteria was 0.614, being higher than 0.5 as indicated for Hair et al. (2009).

The first axis explained 67.4% of variation among the variables (eigenvalue = 10.11), and demonstrated effects of the seasonality. In the same period, the temperature, conductivity and other ions were higher, the values of pH, resistivity and DO were lower. The second axis demonstrated an explicability of 18.6% of data variation (eigenvalue = 2.79), the high values of turbidity were directly related to CAF, DOC and nitrite (Table 4 and Figure 2).

Table 4. Factor loadings of the two first principal components of the water physical and chemical data.

	F1	**F2**
Temperature	0,986	0,032
pH	-0,905	0,080
DO	-0,940	-0,054
Conductivity	0,982	-0,025
ORP	0,935	-0,163
TDS	0,956	-0,098
Resistivity	-0,985	-0,059
Salinity	0,922	-0,340
$N\text{-}NH_3$	0,712	-0,227
$N\text{-}NO_3^-$	0,982	-0,131
$P\text{-}PO_4^{3-}$	0,970	-0,177
DOC	0,474	0,725
CAF	0,210	0,794
$N\text{-}NO_2^-$	0,370	0,805
Turbidity	0,199	0,853

The association of biomarkers was statistically significant (Bartlett test; x^2=153.38; *p*<0,0001), being the KMO criteria 0.707.

The first PCA axis had 31.6% of explanation to the data variation (eigenvalue=2.85). It's possible to note that when the LPO, GSH and SOD were higher, the cholinesterase and GST activities were lower. The second axis had an explanation of 14.46% of data variation (eigenvalue=1.30). It was observed when the GPx were higher, CAT activity and genetic damage were low (Table from the Supplementary information and Figure 3).

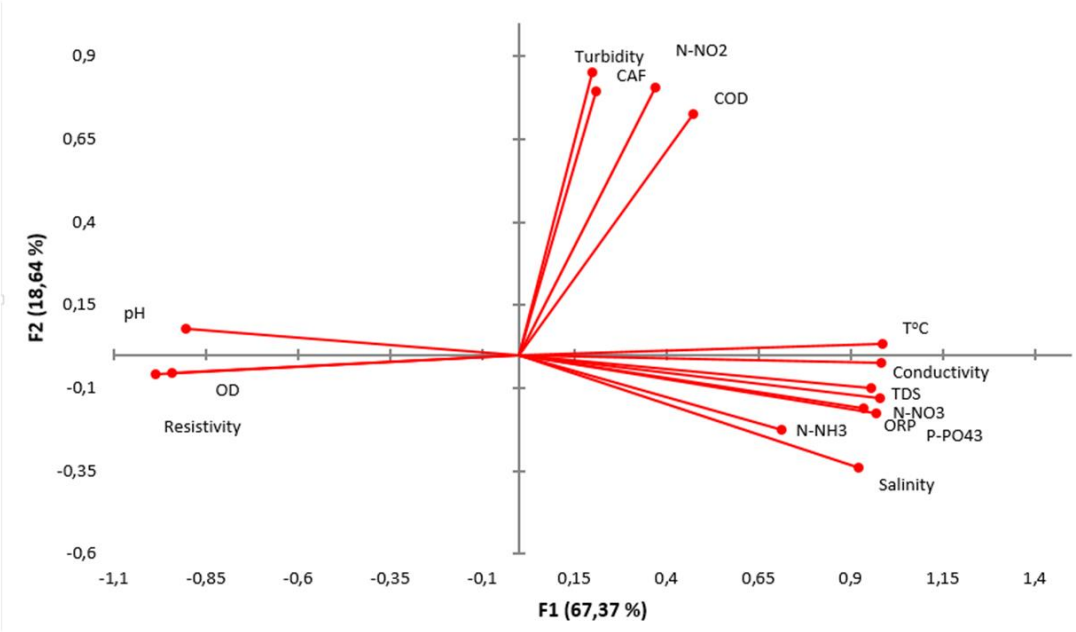

Figure 2. Seasonal pattern. Association of physical and chemical data among the sample points during winter, spring and summer.

The association of the two factorial loadings of the PCA by the indirect gradient analysis CCA demonstrated that the correlation of the two matrices showed a low explanation to variation data (28.57%). Mantel test also demonstrated that biomarkers data and chemical and physical data were not strongly related (r=-0.03; *p*=0.48).

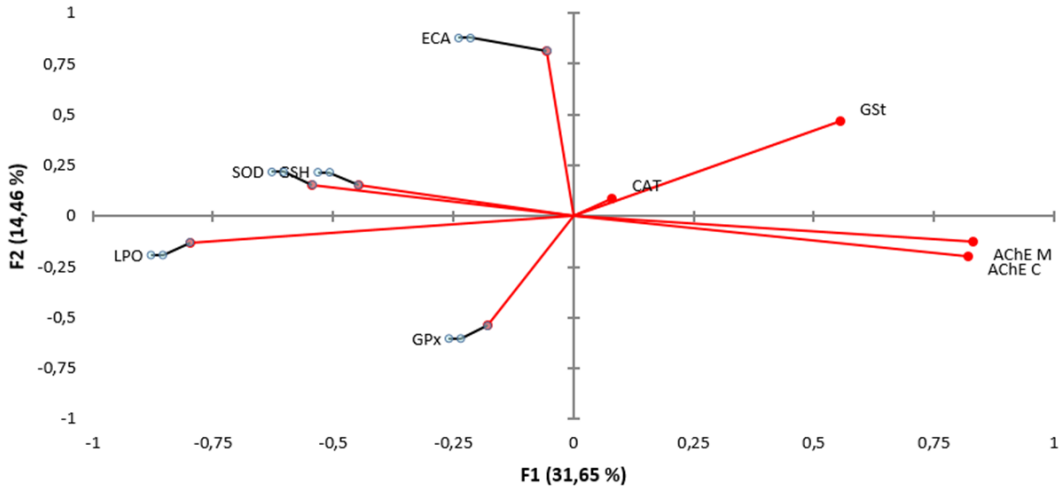

Figure 3. Association of matrices of biomarkers in *Astyanax bifasciatus* in winter, spring and summer.

4 DISCUSSION

This study represents the first assessment of ecotoxicological effects from this region used to water supply for nearly 100 thousand people.

When analyzing fish health through the condition factor, it was noted that there was a trend to increase it over the seasonal periods, except in SP2 in summer and SP1 in spring. Although the CF is a less sensitive biomarker to evaluation of xenobiotic effects (Mayer et al., 1992), it can be affected by illness and availability of nutrients, and support information about energy reserve. SP1 receives sewer and chemical waste contribution from Curitiba city and metropolitan region, with about two million people, which water is considered polluted, beyond the Irineopolis city. The SP2 receives this same contribution added to Pintado River, which water has a source of organic pollution from untreated sewage. Although only caffeine was detected in the water, the CF supports the other biological responses indicating fish health alteration. Other authors related a low CF to xenobiotic presence in aquatic environment (Van Dyk et al., 2007; Linde-Arias et al., 2008).

A similar temporal variation was observed to biochemical and genetic biomarkers responses. Winter was characterized by a reduction of AChE in all

sample points, and this season is a rainy period. This Middle Iguaçu Basin is marked by growing poplar, soy, beans and two tributaries (Pintado River and Vermelho River) bring contribution of inappropriate dumped into sewage water and waste of mineral extractions. The combined effects of these anthropogenic activities are potential factors to the enzymatic response in this seasonal period, which demonstrated that fish are exposed to anticholinesterase agents.

A posterior metal analysis in rainy period demonstrated that aluminum and lead were above the established by CONAMA's resolution (2005) to this class of water use (data not shown). It is not completely understood how aluminum exerts neurotoxic effects, but literature suggest an interaction with cholinergic system, with an action as a cholinotoxin (Gulya et al., 1990) and links between aluminum exposure and degenerative disorders (Flaten, 2001). This is relevant to human health and aquatic biota, mainly due to the observed concentration in SP1 and the links the metals establishes with other contaminants (Carvalho et al., 2013). Urban sewage and atmospheric depositions are important sources of lead presence in hydric bodies (EPA, 1982). This trace metal is very toxic (Türkmen et al., 2008), being able to alter brain morphology and cause edema and hemorrhage in this organ (Adeyemo, 2008), besides the effects on the liver and hematologic parameters (Ates et al., 2008). Some studies developed in Brazil (Ramalho et al., 2000; Guedes et al., 2005) and specifically in Parana state (Santos et al., 2008) detected lead concentrations above the established by CONAMA (Conama, 2005), which sets a concern scenario, because the fishery is also a routine in this region. The increase of metabolism indicated by the hepatic GST activity observed in summer is a response to detoxification process, and is related to maintain the cellular protection against lipid peroxidation (Venkateswara Rao, 2006). In this season also occurred a reduction of the cofactor GSH and GPx activity. Probablythe higher GST activity reduced GSH levels and GPx activity favored the low lipid peroxidation. Generally, when the fatty acids are peroxidized, they become more hydrophilic and it disturbs the normal membrane functions, damaging biomolecules (Livingstone, 2001). Therefore the fish presented DNA damage, but low LPO, which indicates that membrane damage and DNA fragmentation are not directly related, and this alteration in genetic material is associated with the environment where fish are chronically exposed (Calliani et al.,

2009), mainly the leaching chemical pollutants from soil in agriculture areas or residuals from waste treatment, paper and pulp industry.

The CAT activity increased in summer, as the other observed antioxidant enzymes, and it is according to other studies (Kong et al., 2012). This increasing can reduce the stress resulted from reactive oxygen species and reduce lipid damage (Cazenave et al., 2014), although the genetic damage persisted, and, probably there is a direct interaction between contaminant and DNA.

It is complex to interpret data from environmental biomonitoring, especially because of the multiple present xenobiotic sources in freshwater ecosystems. The multivariate analysis provided an integrated view of the overall condition, demonstrating the differences of seasonal periods and, also, monitoring the rivers as temporal and spatial variations and respective effects (Wunderlin et al., 2001; Monferrán et al., 2011). In this study, this kind of analysis permitted to notice that the fish had distinctly response over the seasonal periods, observed mainly by the availability of dissolved solids.

5 CONCLUSION

In summary, the Middle Iguaçu region did not evidence a pronounced pollution according to the chemical and physical parameters but the biochemical and genetic biomarkers demonstrated alteration in fish's health condition over a temporal pattern. The biota probably is in contact to anticholinesterasic and oxidant agents, and the water of this part of the river is used by human population. This is a concern for aquatic and human population.

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CAPÍTULO II

Artigo a ser submetido para a revista Ecotoxicology and Environmental Safety.

EFFECTS OF IGUAÇU RIVER WATER IN BIOMARKERS RESPONSE IN *Astyanax bifasciatus* (Pisces, Teleostei)

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ABSTRACT

The main aim of this study was to investigate if the exposure of healthy fish to Iguaçu river water would cause biomarker responses. Adults of *Astyanax bifasciatus* were sampled from a non-polluted river and, were exposed to Iguaçu river water from three sample points: sample point 1 (SP1) at the region where the water is collected to public supply, to be treated for drinking water; the sample point 2 (SP2) at a region with a massive urban occupation, and the sample point 3 (SP3) at a region with sewage treatment discharge. Experimental and control groups were maintained 96 hours exposed in these waters, and during the bioassay water parameters were measured each 24 hours. Water and muscle concentration of metals were analyzed in each group. Samples of liver, brain, muscle and blood were collected for biochemical and genotoxic biomarkers measurement. Fish exposed to the three water points demonstrated reduction in muscle acetylcholinesterase as well oxidative stress response. Fish muscle demonstrated values of aluminum in concentrations higher than the found to fish species in polluted areas. The results suggested that the three points need attention and monitoring due to the river water is used to water public supply.

Keywords: aluminum, biomarker, *Astyanax bifasciatus*, bioassay

1 INTRODUCTION

The importance of aquatic ecosystems should guarantee a sensitive control from unsafe industrial, agricultural and other anthropogenic activities (Chakraborty and Owens 2014) that generates anthropic pressure (Schwarzenbach, 2006) and compromises the ecosystem's integrity. One of the current challenges about aquatic ecosystems is to understand how the mixture of chemical presented in rivers can alter biota health (Ebrahimi and Taherianfard, 2011) presenting responses as oxidative stress (Oakes and Van der Kraag, 2003), neurotoxicity (Payne et al., 1996; Fasulo et al., 2010; Nunes et al., 2014) and genotoxicity (Fuzinatto et al., 2015).

A xenobiotic mixture involves availability of metals, metalloids, inorganic and organic compounds, synthetic detergents and pesticides, which can be accumulated

in toxic levels in hydric bodies (Rand et al., 1995), and as it can play a role in trophic levels (Franco- Uría et al., 2010) establishes a direct link with the human health (Santos et al., 2002). The toxicity of pollutants mixture in rivers can be investigated through biomarkers in fish, even at low concentrations of xenobiotics and the pollution is not noticeable.

Biomarkers can be a sensitive tool to assess adverse xenobiotic effects on fish because they exhibit if the physiological pattern of the animals is normal or not. So, biomarkers permit a link between animals' health and environmental conditions (Van der Oost et al., 2003) and, for this, are early indicator of contamination (Walker et al., 1996). When biota is exposed to xenobiotics, it can be induced to oxidative stress because of the inadequate balance between antioxidant system and reactive oxygen species (ROS) (Quinn et al., 2011). Generally, the organ which better demonstrates this effect is the liver, being an indicator of injuries in hydric bodies, and to investigate different xenobiotic concentrations available to fish improves interpretation about metabolism and behavior when exposed, even in low concentrations (Atli and Canli, 2010). Moreover, the measurement of acetylcholinesterase (AChE) activity is one of the most used biomarkers. AChE is a key enzyme to nervous system and plays a role removing the neurotransmitter acetylcholine within the synapse through hydrolysis (Durieux et al., 2011). The activity reduction of this enzyme is associated to different xenobiotic that usually are part of a river mixture pollutants (Payne et al. 1996, Moraes et al. 2007) and is relevant to investigate neurotoxic contaminants in hydric bodies (Lionetto et al. 2005). The detection of reduction in activity of this enzyme is also an early sign of pollution. Genotoxicity is associated with environmental contamination in rivers, mainly because it characterizes a chronically exposure (Calliani et al., 2009) and can be always associated to oxidative stress effects (Simonato et al., 2016) when evaluating biota integrity.

The *Astyanax bifasciatus* species (Teleostei:Characidae) is endemic from the Iguaçu River Basin. This fish species is strictly occurring in freshwater, are small (approximately 10 cm of total length) and play role as foragers in all trophic levels, and change their trophic guilds according to the environment availability as a response to ecosystem changes (Lobón-Erviá and Bennemann, 2000), which evidences their ecological plasticity. The foraging is essential in aquatic ecosystems balance, because they participate in mineralization process to the top trophic groups.

A previous biomonitoring in 2012-2013 in Iguaçu River Basin demonstrated during rainy period that aluminum and lead were above the established by Brazilians legislation (CONAMA, 2005) as well residue of caffeine in water, and fish showed neurotoxicity and oxidative stress caused by this mixture of pollutants during a seasonal period (Bueno-Krawczyk et al., 2015). As bioassays allow to link physical and chemical data to fish exposure and subsequent analyses of the effects (Ossana et al., 2013) we considered that expose healthy fish to Iguaçu River water would demonstrate if fish would keep the response of oxidative stress and neurotoxicity as in field biomonitoring.

Considering the ecological role of *A. bifasciatus* and its use for local populations in feed, this fish species was used as bioindicator. In this way, the aim of the study was to expose healthy fish to Iguaçu River water and identify if the water is contaminated by relevant xenobiotics that can alter fish health after 96 hours of exposure.

2 MATERIAL AND METHODS

2.1 Water sampling to the experiments

Water samples were collected in Iguaçu River in three pre-selected sample points, being sample point 1 (SP1) (26º9’032.08”S and 51º4’47.83”W) at the region where the water is collected to public supply, to be treated for drinking water; the sample point 2 (SP2) (26º14’27.61”S and 51º2’53.99”W) at a region with a massive urban occupation, and the sample point 3 (SP3) (26º14’58.25”S and 51º6’31.80”W) at a region characterized by a massive urban occupation with the sewage treatment discharge (Figure 1). These sample points were chosen to this bioassay because of a previous study which indicated aluminum and lead in water, especially in SP1 (Bueno-Krawczyk et al., 2015).

All the water samples were collected in the morning at the same day (between 7 and 10 am), and taken to the aquaria in the laboratory. The pH, water temperature (WT) (ºC), dissolved oxygen (DO) ($mg.L^{-1}$), percentage of oxygen saturation (% O_2), ammonia concentration (NH_3) ($mg.L^{-1}$), hardness (WH) ($mg.L^{-1}$ $CaCO_3$) were

measured during the 96 hours of bioassay in the experiment and control group (filtered water). Metal concentrations was analyzed in the water collected from each aquarium at the end of the bioassay.

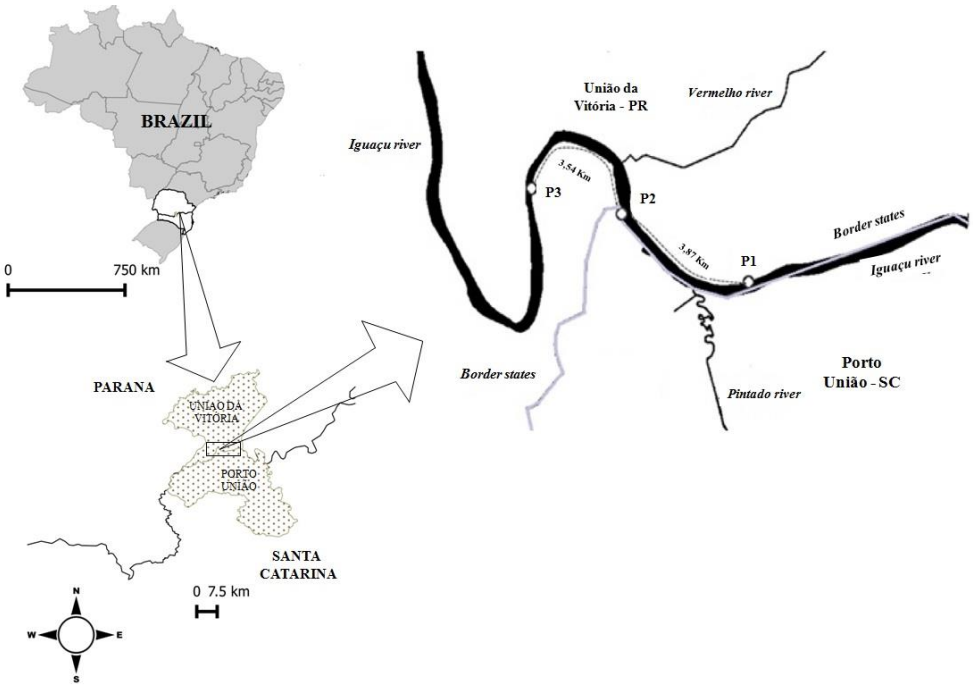

Figure 1. Study area in Middle Iguaçu Basin region in South Paraná state, Brazil, where the samples of water were taken to the bioassay. SP1: sample point 1, at region where the water is collected to public supply; SP2: sample point 2, urbanized area; SP3: sample point 3, urbanized area with sewage treatment discharge.

2.2 Metal water analysis

The water samples from the three experimental groups and the control were frozen at -20º C. The samples were centrifuged (10 mL) and the concentrations were determined by inductively-coupled optical emission spectroscopy (ICP-OES) a Thermo Scientific model ICAP 6500. Mixed metal standards were prepared and analysed at wavelengths such that spectral interferences with other constituent elements were minimal. Water samples were analysed without dilution. The instrument was calibrated from 0.001 to 1 mg. L^{-1} in the axial configuration. All the waters were analysed to: Aluminum (Al), barium (Ba), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese

(Mn), molybdenum (Mo), nickel (Ni), lead (Pb), vanadium (V), and zinc (Zn). The metal concentration in water was compared to Brazilian legislation (CONAMA, 2005) class 3 and 4 of water, which involves water classes to public use.

2.3 Experimental design

It was used a total of 40 fish *Astyanax bifasciatus* (lambari; weight: 17.7 ± 4.9 g, length: 10.76 ± 0.81 cm). The animals were obtained from a clean water site with no anthropic direct influence, no pollution source, under license number 42950-1 of the IBAMA-Sisbio. The animals were acclimatized in tanks of 120 L with a photoperiod of 12 h light: 12 h dark, constant aeration and water temperature of 17.08 °C ± 1.37 °C for 60 days. The fish were fed with commercial food every 24 h.

After the acclimatization period, the fish were divided randomly in groups of 10 organisms. Each group was held in aquariums of 60 L with water from the three sample points SP1, SP2, SP3, which corresponded, respectively, to the experimental groups AQ1, AQ2 and AQ3, in a longitudinal segment in Middle Iguaçu Basin region, which is a part of Iguaçu River from South of Brazil.

This study was approved by the Ethics Committee for Animal Research of the State University of Paraná, under the license 6322 and all protocols were realized in accordance with International Guidelines for Animal Use.

After 96 hours of exposure in Iguaçu River water, the animals were anesthetized with clove oil and the blood was taken from caudal vein to genetic biomarkers. The fish were then killed by medullar section. The animals were weighed and liver, brain and axial muscle (a part of edible tissue) were taken. The tissues were stored at -80 °C for biochemical analysis. A part (5mg) of axial muscle of each fish was separated to metals analyses, and stored in -80 ºC .

2.4 Metal analysis

Approximately 5 mg of muscle was digested at 60 ºC. After the complete drying, the material was digested in 5 N HNO_3 (Suprapur, Merck), centrifuges (3,600 g, 20 min) and the supernatant was used to analysis for nickel (Ni), copper (Cu), lead (Pb) and aluminum (Al). These metals were chosen based on previous study at Middle Iguaçu River Basin, which indicated presence of Al and Pb (Bueno-Krawczyk et al., 2015), as well the relevance of these metals in trophic chain. Tissue digest was analyzed for metals using graphite furnace atomic absorption spectrometer (AAnalyst 700, Perkin Elmer, USA) against a reference metals standard solution (Specsol, Brazil). Tissue concentrations are represented as mg metal per wet weight of muscle (mg metal g wet weight^{-1}). This methodology was modified from Alves and Wood (2006).

2.5 Biochemical biomarkers

Brain and axial muscle were homogenized in phosphate buffer (0.1M) at pH 7.5 in a proportion of 1:10 (mass/volume) and centrifuged at 10,000x g during 20 min, at 4ºC. Acetylcholinesterase activity (AChE) in brain (AChE B) and in muscle (AChE M) were measured spectrophotometrically at 405nm, as proposed in Ellman et al. (1961), modified to microplate by Silva de Assis (1998). Each enzymatic assay was performed in triplicate.

Liver samples were homogenized in phosphate buffer (0.1M) at pH 7.0, and centrifuged at 15,000xg during 30 min, at 4ºC. Catalase (CAT) activity was measured at 240 nm based on Aebi (1984). Superoxide dismutase (SOD) activity was measured at 440 nm according to Gao et al. (1998). Glutathione peroxidase (GPx) activity was measured at 340 nm according to Paglia and Valentine (1967). GST activity was measured at 340nm (Keen et al., 1976). GSH concentration was obtained according to Sedlak e Lindsay (1968) and was measured at 415 nm. Lipoperoxidation analysis (LPO) was carried out using the ferrous oxidation xylenol assay and measured at 570 nm (Jiang et al., 1992).

The protein concentration in liver, brain and axial muscle were carried out according to Bradford's method (Bradford, 1976), using bovine serum albumin as standard.

2.6 Genetic biomarkers

The Comet assay with peripheral blood (erythrocytes; ECA) was performed according to Speit and Hartmann (1999), modified by Cestari et al. (2004) and Ferraro et al. (2004). One hundred nucleoids were analyzed for each fish according to the visual classification based on the migration of DNA fragments from the nucleus. The results were categorized into classes according to Ramsdorf et al. (2009).

The Piscine Micronucleus Test was performed according to the technique described by Hooftman and De Raat (1982). Blood samples obtained from the caudal vein of the specimens were spread on clean slides. After fixation in ethanol for 30 min, the slides were air-dried, and stained with 10% Giemsa solution for 10 min. For each fish, 2.000 erythrocytes were examined under 1,000 × magnification and scored for the presence of both typical micronuclei and nuclear morphologic alterations manifested as changes in the normal elliptical shape of the nuclei. The morphological abnormalities and micronuclei frequencies were observed according to Carrasco et al. (1990).

2.7 Statistical analysis

Initially, the physical and chemical data, metal analysis in water and muscle, biochemical and genetic biomarkers were evaluated according to the pattern of data distribution by Shapiro-Wilk test, as well the homogeneity of variances by Levene's test. The physical and chemical data, metals in water, biomarkers as SOD, CAT, LPO e AChE M and ECA were according to the statistical assumptions, and, therefore, were compared among the experimental and control groups by single factor analysis of variance, followed by Dunnet's test, comparing control and experimental groups. Metals in muscle and the biomarkers (MN, GSH, GPx, GST

and AChE B) were compared among the groups by nonparametric Kruskal-Wallis followed by Dunn's test.

All the statistical analyzes were performed at Statistica and XLStat2015 software, using the level of significance when $p \leq 0.05$.

3 RESULTS

3.1 Experimental conditions

Water parameters values (mean ± standard error of mean) were measured in experimental and control groups each 24 hours during the bioassay. Dissolved oxygen and water temperature were according to Brazilian legislation. The pH in AQ1 was according to legislation, and in control and other experimental groups was lower. There was no significant variation among control and the experimental groups to all analyzed parameters (Table 1). The water hardness was of 38 $mg.L^{-1}$ $CaCO_3$.

One fish died 72 hours after the exposure, and the other animals did not presented any behavior alteration. The fish did not demonstrate significant statistical differences about total length (10.75 ± 0.01, $p = 0.06$), standard length (8.85 ± 0.22, $p = 0.85$) and weight (17.7 ± 4.9 g, $p = 0.42$) among the groups.

Table 1. Water physical and chemical data related to the different Iguaçu River water samples during the 96 hours of the bioassay. C = control group; AQ1 = experimental group 1; AQ2 = experimental group 2; AQ3 = experimental group 3. pH = hidrogenionic potential (Units), DO = dissolved oxygen ($mg.L^{-1}$), OS = percentage of oxygen saturation, and WT = water temperature (ºC), NH_3 = ammonia concentration ($mg.L^{-1}$).

	C	AQ1	AQ2	AQ3	*p*
pH	5.70 ±0.11	6.08 ± 0.11	5.77 ± 0.18	5.93 ± 0.02	0.05
DO	11.19 ± 0.73	11.65 ± 0.51	11.15 ± 0.34	9.76 ± 1.07	0.06
OS	113.82 ± 5.12	120.42 ± 7.73	118.40 ± 4.71	102.12 ± 12.95	0.10
WT	17.06 ± 0.65	17.22 ± 0.71	17.28 ± 0.71	17.44 ± 0.74	0.74
NH_3	0.81 ± 0.24	0.81 ± 0.55	0.56 ± 0.41	0.68 ± 0.50	0.84

Values are demonstrated in mean and standard error of the mean (*X* ± SEM).
p- value of Variance Analysis single factor.

3.2 Metal concentration in water

The metal concentration in water did not indicate higher values than the permitted by Brazilian legislation and only Al, Ba, Co and Pb were statistically significant among the control and experimental groups (Table 2).

Table 2. Mean of total metal concentration in water in experimental groups and control. DL = instrument detection limit; LL = legislation limit to water classes*; C = control group; AQ1 = experimental group 1; AQ2 = experimental group 2; AQ3 = experimental group 3.

	DL	LL	C	AQ 1	AQ 2	AQ 3	*p*
	$\mu g.L^{-1}$	**$\mu g.L^{-1}$**	**$\mu g.L^{-1}$**	**$\mu g.L^{-1}$**	**$\mu g.L^{-1}$**	**$\mu g.L^{-1}$**	
Al	0.12	100	0.17 ± 0.00^{a}	0.15 ± 0.00^{b}	0.16 ± 0.00^{b}	0.17 ± 0.00^{a}	0.00
Ba	0.03	700	0.03 ± 0.00^{a}	0.03 ± 0.00^{b}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}	0.00

Co	0.51	50	0.03 ± 0.00^{a}	0.03 ± 0.00^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.04
Pb	1.06	10	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}	0.00

* Brazilian Legislation to class 3 and 4 of water. The use of the water is to public supply, irrigation, culture and recreation activities.

3.3 Muscle metal concentration

Concentrations of the analyzed metal in axial muscle are expressed in $\mu g.g^{-1}$ wet weight basis and are given in Table 2.

Nickel (Ni) concentration in axial muscle was higher in the following order: AQ2>AQ3>AQ1>C, which demonstrated significant difference between control and experimental groups (H = 8.01; *p* = 0.045).

Copper (Cu) concentration in axial muscle was higher in the following order: AQ1>AQ2>C>AQ3, and the groups demonstrated statistical significant difference (H = 9.49; *p* = 0.02). The experimental group AQ1 showed the highest concentration to this metal, while AQ3 showed the lowest in tissue (*p* = 0.01).

Lead (Pb) concentration in axial muscle was higher in following order: AQ2>AQ3>AQ1>C. It was not verified concentration to this metal in control, which was different to other groups (H = 8.01; *p* = 0.04), specially the experimental group AQ2, whose fish showed the highest values to this metal concentration.

Aluminum (Al) concentration in axial muscle was higher in following order: AQ2>AQ1>C>AQ3. This was the one of a kind that did not show difference between experimental groups and control.

Table 2. Total metal concentration in fish axial muscle in experimental groups and control. C = control group; AQ1 = experimental group 1; AQ2 = experimental group 2; AQ3 = experimental group 3. TUL = tolerable upper limit in Brazil.

Metals	**TUL**	**C**	**AQ1**	**AQ2**	**AQ3**	***p***
	$\mu g.g^{-1}$	**$\mu g.g^{-1}$**	**$\mu g.g^{-1}$**	**$\mu g.g^{-1}$**	**$\mu g.g^{-1}$**	
Ni	5.0	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04
Cu	30.0	0.17 ± 0.01^{ab}	0.29 ± 0.04^{a}	0.22 ± 0.03^{ab}	0.15 ± 0.02^{b}	0.02
Pb	2.0	0.00 ± 0.00^{a}	0.01 ± 0.01^{ab}	0.07 ± 0.02^{b}	0.03 ± 0.01^{ab}	0.03
Al	----	6.73 ± 3.86	8.35 ± 2.79	10.09 ± 2.77	6.62 ± 2.06	0.52

Values are demonstrated in mean and standard error of the mean (X ± SEM).

Different letters indicate statistical significance among the groups.

3.4 Biomarkers

The activity of SOD were statistically significant among the groups, but, in general, experimental groups were similar from control group (H = 10.20; p = 0.01), and demonstrated that fish exposed to Iguaçu River water in AQ2 and AQ3 had lowest activity to this enzyme than the control and the AQ1. Also, AQ1 and AQ2 were statistically different (p = 0.02), because they showed, respectively, higher and lower activity to this enzyme.

The GPx activity also demonstrated significant difference between control and experimental groups ($F_{3.32}$ = 18.81; p = 0.00), the activity in experimental groups was higher than in control. AQ2 and AQ3 showed the highest values to this enzyme.

The LPO demonstrated difference between experimental groups and control (H = 9.32; p = 0.025), especially between control and AQ1, the fish in AQ1 showed the highest values of membrane damage.

The Iguaçu River water decreased the activity of AChEM in fish (H = 9.94; p = 0.019), and in AQ1 the activity to this enzyme was the most reduced (p = 0.02) (Table 3).

The other analyzed biomarkers did not demonstrate statistical significance (p> 0.05) when groups were compared.

Table 3. Biomarkers in *Astyanax bifasciatus* in control and experimental groups. C = control group; AQ1 = experimental group 1; AQ2 = experimental group 2; AQ3 = experimental group 3.

Biomarkers	**C**	**AQ1**	**AQ2**	**AQ3**	***p***
ECA	188.44 ± 13.50	185.75 ± 18.59	191.25 ± 17.37	212.86 ± 12.41	0.64
AMN	1.56 ± 0.29	2.78 ± 0.52	2.40 ± 0.88	1.11 ± 0.35	0.17
GSH	19.28 ± 2.35	16.99 ± 1.66	18.11 ± 2.75	11.53 ± 3.29	0.18
LPO	3.22 ± 1.07^{a}	6.20 ± 0.51^{b}	4.80 ± 1.00^{ab}	2.95 ± 0.83^{ab}	0.02*
SOD	220.55 ± 19.05^{ab}	250.64 ± 25.78^{a}	172.29 ± 12.72^{b}	184.42 ± 11.30^{ab}	0.01*
GPx	41.86 ± 2.60^{a}	86.69 ± 6.09^{b}	112.33 ± 10.10^{b}	114.45 ± 7.64^{b}	0.00*
GST	159.42 ± 16.49	151.02 ± 14.38	142.09 ± 16.55	172.29 ± 12.14	0.58
CAT	30.03 ± 2.89	37.98 ± 5.59	45.02 ± 5.42	35.57 ± 5.39	0.13
AChE M	79.53 ± 14.06^{a}	42.00 ± 4.33^{b}	54.56 ± 5.59^{ab}	55.91 ± 3.79^{ab}	0.01*
AChE B	94.83 ± 6.41	108.73 ± 9.14	99.01 ± 11.86	106.89 ± 5.32	0.63

The values are demonstrated in mean and standard error of the mean ($X \pm SEM$).

Different letters (a, b, c) indicate significant differences among experimental groups and control when $p < 0.05$.

M = muscle, B = brain.

Comet assay (ECA) = score of DNA damage; piscine micronucleus test and morphological abnormalities (AMN) = score of morphological abnormalities and micronucleus frequency; reduced glutathione (GSH) = µg.mg protein^{-1}; lipid peroxidation (LPO) = nmol.mg protein^{-1}; superoxide dismutase (SOD) = U.mg protein^{-1}; glutathione peroxidase (GPx); glutathione S-transferases (GST); catalase (CAT) = µmol.min^{-1}.mg protein^{-1}, and acetylcholinesterase (AChE) = nmol.min^{-1}.mg protein^{-1}.

4 DISCUSSION

Some metals can induce oxidative stress because of the free radical production causing membrane damage and genotoxicity. These effects can be observed in biota, especially in rivers with no evident pollution. Thus, although chemical data in water and metal concentration in axial muscle of *A. bifasciatus* did not show a pattern, the results indicated highest concentration in fish tissue of aluminum in AQ2 and AQ1, respectively.

There are not established limits to this metal in Brazilian legislation to fish tissue, but some studies have already related aluminum and oxidative damage to lipoperoxidation (Yoshino et al., 1999) with consequent genotoxic effects (Galindo et al., 2010). Our results demonstrated higher LPO in AQ1 only, but SOD was high, and this antioxidant enzyme is responsible for counteracting the effects of ROS (van der Oost et al., 2003). It was already associated to aluminum it facilitates the elimination of the metal (Fernández-Dávila et al., 2011).

The association of aluminum and oxidative stress is because this metal potentiates the increase of ROS production (Crichton et al., 2002). Brazilian legislation permits 0.2 $mg.l^{-1}$ of this metal in hydric bodies used to water public supply, but Fernández-Dávila et al. (2012) found membrane damage in a study with cap exposed to 0.1 $mg.l^{-1}$, demonstrating the need of more investigation about the effects of exposure to this kind of pollution, and, also, that Brazilian legislation needs to be reformulated in order to guarantee the maintenance of healthy biota in hydric bodies.

The few available data about aluminum in freshwater fish axial muscle, especially in *A. bifasciatus*. Fish in Turkey showed mean concentration of this metal between 2.23 and 4.93 $mg.kg^{-1}$ (Yilmaz et al., 2010), in Rio de Janeiro the lowest concentration found was 3.0 $mg.kg^{-1}$ (Medeiros et al., 2014) and in France, the lowest concentration reported was 0.53 $mg.kg^{-1}$ to aluminum (Guérin et al., 2011), whereas we found 6.62 and 10.09 $mg.kg^{-1}$ of this metal concentration in axial muscle, whose values are higher than some lowest found in the literature to sold species from evidenced polluted areas.

The general approach in this study demonstrated that the difference to metal in axial muscles occurred to Pb and Al, mainly in experimental groups AQ1 and AQ2,

showing that animals were exposed to similar conditions in this bioassay. It is a concern considering that the water which is collected to water public supply has similar conditions to water which receives sewage and industrial residue, especially because this treatment station offers simplified and limited treatment, favoring metal residue (Ongley et al., 2010). This perspective is commonly verified in rivers used for public supply, with agricultural and aquaculture practices, which increase metallic composts in water, with consequences to biota and human population (Yao et al., 2014; Islam et al., 2015). These results suggest that no parameter in legislation to this metal in fish muscle must be reconsidered, because the high concentration and possible effects to biota and human health.

After the exposure to Iguaçu river water, fish demonstrated oxidative stress and neurotoxicity, and these both responses were in experimental group AQ1, in which the water is used to water public supply after simplified treatment. It was evidenced by the results of SOD, LPO and AChEM and these effects suggested that this sample point is polluted with some xenobiotics that potentially alter cellular metabolism in fish.

The pattern observed in LPO, GSH, GPx and GST presented a cellular effort in preserving membranes from damages. The GST and GPx activities were high while GSH concentration and LPO were lower. This cellular pattern was previously verified to this species in biomonitoring during the seasonal period with higher oxidative stress and with elevated DNA damages (Bueno-Krawczyk et al., 2015). This pattern suggests that the increased GST activity reduced GSH levels and the more intense GPx activity reduced the LPO, which could disturb the normal membrane functions, damaging biomolecules (Livingstone, 2001). We suggest the DNA damage verified is due to exposure in polluted water and not directly related to LPO results (Calliani et al., 2009), because fish demonstrated DNA damage without membrane damage.

Organophosphates and carbamates are not evidenced in chemical analysis because of the high volatility and low persistence in environment. However, as the pesticides have to be often applied, they are always available in water. The acetylcholinesterase in fish is usually inhibited when these chemicals are present (Guiloski et al., 2013) and our results demonstrated reduction in this enzyme in fish exposed to river water. We reaffirm the effectiveness of this enzyme as biomarker, and the need that it be used in integrity evaluation.

Freshwater chemical and physical data are difficult to interpret and also the multiple stressors that are available to biota. In this way, the exposure of healthy fish to river water could demonstrate that this species generate response when exposed. Also, isolating some metal analysis in axial muscle permitted a new perspective with aluminum concentration and a concern about pollution, which is not evidenced in this hydric body.

Neurotoxicity effects, oxidative stress and membrane damage and levels of metal in axial muscle that are not considered in Brazilian legislation, the fish species is useful in biomonitoring because can reflect local pollution and incorporates bioavailability of some xenobiotics. Extended monitoring should be considered to this aquatic ecosystem to fully comprehend fish behavior and other ecotoxicological effects and xenobiotics in water, mainly for the human health relevance of the Iguaçu river in Middle Iguaçu river Basin.

5 CONCLUSION

According to our results, the Iguaçu river Basin water can potentially cause oxidative stress and neurotoxicity in fish. Probably these effects are due to the mixture of xenobiotics (metals and other contaminants), and we suggest a special attention and more investigations to the region due to the water is used to water public supply.

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4 CONSIDERAÇÕES GERAIS

Os resultados obtidos neste estudo indicaram que os efeitos de estresse oxidativo e neurotoxicidade em peixes da espécie *Astyanax bifasciatus*, o que conduziu a discussão de que há xenobióticos na água do Rio Iguaçu, o que é previsível em termos de qualidade da água quando ocorre o crescimento urbano desacompanhado de saneamento básico efetivo, além das práticas agrícolas que geram resíduos que lixiviam para o corpo hídrico e os resíduos da indústria de papel e celulose que, da mesma forma, adicionam xenobióticos à água. Estes resultados ressaltam a necessidade de investigação das fontes poluidoras nos municípios de Porto União (SC) e União da Vitória (PR), mas também as possíveis fontes externas a estes limites jurisdicionais.

Este quadro de aumento das práticas industriais e agrícolas aliadas ao crescimento urbano desacompanhado de tratamento adequado de esgoto têm deteriorado a qualidade ambiental das águas e garantido impactos negativos aos ecossistemas aquáticos (LE-MINH et al., 2010). Os contaminantes destas diferentes fontes poluidoras garantem acúmulo de substâncias orgânicas e inorgânicas na água e, ao longo do tempo, são depositadas no sedimento, ficando disponíveis à biota. Para avaliar as condições do ecossistema hídrico e dos efeitos dos xenobióticos, podem ser mensuradas as características físicas e químicas da água (APHA, 2005).

Neste estudo, os parâmetros físicos e químicos não demonstraram variação sazonal ou espacial, e a cafeína foi o único fármaco que pôde ser detectado durante a avaliação sazonal. Sua ocorrência é indício de esgoto doméstico, pois é um composto consumido exclusivamente pelo ser humano e tem sido verificado em várias áreas com impacto ambiental (PELLER et al., 2006; VERENITCH et al., 2006; KURISSERY et al., 2012), sendo, por isso, considerado um indicador de poluição para ecossistemas aquáticos. Apesar do seu tempo de meia-vida ser inferior a 20 horas, a biodegradação é um importante processo no destino desse composto na água (TOXNET, 2016), e as constantes entradas no meio fazem com que a concentração na água não diminua, e isso aliado à falta de tratamento adequado de esgoto permite que seja um composto persistente.

Além dos parâmetros físicos e químicos tradicionais para avaliar a qualidade da água, o uso de bioindicadores é importante porque permite o acompanhamento dos efeitos a longo prazo, demonstrando se o ambiente está impactado bem como os riscos aos organismos (KNIE e LOPES, 2004). Os resultados do presente estudo demonstraram que os peixes são ferramentas úteis no biomonitoramento, visto que foram mais sensíveis às variações ocorrentes na região do Médio Iguaçu do que outros bioindicadores como os *D. magna* e *D. subspicatus.* Os biomarcadores analisados nos peixes demonstraram variação sazonal e espacial, sugerindo, inclusive, efeitos de neurotoxicidade e estresse oxidativo, enquanto os parâmetros físicos e químicos bem como os outros bioindicadores não demonstraram variação ou outras formas de efeito. Esta observação é importante, pois mesmo que alguns xenobióticos estejam presentes em concentrações permitidas e mesmo que os parâmetros físicos e químicos sejam considerados aceitáveis, pode haver alteração na saúde da biota residente. Assim, avaliando os possíveis agentes de poluição juntamente com efeitos na biota é possível fornecer subsídio para o gerenciamento, proteção e tomada de decisões em relação aos ecossistemas aquáticos, em especial os ambientes explorados para abastecimento público.

Além do mais, as respostas subletais juntamente com a quantificação de alguns xenobióticos no tecido dos animais respondem sobre as alterações ambientais e permitem melhor entendimento sobre níveis de degradação, em especial quando a ocorrência é em áreas com poluição considerada moderada ou pouco evidente. Como não houve conexão entre a quantificação de metais na água e nos tecidos dos peixes, pôde-se concluir que uma interpretação equivocada dos resultados seria a consequência de uma análise isolada dos parâmetros químicos usados. Isto reforça a necessidade de uma avaliação integrada das condições do ecossistema aquático (avaliação biológica e avaliação química e física da água), principalmente em áreas com poluição pouco evidente. Entretanto, os resultados sugerem concentração de alumínio no tecido dos animais, com valores não previstos na legislação brasileira, e como este metal é associado a efeitos neurotóxicos na literatura (GALINDO et al., 2010; VIEIRA et al., 2013), sua presença é preocupante visto o uso da água do Rio Iguaçu para abastecimento público. Como ainda há uma lacuna entre o entendimento dos xenobióticos e as respostas biológicas, precisa-se avaliar com mais clareza os efeitos que este metal possa garantir à biota local.

A questão central dos resultados de metais nos tecidos é que há considerações sobre a toxicidade do alumínio para as populações de peixes (ALSTAD et al., 2005), principalmente porque o metal se torna mais solúvel conforme o pH for ácido, que é uma característica verificada para a água do Rio Iguaçu. Assim, se este metal é disponibilizado aos organismos, pode causar dano ecológico e risco à saúde humana pela cadeia trófica (GÖTZE et al., 2014), tornando urgentes as diferentes abordagens de estudos que investiguem concentração e efeitos deste metal nos peixes e na água.

Estudos mais frequentes são necessários para monitoramento de xenobióticos e seus efeitos na biota. Entretanto, essas análises iniciais mostram que os lambaris são bons bioindicadores e apresentam efeitos subletais quando expostos a água do Rio Iguaçu.

5 CONCLUSÕES

Os efeitos biológicos observados nos peixes durante o biomonitoramento bem como no bioensaio demonstram que a biota do Rio Iguaçu está exposta a uma mistura de contaminantes que alteram a integridade ambiental do corpo hídrico e podem influenciar a biota.

Os biomarcadores foram úteis para demonstrar um padrão de efeito de neurotoxicidade e de estresse oxidativo durante o biomonitoramento bem como no bioensaio. Neste caso, embora a poluição neste corpo hídrico seja considerada moderada, ou tenha presença de xenobióticos a uma concentração permitida em legislação, pode afetar a biota residente e prejudicar seu ajuste ecológico.

Os resultados de alumínio no tecido dos animais é alarmante por não ser preconizado em legislação. Foi demonstrado que, embora não haja poluição evidente no corpo hídrico, os resultados do metal nos peixes é maior do que o registrado na literatura para áreas consideradas fortemente poluídas.

É necessária atenção especial ao Rio Iguaçu nesta região por causa da população local, pois ocorre a captação de água para abastecimento público bem como a pesca frequente de peixes para o consumo. As análises iniciais do estudo demonstraram que a água apresenta cafeína e chumbo em alguns períodos do ano, além de alumínio no tecido dos animais, o que reforça a necessidade de não dissociar a importância da população humana em próximas abordagens, pois pode envolver em risco à saúde pública.

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