

UNIVERSIDADE FEDERAL DO PARANÁ

FLÁVIA YOSHIE YAMAMOTO

**AVALIAÇÃO DA QUALIDADE DA ÁGUA E DETECÇÃO DE DESREGULADORES
ENDÓCRINOS EM CINCO RESERVATÓRIOS DO RIO IGUAÇU**

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, Departamento de Biologia Celular, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção de título de Doutor em Biologia Celular e Molecular

Orientador: Prof. Dr. Ciro Alberto de Oliveira Ribeiro
Co-orientador: Prof. Dr. Daniel Schlenk

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Departamento de Biologia Celular
Setor de Ciências Biológicas
Universidade Federal do Paraná



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*Aos meus pais pelo amor, dedicação e educação, e
por me mostrarem a importância da busca pelo conhecimento*

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*Se você quer transformar o mundo,
experimente primeiro promover o seu
aperfeiçoamento pessoal e realizar
inovações no seu próprio interior*
Dalai Lama

RESUMO

Milhares de compostos introduzidos nos ambientes aquáticos podem causar efeitos adversos tanto para a saúde humana como para a biota. Entre eles, os compostos capazes de interferir no funcionamento normal do sistema endócrino despertam grande interesse e preocupação científica. Com o objetivo de avaliar a qualidade da água dos reservatórios do Rio Iguaçu e detectar desreguladores endócrinos, foram realizadas análises químicas e utilizados diferentes biomarcadores em três espécies de peixes nativas (*Astyanax bifasciatus*, *Geophagus brasiliensis* e *Crenicichla iguassuensis*) coletados em cinco reservatórios do Rio Iguaçu. Para avaliação dos efeitos endócrinos nos peixes foi desenvolvido um anti-soro policlonal anti-vitelogenina para *G. brasiliensis* como uma ferramenta a ser utilizada no biomonitoramento de ambientes aquáticos. Além disso, exemplares de tilápias (*Oreochromis niloticus*) foram mantidos em tanques redes montados em quatro reservatórios para avaliação de respostas precoces relacionadas à presença de desreguladores endócrinos. Em três reservatórios foram detectadas concentrações de Cr na água, acima do limite permitido pelo CONAMA. Outros metais com potencial tóxico, como Cd, Cu, Mn, Pb, foram detectados em todos os reservatórios. A integração dos dados, associando a presença dos metais e a relevância das respostas biológicas (biomarcadores moleculares, bioquímicos, genotóxicos, morfológicos), demonstrou um maior impacto dos reservatórios Foz do Areia (FA), Segredo (SE) e Salto Caxias (SC). Através do ensaio de ELISA padronizado foi possível detectar níveis de vitelogenina nos machos acima dos níveis basais, enquanto a técnica de *Western Blot* demonstrou a expressão da vitelogenina em fêmeas e em poucos indivíduos machos. Ambas as técnicas confirmaram a especificidade e sensibilidade do anti-soro produzido. Observou-se uma menor concentração de vitelogenina nas fêmeas de *G. brasiliensis* do reservatório FA associada a um menor grau de maturação das gônadas, indicando a presença de desreguladores endócrinos neste reservatório. Já nas tilápias verificou-se maior atividade das enzimas citocromo P450 (CYP1A e CYP3A) em indivíduos machos do reservatório Salto Santiago (SS), onde foram encontrados maiores níveis de PCBs na água. A atividade deste sistema enzimático foi correlacionada com seu papel no metabolismo de xenobióticos e moléculas endógenas. A presença de pesticidas organoclorados predominou nos reservatórios SS, SO e SC, áreas com intensa atividade agrícola na região. De forma geral, foi possível concluir neste estudo que o Rio Iguaçu apresenta-se impactado por diferentes fontes de atividades antrópicas ao longo do percurso do rio: i) Desde a região metropolitana de Curitiba, principal fonte de poluentes para o rio, especialmente para o reservatório FA; ii) Pela atividade agrícola e pecuária, principalmente nos reservatórios SS, SO e SC. Portanto, não se verificou um gradiente de poluição nítido ao longo do percurso do rio, já que, além da poluição oriunda da região metropolitana de Curitiba e demais cidades, observam-se outras fontes de poluição difusas. Considerada a grande importância do Rio Iguaçu, é necessário que uma maior atenção seja dada a esta bacia por parte da comunidade e autoridades públicas, com o intuito de mitigar os impactos causados pelas diversas atividades humanas.

Palavras chave: Biomarcadores; peixes nativos; vitelogenina; metais; pesticidas; atividades antrópicas

ABSTRACT

Several compounds introduced into aquatic environments may cause adverse effects over human health and biota. Among them, compounds able to interfere in normal function of endocrine system arise great scientific concern. With the aim to evaluate the water quality of the reservoirs of Iguaçu River and detect endocrine disruptors were performed chemical analysis and used different biomarkers in three native fish species (*Astyanax bifasciatus*, *Geophagus brasiliensis* and *Crenicichla iguassuensis*) captured in five reservoirs of Iguaçu River. For evaluation of endocrine effects in fish was developed a polyclonal antiserum anti-vitellogenin of *G. brasiliensis* as a tool for biomonitoring studies in aquatic environments. Finally, tilapias (*Oreochromis niloticus*) were maintained in cages mounted in four reservoirs for evaluation of earlier responses related to the presence of endocrine disruptors. In three reservoirs were detected Cr in water above the concentrations permitted by CONAMA legislation. Others metals with toxic potential, as Cd, Cu, Mn, Pb, were detected in all reservoirs. The integration of data associating the presence of metals and the relevance of biological responses (molecular, biochemical, genotoxic, morphological biomarkers), demonstrated a higher impact of the reservoirs Foz do Areia (FA), Segredo (SE) e Salto Caxias (SC). Through the standardized ELISA assay detectable levels of vitellogenin in male fish were higher than basal levels. Western blot demonstrated the expression of vtg in females and few male individuals expressing the protein. Both assays validated the specificity and sensitivity of the produced antiserum. Lower concentration of vitellogenin in female *G. brasiliensis* from FA associated to a lower gonad maturation degree, indicated the presence of endocrine disruptors in this reservoir. On the other hand, in tilapias was verified higher activity of CYPs (CYP1A and CYP3A) in male individuals from Salto Santiago (SS), where were found the highest levels of PCBs in the water. Higher activity of this enzyme showed its key role responsible for the metabolism of xenobiotics and endogenous molecules. The presence of organochlorine pesticides predominated in the reservoirs of SS, SO and SC, where there is intense agricultural activity. Overall it was possible to conclude in this study that Iguaçu River is impacted by different sources of human activities along the river course: i) The metropolitan region of Curitiba represents the main source of pollutants for the river, specially for FA reservoir, ii) Agriculture and livestock activities are impacting mainly the SS, SO and SC reservoirs. Therefore, no gradient of pollution was seen along the river course, since, besides the pollution from RMC, there are other diffuse sources of pollution. Considering the importance of Iguaçu River, it is necessary higher attention for this river from community and public authorities, with the aim of mitigating the impacts caused by different human activities.

Key words: Iguaçu River; Water quality; endocrine disruptors; native fish; vitellogenin; metals; pesticides

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LISTA DE ABREVIATURAS E SIGLAS

AChE - Acetilcolinesterase
BPA – Bisfenol A
CAT – Catalase
CYP – Citocromo P450
COPEL – Companhia Paranaense de Energia
DEs – Desreguladores endócrinos
DDT – Diclorodifeniltricloreto
E2 – 17 β -estradiol
EDC – *Endocrine disruptors chemicals* (Químicos Desreguladores endócrinos)
EE2 – 17 α -etinilestradiol
ELISA – *enzyme-linked immunosorbent assay*
EROs – Espécies reativas de oxigênio
ERN - Espécies reativas de nitrogênio
FA – Foz do Areia
GPx- Glutathione Peroxidase
GR – Glutathione Redutase
GSH – Glutathione reduzida
GSSG – Glutathione dissulfeto (glutathione oxidada)
GST – Glutathione S-transferase(s)
H₂O₂ – peróxido de hidrogênio
HPA – Hidrocarboneto policíclico aromático
HRP – peroxidase de raiz forte
IAP – Instituto Ambiental do Paraná
IHS – Índice hepatossomático
IGS – Índice gonadossomático
LPO – peroxidação lipídica
MM – Marcador de Peso Molecular
O₂⁻ - (radical) ânion superóxido
PBS - tampão fosfato salino
PBDEs- bifenilas polibromadas
PCBs – bifenilas policloradas
PCO – carbonilação de proteínas
PMSF – ácido fenil-metil-sulfonil fluorídrico
PXR – Receptor X pregnano
RE – Receptor estrogênico
ROS – *Reactive oxygen species* (Espécies reativas de oxigênio)
SC – Salto Caxias
SE – Segredo
SS – Salto Santiago
SO – Salto Osório
SDS-PAGE – Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis
SOD- Superóxido dismutase
USEPA – Agência de Proteção Ambiental dos Estados Unidos
VTG – Vitelogenina
WHO – *World Health Organization* (Organização Mundial da Saúde)

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1 INTRODUÇÃO

1.1 AVALIAÇÃO DO RISCO DE EXPOSIÇÃO E A ECOTOXICOLOGIA

O crescimento urbano e industrial nas últimas décadas tem como consequência um aumento na produção mundial dos milhares de compostos desenvolvidos pelas atividades antrópicas. Dentre as várias substâncias liberadas no ambiente cotidianamente, muitas apresentam potencial tóxico, como: os pesticidas (DDT, endosulfan, metoxicloro), organoclorados (PCBs, dioxinas, furanos), metais de transição (Hg, Cd, Pb, Cu), hidrocarbonetos policíclicos aromáticos (HPAs), nanopartículas, plastificantes (bisfenol A, ftalatos) e produtos farmacêuticos (etinilestradiol, tamoxifeno). Muitas destas substâncias são consideradas potencialmente perigosas não somente pela toxicidade que apresentam, mas também pela sua persistência no ambiente (ATSDR, 2008).

De forma geral, não há um controle adequado da introdução destes compostos no ambiente natural bem como sua remoção, principalmente em países subdesenvolvidos (FREEMAN et al., 2015). Assim, a liberação contínua destes contaminantes, provenientes de descargas de resíduos industriais e urbanos, pode aumentar os riscos de exposição e causar efeitos adversos tanto para a saúde humana como para a biota como um todo (LIU et al., 2016).

Desta forma surge a necessidade de desenvolver estudos que avaliem estes riscos, a fim de prevenir efeitos tardios antes que alcancem estágios mais críticos ou até mesmo irreversíveis. As avaliações de risco têm como objetivo analisar as probabilidades e magnitudes das consequências e efeitos adversos das atividades humanas ou catástrofes naturais (BROWNING; THOMAS, 2016; BUI et al., 2016; GUNASEKERA et al., 2015). Este processo consiste em diferentes etapas, incluindo a caracterização dos riscos de exposição (identificação do potencial tóxico dos poluentes e seus efeitos adversos), classificação e redução dos riscos e o biomonitoramento (CACHADA et al., 2016; KÜHNEL; NICKEL, 2014).

Os estudos ecotoxicológicos são fundamentais para a avaliação dos efeitos destas substâncias tóxicas nos diferentes níveis de organização biológica, que incluem desde os moleculares até os ecossistemas (HOFFMAN et al., 2003; NEWMAN, 2014; WALKER et al., 2005). Testes de toxicidade são comumente utilizados para identificar a relação entre dose e efeito de um produto específico,

para então estabelecer níveis toleráveis de exposição humana. Apesar da importância dos dados obtidos em estudos laboratoriais, os efeitos consequentes da situação real de exposição podem ser tanto superestimados como subestimados. Geralmente estes estudos são baseados em experimentos onde os organismos são mantidos sob condições laboratoriais otimizadas e expostos a diferentes concentrações de um único composto. Entretanto, no ambiente, os organismos estão susceptíveis a estressores naturais (temperatura, alimento e oxigênio) que podem interagir sinergicamente com os poluentes (HOLMSTRUP et al., 2010).

Entre os obstáculos que dificultam a compreensão dos reais riscos de exposição humana, também pode-se citar a complexidade das interações entre misturas químicas existentes no ambiente (DING et al., 2016; GAUTHIER et al., 2014; JARVIS et al., 2014). Estas misturas podem ter um efeito tóxico significativo mesmo que em baixas concentrações (COBBINA et al., 2015) e normalmente são maiores em relação ao efeito individual de cada componente (BACKHAUS; BLANCK, 2010). As concentrações dos xenobióticos aos quais a biota vai estar exposta pode mudar de acordo com a distribuição das fontes dos contaminantes assim como às mudanças nas características do ambiente (BATIHA et al., 2010; LINDIM et al., 2016; ZHU et al., 2014), incluindo fatores como a sazonalidade (NIE et al., 2012).

1.1.1 Ecotoxicologia aquática e o uso da água

A maior parte das milhares de substâncias introduzidas pela atividade humana são transportadas e destinadas aos corpos hídricos, seja por processos hidrológicos ou atmosféricos (GRILL et al., 2016; PRAMANIK; ROY, 2013). Portanto, faz-se necessário a detecção destes compostos em ecossistemas aquáticos assim como os efeitos em organismos que dependem destes habitats (AMIARD-TRIQUET et al., 2015).

A água é um recurso necessário para a sobrevivência de diversas formas de seres vivos, sendo essencial para a manutenção das condições fisiológicas normais em um organismo. Porém, a disponibilidade de água potável é cada vez mais limitada devido à maior demanda pelo consumo deste recurso natural, decorrente do aumento populacional (HAN; ZHAO, 2012; WANG et al., 2014), assim como à crescente degradação na sua qualidade (SUDHIR-YADAV et al., 2013). Além da sua

importância para o próprio consumo, a água pode proporcionar outras funções relevantes para os seres humanos como: a higiene, lazer, transporte, irrigação, pecuária e produção de energia elétrica (PRICE et al., 2016; RAN et al., 2016; VESTERINEN et al., 2010), representando vias alternativas de exposição (DOREVITCH et al., 2015, 2011).

De fato, uma das formas mais eficientes para a produção de energia elétrica é através da construção de usinas hidrelétricas (UHEs), principalmente no Brasil, privilegiado pela riqueza dos seus recursos hídricos (CORDOVA et al., 2014; FERREIRA et al., 2016). Além do elevado rendimento energético, outras vantagens incluem o fato de não ser poluente, ser renovável e permitir o controle de vazão dos rios (PÉREZ-DÍAZ et al., 2015; SACHDEV et al., 2015). Por outro lado, a construção de grandes UHEs pode apresentar desvantagens como o elevado custo e o impacto ambiental causado (MANDERS et al., 2016; MORALES et al., 2015). Além de comprometer a moradia de habitantes próximos ao rio, há a interferência no equilíbrio destes ecossistemas, podendo influenciar na fauna e flora local (YU; XU, 2016). Por exemplo, a modificação dos cursos d'água pode levar ao isolamento de populações de peixes, prejudicando a migração e reprodução destes.

1.2 ÁREA DE ESTUDO: O RIO IGUAÇU

Dentro deste contexto, o Rio Iguaçu representa um ecossistema aquático de grande relevância tanto socioeconômica quanto ambiental. Localizada, em sua maior parte, na região sul do estado do Paraná, a bacia do Rio Iguaçu apresenta o principal rio gerador de energia do sul do País. Drenando uma área de aproximadamente 70.800 km², a bacia também é responsável pelo abastecimento de água de até 28% de todo o consumo no estado (SEMA, 2010).

A nascente do rio se encontra na região metropolitana de Curitiba (RMC), surgindo da confluência do Rio Atuba com o Rio Iraí. Após atravessar todo o estado, com um percurso de 1320 km de extensão, o Rio Iguaçu desemboca no Rio Paraná onde são encontradas as Cataratas do Rio Iguaçu. Estas barreiras naturais são responsáveis pelo isolamento de populações de peixes com consequente elevada taxa de endemismo (70%) da fauna ictiológica do rio (BAUMGARTNER et al., 2012). Porém devido à influência de vários fatores antrópicos neste ecossistema, como a construção de usinas hidrelétricas, poluição e atividade de aquicultura, o risco de

extinção destas espécies de peixes tem aumentado significativamente (DAGA; GUBIANI, 2012).

A poluição no Rio Iguaçu representa de fato uma questão de grande preocupação, já que este é considerado o segundo rio mais poluído do Brasil (IBGE, 2010). Esta classificação se deve à enorme carga de poluentes que são liberados na origem do rio, consequência da atividade antrópica na Região Metropolitana de Curitiba. Esta região, denominada como Alto Iguaçu por se localizar no primeiro Planalto Paranaense, representa a principal fonte de contaminantes provenientes de descargas de resíduos urbanos e industriais. No trecho do médio Iguaçu, localizado aproximadamente a 100 km da origem do rio, a qualidade da água tende a melhorar de acordo com parâmetros físico-químicos avaliados pelo Instituto Ambiental do Paraná (IAP, 2008), assim como outro estudo recente demonstrou o mesmo padrão (Ide et al., 2016).

Após o município de União da Vitória, mais de 200 km distante de Curitiba, são encontradas as cinco principais usinas hidrelétricas no trecho denominado Baixo Iguaçu (FIGURA 1). A primeira, com maior capacidade geradora de energia elétrica (1.676 MW de potência) é a Usina Hidrelétrica Governador Bento Munhoz da Rocha Netto (Foz do Areia), a qual começou a operar em 1980 pela Companhia Paranaense de Energia (COPEL). A segunda Usina Hidrelétrica, localizada aproximadamente a 285 km de Curitiba, Governador Ney Aminthas de Barros (Segredo), também é administrada pela COPEL, possui capacidade geradora de 1260 MW e foi inaugurada em 1992. A terceira, Salto Santiago, e a quarta, Salto Osório são ambas administradas pela empresa Tractebel Energia e possuem capacidade de gerar 1420 e 1078 MW, respectivamente. E por fim a última, a 600 km da origem do rio, Usina Hidrelétrica José Richa (Salto Caxias), foi inaugurada em 1999 pela COPEL e apresenta capacidade de 1240 MW de potência. Nesta região do baixo Iguaçu as principais atividades humanas realizadas próximo aos reservatórios são a agricultura e pecuária, assim como a aquicultura.



FIGURA 1. Mapa do Rio Iguaçu e as imagens dos cinco reservatórios do Rio Iguaçu com as atividades antrópicas realizadas próximas aos reservatórios.
FONTE: O autor (2013).

Segundo o relatório “Qualidade das águas dos reservatórios do estado do Paraná” desenvolvido pelo IAP (2008), a qualidade da água no reservatório Foz do Areia é a pior entre as hidrelétricas do Rio Iguaçu, sendo considerada como “moderadamente degradada” (índice da qualidade da água = 3,3). Provavelmente este índice esteja relacionado à poluição oriunda da Região Metropolitana de Curitiba, visto que é o reservatório mais próximo da RMC. Já os reservatórios na sequência, apresentam índices que demonstram a melhora na qualidade da água (Segredo = 2,6 moderadamente degradado; Salto Santiago = 2,4; Salto Osório = 2,3 e Salto Caxias = 2,2, pouco degradado).

O critério de Qualidade da Água (cQA), formado a partir de dados científicos, estabelece níveis seguros dos poluentes ou parâmetros na água que devem ser considerados a fim de evitar efeitos tóxicos em humanos e organismos aquáticos através do uso da água (CONAMA, 2012; EPA, 2002). Entre os principais parâmetros avaliados por agências governamentais (ANA, 2004; IAP, 2008), incluem-se: concentrações de oxigênio dissolvido na água, turbidez e transparência, pH e Alcalinidade, Matéria orgânica (nitrogênio amoniacal, nitrato, nitrito, fosforo total, Clorofila **a** e Densidade de cianobactérias). Para o cálculo do Índice de qualidade da água, os parâmetros analisados apresentam pesos distintos, sendo o déficit de oxigênio dissolvido, seguido pela clorofila **a**, considerados os fatores mais influentes no resultado. De acordo com o índice determinado pelo IAP (2008), o Reservatório de Foz do Areia apresenta pior qualidade da água devido à elevada concentração de clorofila **a** em relação aos demais reservatórios. Isto se deve à ocorrência de florações de cianobactérias neste reservatório, como demonstrado em estudos prévios (CARNEIRO et al., 2014; SERPE, 2014).

Apesar destas análises permitirem avaliar um cenário geral, indicando a poluição causada pelo lançamento de esgotos, estas apresentam limitações por não informarem o real risco de exposição a misturas químicas e seus potenciais efeitos na biota. Portanto, um diagnóstico subestimando a qualidade da água pode ser fornecido se apenas estas análises forem consideradas. Assim, uma avaliação de risco ideal representaria a integração de dados obtidos através da detecção de diversos poluentes no ambiente e sua consequente bioacumulação nos organismos aquáticos, bem como os efeitos que estes compostos possam estar causando à biota (VAN DER OOST et al., 2003). As análises químicas são importantes para a identificação e quantificação dos compostos presentes no ambiente, porém não são

suficientes para prever as interações de misturas complexas e seus efeitos nos seres vivos, sendo necessária também uma avaliação das respostas biológicas (ZAGATTO & BERTOLETTI, 2006).

1.3 PEIXES COMO MODELOS ANIMAIS DE ESTUDO

Os peixes são considerados bons modelos animais para este tipo de estudo, pois apresentam características que colaboram com seu uso em diferentes desenhos experimentais, como a fácil adaptabilidade (KAPOOR AND KHANNA, 2004). Além disso, estão em contato direto com o ambiente aquático, podendo apresentar diferentes níveis de concentração e diversidade de poluentes.

Em uma mesma situação de exposição, os peixes podem naturalmente apresentar diferentes respostas entre as espécies, assim como estão sujeitos a diferentes processos de bioacumulação¹ e biomagnificação² dos poluentes (GRAY, 2002; LEWIS et al., 2011). Portanto, de forma ideal, estudos de biomonitoramento devem considerar análises em mais de uma espécie de peixe e preferencialmente pertencendo a níveis tróficos distintos (TABELA 1).

¹ Bioacumulação – Absorção de um químico no organismo proveniente do meio abiótico/biótico

² Biomagnificação – Processo onde uma substância química é transferida para um organismo via

² Biomagnificação – Processo onde uma substância química é transferida para um organismo via trófica, resultando numa concentração maior em relação à sua fonte (GRAY, 2002)

TABELA 1. Espécies de peixes do Baixo Rio Iguaçu utilizados como bioindicadores e modelo biológico no estudo.

Espécie	Características
 Lambari (<i>Astyanax bifasciatus</i>, Characidae)	Distribuição: Endêmico, abundante no Rio Iguaçu Hábito alimentar: Herbívoro (vegetais, alternativamente algas e insetos) Período Reprodutivo: Durante o ano todo (principalmente na primavera e verão) Características: Pequeno porte.
 Cará (<i>Geophagus brasiliensis</i>, Cichlidae)	Distribuição: Nativo, Ampla distribuição, bacias costeiras do leste, sudeste e sul do Brasil Hábito alimentar: Onívoro (insetos, outros invertebrados, vegetais, peixes) Período Reprodutivo: Setembro a Janeiro Características: Médio porte, hábito diurno, comportamento solitário, preferência por ambientes lânticos.
 Joaninha (<i>Crenicichla iguassuensis</i>, Cichlidae)	Distribuição: Endêmico, distribuição restrita Hábito alimentar: Piscívoro (alternativamente crustáceos) Período Reprodutivo: Outubro a Dezembro Características: Grande porte
 Tilápia (<i>Oreochromis niloticus</i>, Cichlidae)	Distribuição: Origem africana, distribuição ampla com introdução em vários países. Hábito alimentar: Onívoro Período Reprodutivo: Ano todo Características: Grande porte, rápida taxa de crescimento, fácil adaptabilidade em diferentes ambientes, cuidado parental, hábito diurno.

(BAUMGARTNER et al., 2012)
FONTE: O autor (2013).

1.4 BIOMARCADORES

Além das respostas distintas entre as espécies, vários parâmetros biológicos podem estar sendo influenciados devido à exposição química, sendo importante uma avaliação em diferentes subníveis de organização biológica. Uma estratégia adequada e amplamente utilizada é uma abordagem com o uso de multi-biomarcadores, onde podem ser considerados diferentes órgãos-alvo assim como várias respostas biológicas (BRITO et al., 2012; SANTOS et al., 2014; SANTOS et al., 2016; OSÓRIO et al., 2014).

De modo geral, um biomarcador pode ser definido como uma resposta mensurável que reflete a interação entre um sistema biológico e o potencial estressor, seja este químico, físico ou biológico (JAIN, 2010). Dentro do contexto ecotoxicológico os biomarcadores representam mudanças nas respostas biológicas a nível de sub organismo, desde moleculares, celulares, fisiológicas ou comportamentais, relacionadas à exposição e efeitos tóxicos dos poluentes ambientais (GUPTA, 2014). Os biomarcadores podem ser classificados em três classes distintas (VAN DER OOST et al., 2003):

- **Biomarcadores de exposição:** Detecta e quantifica uma substância exógena ou seu metabólito e sua distribuição em um compartimento do organismo, estabelecendo uma relação entre a exposição externa e as concentrações internas. Níveis de um composto que indiquem bioacumulação não representam biomarcadores de exposição;
- **Biomarcadores de efeito:** Inclui a quantificação de alterações bioquímicas, fisiológicas, ou outras respostas nos tecidos ou fluídos corporais de um organismo que podem ser associados a um possível ou estabelecido efeito adverso;
- **Biomarcadores de susceptibilidade:** Indicam a capacidade inerente ou adquirida de um organismo responder a uma substância xenobiótica específica;

Biomarcadores ideais são indicadores sensíveis e específicos de uma exposição química, refletindo a interação entre uma substância e um sistema biológico (CAMPION et al., 2013). Em outras palavras, um biomarcador deve ser

capaz de detectar um efeito precocemente em um organismo antes que se torne irreversível ou em um nível mais complexo de organização biológica, por exemplo, levando à redução ou mesmo extinção de determinada população naquele ambiente (DWYER et al., 1997).

1.4.1 Enzimas de biotransformação

Após a exposição e absorção de um xenobiótico, o organismo pode ter a capacidade de eliminá-lo mais facilmente na sua forma original (composto parental), caso esta seja hidrofílica, assim evitando sua acumulação (VAN DER LINDE et al., 2001). Porém, quanto mais hidrofóbico for o xenobiótico maior a dificuldade de excretá-lo via excreção renal. Entre os numerosos compostos sintéticos produzidos pelo homem, os poluentes orgânicos persistentes (POPs) apresentam alta hidrofobicidade e consequente resistência à degradação além da toxicidade inerente (MACKAY; FRASER, 2000; BEYER; BIZIUK, 2009). Desta forma as células apresentam estratégias para converter enzimaticamente os compostos lipofílicos em uma forma mais hidrofílica. Este processo de metabolismo dos xenobióticos, também conhecido como biotransformação, ocorre principalmente no fígado e pode ocorrer através de duas principais etapas:

- 1 – Fase I, onde as enzimas introduzem ou fazem a quebra de um grupo funcional (-OH, -COOH, -NO₂) dos compostos, envolvidas em reações de oxidação, redução ou hidrólise por enzimas monooxigenases microsomais (MO);
- 2 – Fase II, onde as enzimas conjugam um substrato endógeno, como a glutationa, ao xenobiótico (ou seu metabólito primário, derivado da Fase I).

Ambas as reações objetivam aumentar a solubilidade em água do xenobiótico e então facilitar sua excreção, podendo ocorrer de forma independente, ou seja, a fase II pode ocorrer sem haver a fase I e vice-versa (BOELSTERLI, 2003). Entretanto, o metabolismo nem sempre levará a detoxicação ou bioinativação de um poluente, podendo convertê-lo para uma forma ainda mais reativa e tóxica. Em muitos casos, o composto parental não confere toxicidade, enquanto seu metabólito formado apresenta maior reatividade, devido à sua bioativação (ATTIA, 2010).

As enzimas do citocromo P450 constituem uma superfamília de monooxigenases, heme proteínas, que estão envolvidas com a fase I de biotransformação de uma grande variedade de compostos tanto endógenos como

exógenos e são encontradas tanto em organismos eucariontes como procariontes (POULOS; JOHNSON, 2005). Em mamíferos, isoenzimas pertencentes às primeiras quatro famílias (CYP 1, 2, 3 e 4) são geralmente associadas com o metabolismo xenobiótico, enquanto que em outros organismos ainda falta esclarecer as principais vias de metabolismo das drogas (GUENGERICH, 2008; ORTIZ DE MONTELLANO, 2005). Compostos lipofílicos, como os POPs, podem levar à indução do sistema de metabolização em peixes, sendo o mecanismo de indução ou inibição de isoenzimas P450 extensamente estudado (TABREZ; AHMAD, 2010; ZHOU et al., 2013). A indução da CYP1A representa um dos biomarcadores mais estudados para contaminação de ecossistemas aquáticos, pois seus níveis e atividade são relacionados à exposição a hidrocarbonetos aromáticos de forma dose-dependente, tanto em peixes como mamíferos (SMEETS et al., 2002). Outra isoforma abundante no fígado é a CYP3A que apresenta importante papel metabólico de compostos exógenos e endógenos (EICHELBAUM; BURK, 2001; WILKINSON, 1996).

1.4.2 Biomarcadores de estresse oxidativo

As alterações bioquímicas, incluindo as enzimas de biotransformação, representam biomarcadores de efeito que fornecem uma resposta rápida após a exposição a um contaminante, sendo, portanto sensíveis a um estímulo estressor (VAN DER OOST et al., 2003). Entre as diversas reações enzimáticas que podem ser desencadeadas frente à exposição a um químico ambiental, encontram-se àquelas relacionadas à indução do estresse oxidativo. Pode-se definir o estresse oxidativo como o efeito prejudicial desencadeado pela produção excessiva de espécies reativas de oxigênio, onde o sistema de defesa é incapaz de contrabalanceá-los, ou restaurar um status redox normal da célula (VALKO et al., 2007).

Embora muitos xenobióticos possam ser tanto oxidantes ou redutores, geralmente os principais responsáveis pelo estresse oxidativo, ou oxidoreduutivo, são as espécies reativas de oxigênio (EROs). Isto ocorre, pois a molécula de oxigênio pode ser facilmente reduzida nos sistemas biológicos, principalmente dentro da mitocôndria, tanto em processos metabólicos celulares normais, ou pela indução de estressores xenobióticos, como os metais (LUSHCHAK, 2011). Esta redução parcial

ou em passos pode gerar intermediários do oxigênio com diferentes níveis de reatividade (FIGURA 2).

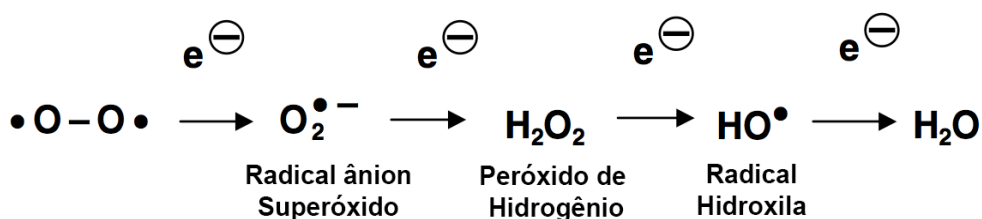


FIGURA 2. Redução da molécula de oxigênio gerando espécies reativas de oxigênio e, por fim, a água.

FONTE: BOELSTERLI (2003)

O ânion superóxido, apesar de não ser extremamente reativo, apresenta uma potencial ameaça para as células, sendo removido pela ação da superóxido dismutase (SOD) (WEYDERT & CULLEN, 2010). Por outro lado, o peróxido de hidrogênio, mesmo não sendo um radical e apresentando baixa reatividade, pode atravessar membranas biológicas e formar o radical hidroxila, o qual é extremamente reativo. Caso o peróxido de hidrogênio escape da degradação pela ação da Catalase (CAT) ou Glutathione peroxidase (GPx), ele pode ser reduzido ao radical hidroxila através da reação de Fenton (THOMAS et al., 2009), na presença de um metal de transição, como o ferro (FIGURA 3). Estas moléculas pró-oxidantes podem causar danos diretamente em macromoléculas alvos como os lipídeos, proteínas e até mesmo o DNA, levando ao surgimento de doenças neurodegenerativas ou até mesmo o câncer (MENA et al., 2009; MIGLIORE; COPPEDÈ, 2009). A detecção de lipídeos peroxidados, proteínas carboniladas e a 8-desoxiguanosina, através de diferentes métodos, representam biomarcadores de efeito muito utilizados tanto em experimentos laboratoriais como em campo (BRITO et al., 2012; OSÓRIO et al., 2014; ZIECH et al., 2010).

Para combater estes agentes químicos prejudiciais à integridade celular, os organismos desenvolveram mecanismos de defesa (FIGURA 3), a fim de manter um ambiente redox ideal, através de um sistema antioxidante que tem como funções: i) eliminar EROs; ii) manter um status redox de tióis na forma reduzida; iii) prevenir e reparar danos em lipídeos oxidados; iv) sequestrar metais redox-ativos para prevenção de reações como Fenton (PISOSCHI; POP, 2015).

A glutathione (GSH), em conjunto com o sistema de enzimas acopladoras, representa uma das principais linhas de defesa antioxidante no organismo. A

glutathiona é um tripeptídeo (glutamato, cisteína e glicina) que apresenta dois papéis importantes: i) pode ser conjugada a xenobióticos eletrofílicos, principalmente pela atividade da Glutathione S-transferase, uma enzima de fase II (KEEN et al., 1976), e/ou ii) age na manutenção do estado redox ideal para as células podendo regenerar proteínas oxidadas pela redução do grupo sulfidríla, responsável pelas suas propriedades antioxidantes (BOELSTERLI, 2003). Neste caso, com a doação do elétron, o grupo tiol é oxidado formando a glutathiona dissulfeto, que serve de substrato para a glutathione redutase reduzi-la novamente a GSH. Por outro lado, a Glutathione peroxidase usa a GSH como substrato para degradação de uma molécula de peróxido de hidrogênio à água e oxigênio, sendo a enzima mais importante para degradação do H_2O_2 extra-peroxissomal. Dentro dos peroxissomos, a Catalase é responsável pela degradação rápida do H_2O_2 (NAGABABU et al., 2003). A presença de xenobióticos pode levar tanto a uma indução como inibição deste sistema de defesa antioxidante essencial para funcionalidade normal da atividade celular. Metais redox-ativos, como o Cu e Cr, estão envolvidos com as reações de Fenton, aumentando a produção do radical hidroxila, enquanto metais redox-inativos, como o Pb e Cd, podem diminuir níveis de GSH (ERCAL et al., 2001).

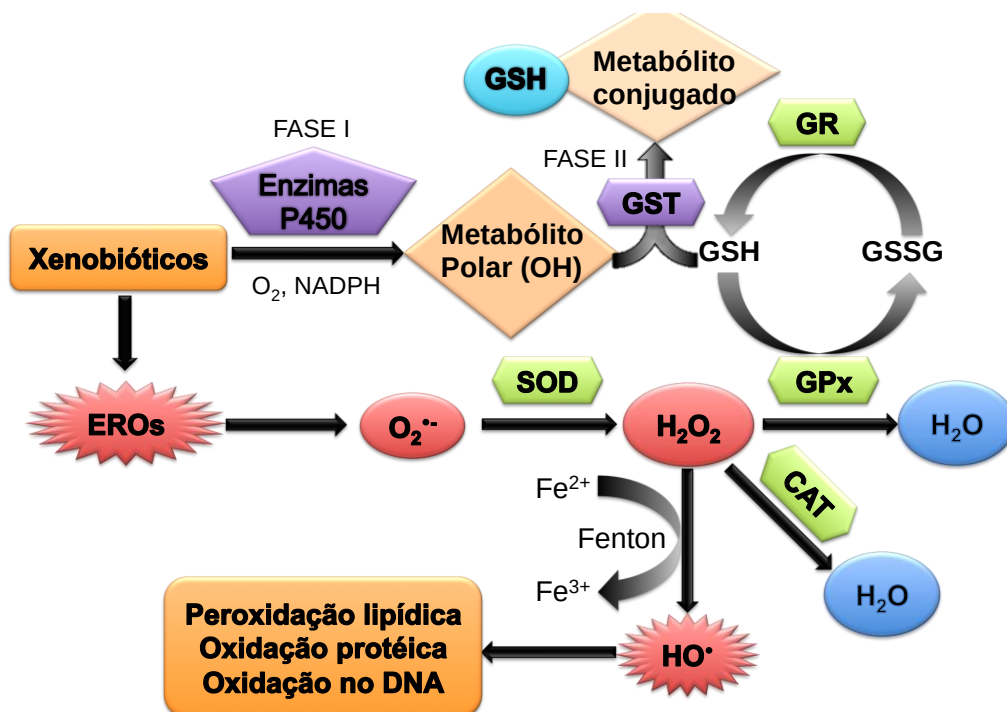


FIGURA 3. Esquema ilustrando o mecanismos de estresse oxidativo induzido por xenobióticos e o papel das enzimas antioxidantes e enzimas de transformação para proteção celular.
FONTE: O autor (2016).

1.4.3 Biomarcadores de neurotoxicidade

O sistema nervoso também apresenta potencial alvo para ação dos xenobióticos. Entre os diversos mecanismos desencadeados, a inibição da atividade da Acetilcolinesterase (AChE), tanto no cérebro como no músculo, é um biomarcador muito utilizado para avaliação de efeitos neurotóxicos em estudos de biomonitoramento (BRITO et al., 2012; FREIRE et al., 2015; OSÓRIO et al., 2014). Os pesticidas, como organofosforados, carbamatos, e alguns metais apresentam grande potencial inibidor para a atividade da AChE (PUNDIR; CHAUHAN, 2012; SCHLENK, 2005). Esta enzima, responsável pela rápida degradação da acetilcolina, é necessária para a transmissão sináptica colinérgica. Portanto, uma alteração na atividade normal pode implicar em efeitos adversos, incluindo respostas relacionadas à coordenação motora, memória e mudança comportamental (COLOVIĆ et al., 2013).

1.4.4 Biomarcadores de genotoxicidade

Outro parâmetro importante a ser avaliado são os efeitos genotóxicos, ou seja, as alterações no material genético que podem ser a nível estrutural do DNA, inicialmente, e causar danos no DNA, resultando em efeitos mutacionais e hereditários à longo prazo (KIRSCH-VOLDERS et al., 2009; SCHRADER, 2016). Análises que detectem a indução destes danos são de extrema relevância, pois podem indicar uma maior susceptibilidade a várias doenças, como o câncer (JAMIN et al., 2013; WEDEBYE et al., 2015). Para a avaliação de alterações no processo mitótico, a presença de micronúcleos ou alterações morfológicas nucleares em células sanguíneas de peixes, representa um biomarcador amplamente utilizado (ARALDI et al., 2015). Quanto à integridade do DNA, o ensaio cometa é eficiente para estimar um aumento na quebra das cadeias de DNA (COLLINS et al., 2014), induzidas por xenobióticos como o benzo[a]pireno (FRENZILLI et al., 2009).

1.4.5 Biomarcadores morfológicos

Além destes efeitos observados, é importante considerar outros aspectos em diferentes níveis de organização biológica, como por exemplo o morfológico, e

associá-lo a outras respostas, como o estresse oxidativo. As análises histopatológicas apresentam várias vantagens como um biomarcador, pois além de ser um método rápido e barato, demonstram de forma objetiva o grau de comprometimento de diferentes tecidos e órgãos alvos. Para estas análises, o fígado representa um órgão alvo adequado, já que possui papel chave no metabolismo e excreção de xenobióticos (MELA et al., 2007; OLIVEIRA RIBEIRO et al., 2002; OLIVEIRA RIBEIRO et al., 2007). Entre as principais alterações encontradas, estas podem variar de facilmente reversíveis, como uma hemorragia, até geralmente irreversíveis, como uma necrose ou neoplasia (BERNET et al., 1999). Da mesma forma, as brânquias, responsáveis pela osmorregulação, também representam um órgão alvo, já que estão susceptíveis a diversos danos por estarem em contato direto com a água (WEI; YANG, 2015).

1.5 DESREGULADORES ENDÓCRINOS

Em relação aos outros efeitos citados, mais recentemente, uma maior preocupação tem sido direcionada aos poluentes ambientais capazes de comprometer o funcionamento normal do sistema endócrino. De acordo com a Organização Mundial da Saúde, estima-se que mais de 800 químicos são suspeitos ou confirmados como desreguladores endócrinos (WHO, 2012). Durante as últimas duas décadas várias definições similares foram propostas para estabelecer um conceito de Desregulador Endócrino (DE):

1 - “ Um DE é um agente exógeno que interfere na produção, liberação, transporte, metabolismo, ligação, ação ou eliminação de hormônios naturais no corpo responsáveis pela manutenção da homeostase e a regulação de processos no desenvolvimento” (KAVLOCK et al., 1996).

2 – “ Um DE é uma substância exógena que causa efeitos adversos na saúde de um organismo intacto, ou sua prole, decorrentes de mudanças na função endócrina. Um potencial DE é uma substância que apresenta propriedades que possam levar a desregulação endócrina em um organismo intacto” (EC-WEYBRIDGE, 1996).

3 – “ DEs são químicos sintéticos que quando absorvidos pelo corpo tanto mimetizam ou bloqueiam hormônios e desregulam as funções normais do corpo através da alteração dos níveis de hormônios, diminuindo ou estimulando a

produção de hormônios, ou mudando a forma como os hormônios são transportados pelo corpo” (NRDC, 1998).

4 – “ A EDSTAC (1998) descreve um desregulador endócrino como uma substância química exógena ou mistura que altera a estrutura ou função do sistema endócrino e causa efeitos adversos em nível de organismo, prole, populações, ou subpopulações de organismos, baseada em princípios científicos, dados, evidência-de-peso, e princípio precatório.

5 - A Sociedade de Endocrinologia descreve os DE como “compostos tanto naturais ou sintéticos, os quais, através de exposições ambientais inapropriadas, alteram o sistema hormonal e homeostático que permitem o organismo se comunicar e responder ao seu ambiente (DIAMANTI-KANDARAKIS, 2009).

6 – “Um DE é uma substância exógena ou mistura que altera a função do sistema endócrino e consequentemente causa efeitos adversos em um organismo intacto, ou sua prole, ou (sub) populações” (WHO; IPCS, 2002).

Apesar de todas estas definições serem consideradas, a mais aceita e completa atualmente é a definição da Organização Mundial da Saúde (WHO; IPCS 2002) que é mantida no seu último relatório (WHO, 2012). Neste conceito, um composto desregulador endócrino é capaz de alterar a função do sistema endócrino causando um efeito adverso no organismo, o que não está implícito em outras definições, como a primeira, a qual foi considerada como principal referência anteriormente. Neste contexto, é importante ressaltar a definição de um efeito adverso como “uma mudança na morfologia, fisiologia, crescimento, desenvolvimento, reprodução ou vida útil de um organismo, sistema, ou (sub) população que prejudica a capacidade funcional, ou seja altera a capacidade de compensar um estresse adicional, ou aumenta a susceptibilidade a outras influências” (WHO; IPCS, 2004). Portanto, apesar de um químico poder causar uma perturbação, ou seja, uma alteração em um modo de ação ou via, na homeostase endócrina não necessariamente ele causará um efeito toxicológico, já que o sistema endócrino é naturalmente dinâmico e responde a diversos estímulos normalmente (DEKANT; COLNOT, 2013). Entretanto, a exposição durante o desenvolvimento representa uma maior “janela de susceptibilidade”, já que estes organismos apresentam maior sensibilidade por não terem a mesma capacidade de compensar as alterações endócrinas (UZUMCU & ZACHOW, 2007).

Entre os principais mecanismos estudados nos quais os DEs podem afetar o sistema endócrino e causar efeitos adversos, pode-se citar:

- 1- Efeitos agonistas - Mimetizando a atividade biológica de um hormônio pela ligação a um receptor celular, levando a uma resposta indesejada e similar ao do hormônio natural em um momento inadequado ou em uma maior extensão;
- 2- Efeitos antagonistas – Ligando a um receptor, mas não o ativando e impedindo a ligação do hormônio natural;
- 3- Ligando a proteínas transportadoras no sangue, interferindo na concentração de hormônios presentes na circulação;
- 4- Interferindo com o processo metabólico, afetando tanto as taxas de síntese como quebra e eliminação dos hormônios naturais;

(KIYAMA; WADA-KIYAMA, 2015)

Particularmente, os desreguladores endócrinos capazes de interferir na ação de hormônios esteroides, os quais controlam processos de reprodução, desenvolvimento e comportamentos sexuais, são amplamente estudados devido aos efeitos deletérios relacionados (HAMPL et al., 2014a, 2014b).

No contexto de reprodução humana alguns estudos indicam que a exposição a estes químicos pode estar associada: 1) a uma desregulação na função reprodutiva, como a redução da qualidade do sêmen e infertilidade; 2) Desenvolvimento fetal alterado, como anormalidade do trato urogenital e 3) Câncer testicular (SCHUG et al., 2011). Outros autores ainda sugerem que alguns efeitos da Síndrome de Disgenesia Testicular (aumento de incidência de criptorquidismo, hipospádias, de câncer testicular e baixa contagem de espermatozoides) podem estar relacionados a uma alteração na função androgênica, durante a diferenciação sexual, devido a uma exposição ambiental (EERTMANS et al., 2003; WITORSCH, 2002; SAFE, 2004). Mulheres expostas a xenoestrógenos, como o dietilbestrol (DES), demonstraram apresentar maior infertilidade, deformidades no útero, câncer vaginal e de mama (SAFE 2004).

Nos animais, assim como em estudos *in vitro*, as evidências demonstrando os efeitos dos desreguladores endócrinos são mais conclusivas, devido à menor limitação da investigação (FRISCHE et al., 2013; KNEZ, 2013; KOLLE et al., 2012). Neste sentido, agências de proteção ambiental têm como objetivo desenvolver

estratégias, padronizando testes para a identificação das propriedades destes químicos e seus efeitos (BARS et al., 2012; BORGERT et al., 2011; GELBKE et al., 2004; KOLLE et al., 2012; OECD, 2012).

De forma geral, as principais conclusões destas pesquisas apresentam um consenso: i) Como já discutido anteriormente, uma interação com o sistema endócrino não necessariamente implica em um efeito adverso; ii) apesar das diversas doenças relacionadas ao sistema endócrino, a ação de desreguladores endócrinos em concentrações ambientais na saúde humana não foi comprovada; iii) nos animais há indícios de efeitos na reprodução após exposição aos DEs em concentrações ambientais, enquanto que em altas doses os efeitos são confirmados; iv) a exposição humana a fitoestrógenos naturais é maior em relação aos poluentes produzidos pelo homem; v) muitos poluentes ainda não foram investigados e a falta de dados traz incertezas sobre a extensão dos reais riscos que os DEs podem causar.

Considerando os distintos modos de ação desencadeados pelos DEs, afetando o sistema endócrino tanto em humanos como em animais, é importante compreender quais os principais alvos estudados no aspecto reprodutivo:

1.5.1 Eixo hipotálamo-hipofisário-gonadal

A reprodução é controlada pela interação entre os hormônios do eixo hipotálamo-hipofisário, onde o hormônio liberador de gonadotrofina (GnRH), produzido no hipotálamo, é responsável por estimular a glândula pituitária anterior na liberação de gonadotrofinas, LH e FSH em mamíferos, análogas ao GTHI e GTHII em peixes (FIGURA 4). As gonadotrofinas, por sua vez, elevam a produção dos hormônios esteróides nas gônadas (estradiol, progesterona, testosterona), os quais regulam a secreção das gonadotrofinas através de um *feedback* nas glândulas pituitárias e hipotálamo (KNOBIL; NEILL, 2006).

Nos peixes e em mamíferos, uma desregulação no funcionamento deste eixo pode, além de afetar a produção dos hormônios reprodutivos, alterar o desenvolvimento folicular, ovulação e função do corpo lúteo nas fêmeas (CRAIG et al., 2011; MLYNARCIKOVA et al., 2014) e a alteração do desenvolvimento testicular e espermatogênese nos machos (ANKLEY et al., 2009; GORE, 2010; JENG, 2014).

1.5.2 Esteroidogênese

Os esteroides são compostos lipofílicos, de baixo peso molecular e derivados do colesterol participando de vários papéis fisiológicos importantes. Estes hormônios sintetizados principalmente em glândulas endócrinas como as gônadas e adrenais, também podem ser produzidos em órgãos como o cérebro, pois eles agem tanto em órgãos periféricos como no sistema nervoso central (LEPHART, 1996).

Assim como o colesterol, os esteroides possuem três anéis de carbono hexagonais e um pentagonal, onde as cadeias laterais de carbono (C 20-27) são atacadas. A remoção de parte das cadeias-laterais pela 20,22-desmolase (P-450_{scc}) origina compostos C-21 pregnanos (progestinas e corticosteróides). Enquanto a remoção total pela 17 β -hidroxiesteróide desidrogenase (17HSD) produz esteroides C-19 andrógenos e a perda do grupo metil-19, catalisada pela aromatase, gera os estrogênios (BROWN et al., 2003). Uma modulação na atividade destas enzimas devido à exposição aos desreguladores endócrinos, principalmente a inibição da aromatase, é bem relatada na literatura (CHESHENKO et al., 2008; MILLS et al., 2014; SANDERSON, 2006).

DEs também podem interferir no transporte dos hormônios esteroides, os quais são liberados na circulação sanguínea após sua síntese e se conjugam com grupamentos hidrofílicos ou proteínas carreadoras devido às suas propriedades hidrofóbicas (DARBRE, 2015). Esta característica também permite que os esteroides, assim como outros químicos lipofílicos, como os PCBs, atravessem as membranas biológicas e desencadeiem um mecanismo através da sua ligação em proteínas intracelulares ou receptores nos órgãos alvos (NORRIS; CARR, 2005). Outra via que desperta interesse são os DEs capazes de interferir na função de enzimas responsáveis pela metabolização dos esteroides em metabólitos ativos, antes de interagirem com seu receptor específico (CHESHENKO et al., 2008b; ZHOU et al., 2013).

1.5.6 Expressão da vitelogenina

Grande parte dos estudos dos mecanismos desencadeados pelos DEs está relacionada à capacidade de interação com receptores hormonais, onde a natureza da resposta pode depender da afinidade da ligação. No cenário reprodutivo dos

peixes, um dos processos mais bem caracterizado e estabelecido é a expressão da vitelogenina (MARIN; MATOZZO, 2004; MATOZZO et al., 2008; SUMPTER; JOBLING, 1995). Este biomarcador molecular é amplamente utilizado no diagnóstico de ambientes aquáticos impactados pela atividade humana, devido à sensibilidade e especificidade na detecção de compostos que apresentam atividade endócrina, particularmente estrogênica (CARBALLO et al., 2005; DESFORGES et al., 2010; SANTOS et al., 2016; GILANNEJAD et al., 2016; REINEN et al., 2010).

A vitelogenina (vtg) é uma lipo-fosfo-glicoproteína de elevado peso molecular, expressa por fêmeas ovíparas em época reprodutiva (SPECKER, 1994). Como precursora de proteínas do vitelo, desempenha um papel essencial como reserva alimentar durante o desenvolvimento do ovócito. A síntese desta molécula complexa depende da presença do hormônio estradiol (ARUKWE; GOKSØYR, 2003), o qual é regulado pelo eixo neuro-endócrino como informado anteriormente. O estradiol atravessa a membrana dos hepatócitos, devido à sua lipofilicidade, e se liga aos receptores estrogênicos intracelulares que agem como fatores de transcrição no núcleo (NAGLER et al., 2010). Este complexo ligante-receptor se liga em elementos responsivos estrogênicos em vários genes que levam a ativação da transcrição do gene da vitelogenina. No fígado sofre modificações (fosforilação e glicosilação e lipilificação) e é transportada via corrente sanguínea para os ovócitos, onde é incorporada por receptores de endocitose e então proteoliticamente clivada em proteínas menores do vitelo (lipovitulina 1 e 2, fosfovítina e β componente) (HIRAMATSU et al., 2005).

Apesar dos machos também possuírem o gene para vtg, sua síntese é normalmente muito reduzida devido aos baixos níveis de E2 circulantes. Entretanto, a exposição a compostos capazes de interagir com receptores estrogênicos, ou seja, agonistas podem levar a uma indução da expressão da vitelogenina em peixes machos (HIRAMATSU et al., 2006; PAIT; NELSON, 2003). Por outro lado, menos comumente avaliada, a expressão da vitelogenina também pode ser afetada nas fêmeas pela ação de compostos anti-estrogênicos, gerando efeitos deletérios até mesmo em populações de peixes (NICOLAS, 1999). Diversos compostos foram identificados como (anti) estrogênicos através da alteração nos níveis de vitelogenina, independente da semelhança estrutural ao 17β -estradiol, como o 17α -etinilestradiol, HPAs, PCBs, nonilfenol, octilfenol, bisphenol-A e metais (CALÒ et al., 2012; KIM et al., 2015; WANG et al., 2015a; YANG et al., 2015).

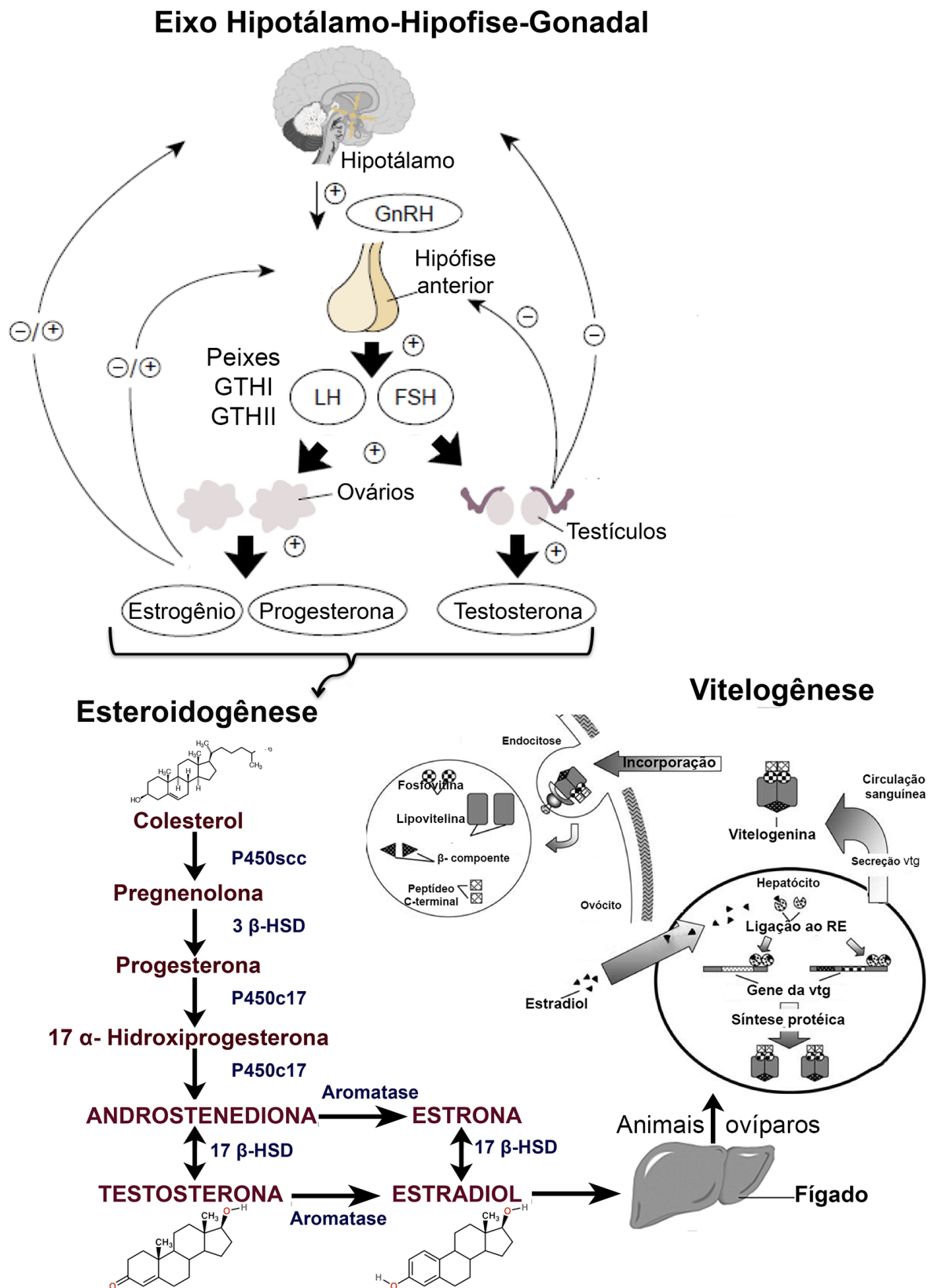


FIGURA 4. Funcionamento do eixo hipotálamo-hipófise-gonadal, a síntese de hormônios esteróides reprodutivos na esteroidogênese e os mecanismos de indução da expressão da vitelogenina em peixes.

FONTE: BROWN et al., (2003), HIRAMATSU et al. (2006), KONG et al. (2014), modificado pelo autor.

Para a detecção e quantificação desta proteína em indivíduos ovíparos, assim como outras proteínas alvos, diferentes análises moleculares são utilizadas, entre elas os imunoenaios (ELISA, radio-imunoensaio, *Western blott*) e qRT-PCR predominam devido à alta sensibilidade e especificidade (BARUCCA et al., 2006; HULTMAN et al., 2015; PALUMBO et al., 2009; WANG et al., 2015b). Atualmente os biomarcadores moleculares (genômica, proteômica, metabolômica) são de grande interesse científico por serem capazes de elucidar mecanismos de ação de um químico antes de causar um efeito adverso (FOWLER, 2012; SCHOLZ; MAYER, 2008; TYLER et al., 2008). Porém, apresentam como desvantagem mão de obra especializada e elevado custo. Uma alternativa para diminuir o custo destas análises é através da produção de anticorpos para uma determinada proteína, podendo ainda conferir maior especificidade de reação quando comparada a um anticorpo comercial. Por estas razões, entre outras, diversas estratégias são utilizadas para produção de anticorpos primários monoclonais e policlonais anti-vitelogenina de várias espécies de peixes (MARX et al., 2001; WANG et al., 2015a; WATTS et al., 2003).

1.6 PRODUÇÃO DE ANTICORPOS

Os anticorpos são glicoproteínas globulares que pertencem a cinco famílias distintas das imunoglobulinas (IgA, IgD, IgE, IgD e IgM), sintetizados por linfócitos B e plasmócitos em resposta ao estímulo imunogênico. Estruturalmente, um anticorpo é formado por duas cadeias leves ligadas por pontes dissulfídicas a cadeias pesadas, ambas idênticas. Para cada cadeia leve e pesada é possível dividi-las em duas partes: uma região constante, que não varia, e outra região variável, que se liga ao antígeno, ou seja, é responsável pelo reconhecimento do epítipo e é diferente entre as imunoglobulinas (JANEWAY et al., 2001).

A produção de anticorpos se refere ao inteiro processo de geração de um anticorpo específico utilizável, através de diferentes etapas, desde a preparação do antígeno, imunização, coleta e até a verificação da sua eficiência (LEENAARS; HENDRIKSEN, 2005). A preparação do antígeno, a molécula responsável por gerar a imunogenicidade e antigenicidade, representa uma etapa crítica para o sucesso na produção do anticorpo e seu uso adequado nas análises (GODING, 1996).

A imunogenicidade, por sua vez, está relacionada à capacidade de induzir uma resposta imune específica. Entre os principais fatores que irão influenciar na resposta pode-se citar: a relação filogenética, tamanho molecular e complexidade química (EDWARDS, 1999). De forma geral, quanto mais distante a relação filogenética entre a fonte do antígeno e o animal receptor, maior a indução da resposta imune. Assim como quanto maior a molécula, maior o número de epítomos e a complexidade química também gera maior imunogenicidade. A antigenicidade representa a capacidade do antígeno interagir com os linfócitos T ou B já sensibilizados, ou seja todas substâncias imunogênicas são também antigênicas. Outros fatores que influenciam diretamente na resposta imune são a dose do antígeno, as vias e escalas de inoculação e o uso de adjuvantes (HARLOW; LANE, 1988; STILS, 2005).

Considerando todas as possíveis ferramentas citadas que podem ser utilizadas para uma avaliação de risco ambiental toxicológico em ambientes aquáticos e a problemática apresentada em relação ao Rio Iguaçu, o presente trabalho teve como principais hipóteses a serem testadas:

- **H1** – Ao longo do Rio Iguaçu ocorre a presença de compostos desreguladores endócrinos (estrogênicos ou anti-estrogênicos/ androgênicos ou antiandrogênicos) que influenciam na saúde dos peixes deste ambiente aquático;
- **H2** – Durante o percurso do Rio Iguaçu ocorrem fontes difusas de poluição, provenientes principalmente da atividade agrícola, que dificultam a depuração da água e acabam sendo acumulados nos reservatórios do Rio Iguaçu.
- **H3** – Peixes nativos de diferentes níveis tróficos convivendo com uma mesma situação de exposição no Rio Iguaçu podem apresentar diferentes respostas biológicas.
- **H4** – Uma exposição subcrônica (gaiolas montadas nos reservatórios) das tilápias gera respostas diferentes em relação a uma exposição crônica.
- **H5** – A produção de anticorpos policlonais contra a vitelogenina e a técnica de ELISA são metodologias eficientes e importantes para avaliação da presença de compostos na água, que exercem atividade endócrina nos peixes.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a qualidade da água e detectar desreguladores endócrinos em cinco reservatórios do Rio Iguaçu através de análises químicas associadas a diversas respostas biológicas em peixes.

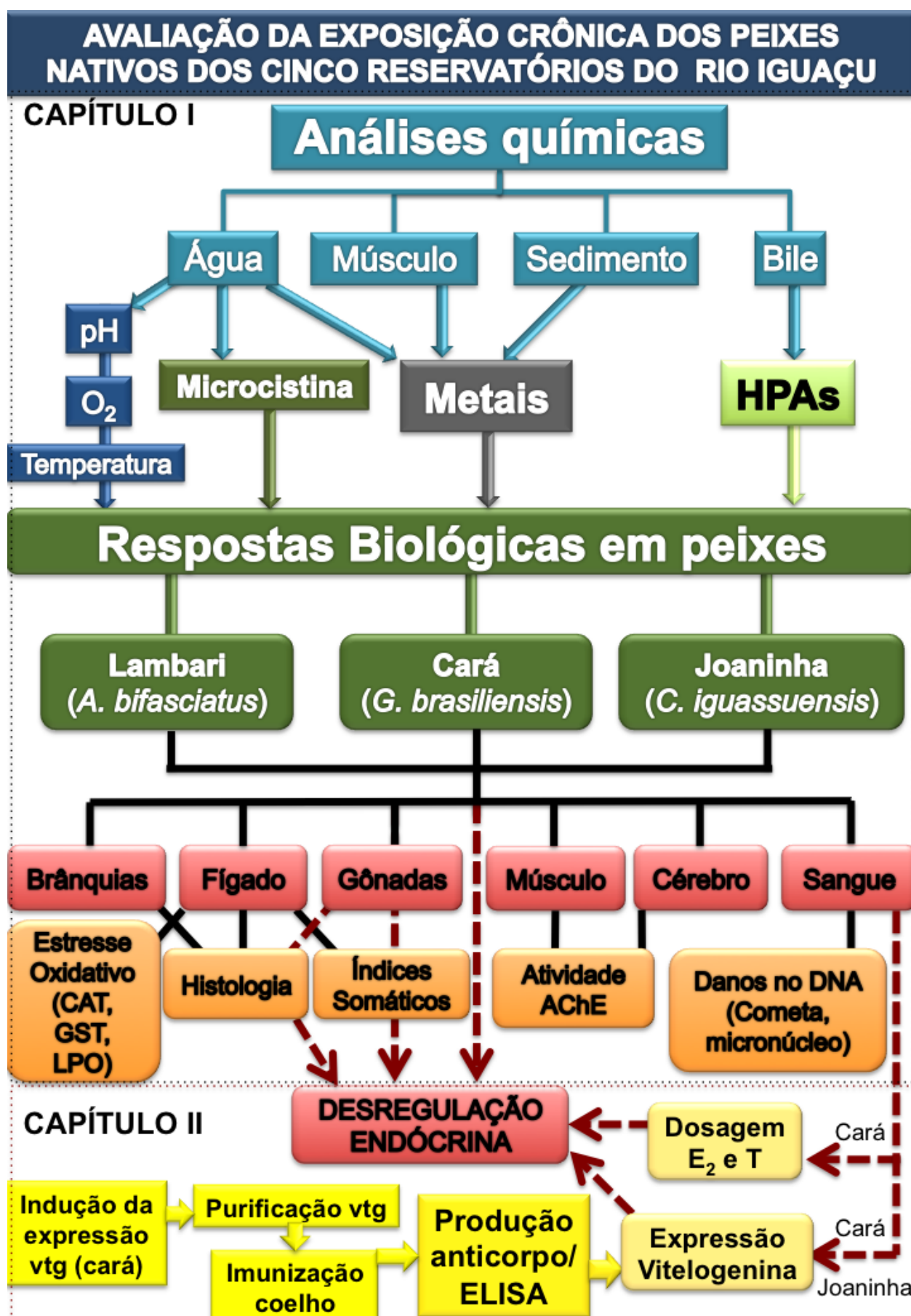
2.2 OBJETIVOS ESPECÍFICOS

Capítulo I – Avaliar múltiplas respostas biológicas (bioquímicas, genotóxicas e morfológicas) em diferentes tecidos-alvos (fígado, cérebro, músculo, sangue e brânquias) de três espécies de peixes nativas (*Astyanax bifasciatus*, *Geophagus brasiliensis*, *Crenicichla iguassuensis*) associadas à presença de metais (água, sedimento e músculo) e hidrocarbonetos policíclicos aromáticos na bile dos peixes dos cinco reservatórios do Rio Iguaçu.

Capítulo II – Produzir antisoros policlonais anti-vitelogenina de *Geophagus brasiliensis*, para verificar sua expressão, através do ensaio de ELISA e *Western blot*, em peixes coletados nos cinco reservatórios do Rio Iguaçu (PR) como uma ferramenta de detecção de atividade (anti) estrogênica à exposição crônica aos desreguladores endócrinos. Associar esta resposta aos níveis de hormônios esteroides (estradiol e 11 keto-testosterona) no plasma de *Geophagus brasiliensis*, assim como à morfologia das gônadas dos peixes nativos coletados nos cinco reservatórios.

Capítulo III – Avaliar os efeitos endócrino-reprodutivos precoces após exposição subcrônica de tilápias (*Oreochromis niloticus*) mantidas em tanques-redes montados nos cinco reservatórios do Rio Iguaçu. Detecção de poluentes orgânicos persistentes (POPs) e avaliação da atividade endócrina através da expressão de diferentes genes-alvos, atividades das CYPs, níveis de hormônios esteróides e análise da morfologia das gônadas.

- Resumo esquemático do desenho experimental adotado nos 3 capítulos:



AVALIAÇÃO DA EXPOSIÇÃO SUBCRÔNICA DAS TILÁPIAS EM QUATRO RESERVATÓRIOS (SE, SS, SO E SC) DO RIO IGUAÇU

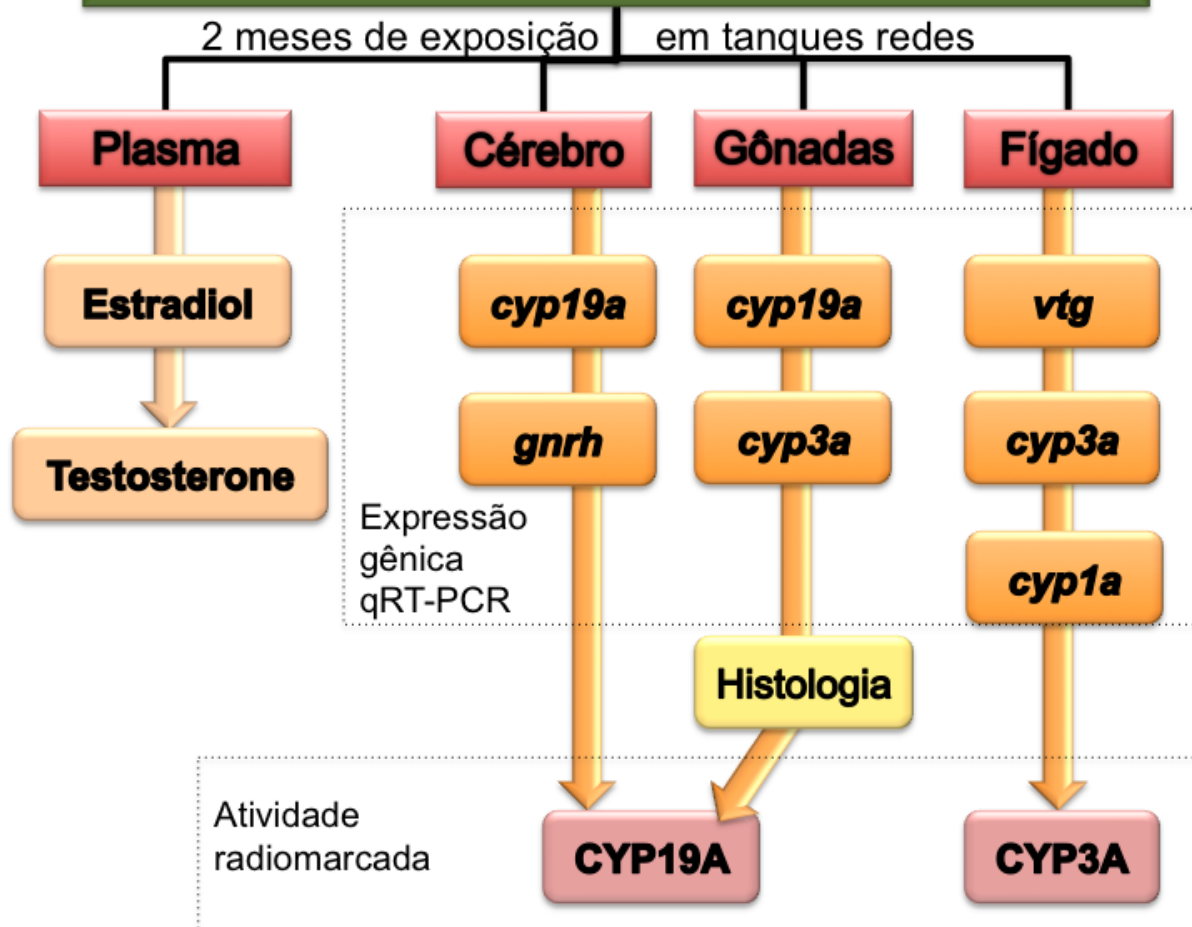
CAPÍTULO III

Análises químicas



Poluentes Orgânicos Persistentes

Respostas biológicas nas tilápias



Protocolos detalhados de metodologias não descritas nos artigos encontram-se nos APÊNDICES.

CAPÍTULO I

Bioavailability of pollutants sets risk of exposure to biota and human population in reservoirs from Iguaçu River (Southern Brazil)

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ABSTRACT

The Iguaçu River, located at the Southern part of Brazil, has a great socioeconomic and environmental importance due to its high endemic fish fauna and its potential to generate hydroelectric power. However, Iguaçu River suffers intense discharge of pollutants in the origin of the river. In a previous report, the local environmental agency described water quality to improve along the river course. However, no study with integrated evaluation of chemical analysis and biological responses has been reported so far for the Iguaçu River. In the current study, three different Brazilian fish species (*Astyanax bifasciatus*, *Chrenicicla iguassuensis*, and *Geophagus brasiliensis*) were captured in the five cascading reservoirs of Iguaçu River for a multi-biomarker study. Chemical analysis in water, sediment, and muscle indicated high levels of bioavailable metals in all reservoirs. Polycyclic aromatic hydrocarbons (PAHs) were detected in the bile of the three fish species. Integration of the data through a FA/PCA analysis demonstrated the poorest environmental quality of the reservoir farthest from river's source, which is the opposite of what has been reported by the environmental agency. The presence of hazardous chemicals in the five reservoirs of Iguaçu River, their bioaccumulation in the muscle of fish, and the biological responses showed the impacts of human activities to this area and did not confirm a gradient of pollution between the five reservoirs, from the source toward Iguaçu River's mouth. Therefore, diffuse source of pollutants present along the river course are increasing the risk of exposure to biota and human populations.

Keywords: Fish; Iguaçu River; Metals; Multi-biomarkers; PAHs; Risk of exposure

1 INTRODUCTION

Aquatic ecosystems receive a diverse set of compounds from many anthropogenic sources. Chemical interactions in complex mixtures represent a challenge for assessing the health risks of organisms (Contreras López 2003; Tian et al. 2015), but represent the real situation of exposure.

Human activities have deteriorated water quality in the last century through discharge of poorly treated urban and industrial waste. A critical consequence is the scarcity of potable water even in regions where freshwater is abundant. With the continuous growth of human population, the demand for potable water will increase.

The Iguaçu River is an important river of Southern Brazil (Fig. 1), due to its potential for hydroelectric power generation, human water supply, and the occurrence of many endemic fish species. Nevertheless, Iguaçu River is described as the second most polluted river in Brazil (IBGE, 2010). It rises in the Metropolitan Region of Curitiba (MRC, 1.8 million inhabitants - IBGE 2010) and receives large amounts of urban and industrial wastes without adequate treatment, resulting in frequent cyanobacteria blooms in reservoirs closest to the city (Carneiro et al., 2014) and high concentrations of polycyclic aromatic hydrocarbons (PAHs) in the sediment (Leite et al. 2011).

Approximately 200 km from MRC, there is a sequence of five major cascading reservoirs used for hydropower plants and intense agricultural activity (e.g., soybeans and wheat farms) developed along the riverbank. After crossing Parana State, approximately 1000 km from the MRC, the Iguaçu river reaches the Iguaçu Falls, which works as a natural barrier and is responsible for generating a high endemic fish fauna in the Iguaçu River (about 70 % of fish species) (Baumgartner et al. 2012). The lower part of the Iguaçu River will then flow into the Parana River. This significant ichthyologic endemism represents an important ecologic issue for the Iguaçu River, since susceptibility of extinction increases with the construction of dams, introduction of non-native species, aquaculture, and pollution (Daga and Gubiani, 2012).

The State Environmental Agency described in its most recent online report that an improvement of the water quality index of the studied sites upgraded from moderately to less degraded (IAP 2008). However, these regulatory monitoring programs usually consider only analysis of physicochemical parameters, which are

inadequate to predict potential toxic effects on the ecosystem (Santos et al. 2016). Correlations among environmental concentrations of contaminants, bioaccumulation, and early biological responses should be integrated to better comprehend the environmental risks (Van der Oost et al. 2003).

In this context, a multi-biomarker approach is a more suitable analysis to detect earlier biological responses triggered by pollutants (Brito et al. 2012; Linde-Arias et al. 2008; Osório et al. 2014). This approach will provide a better ecotoxicological scenario necessary for environmental management (Mills and Chichester, 2005).

Biochemical biomarkers have considerable potential for measuring effects of chemicals under field conditions since they are the earliest sensitive response (Carvalho et al. 2012). Genetic biomarkers inform levels of DNA damage which can be parameter used to assess the susceptibility of an organism to mutations and cancer (Araldi et al. 2015; Collins et al. 2014). Histopathological analysis is another important tool for evaluating lesions in target organs that can detect both acute and chronic exposure (Mela et al. 2007; Oliveira Ribeiro et al. 2006; Rabitto et al. 2005). Evaluation of these biomarkers along with the chemical profile of the water provides a reliable risk assessment for the exposed biota.

In the present study, the water quality of five reservoirs along the Iguaçu River was investigated for the first time through a multi-biomarkers approach in three Brazilian fresh- water fish species. Three different fish species were used to compare their specific responses. The fish used are from different trophic levels: the *Astyanax bifasciatus* (Characidae) or lambari an endemic herbivore and the most abundant fish species, the *Chrenicicla iguassuensis* (Cichlidae) or joaninha an endemic and carnivorous group, and the *Geophagus brasiliensis* (Cichlidae) or cará an omnivorous and widely distributed fish (Baumgartner et al. 2012).

MATERIALS AND METHODS

The Iguaçu River is located in Paraná, a southern state in Brazil. It extends 1.060 km and is the most important river in a basin of approximately 72.000 km² (Baumgartner et al. 2012). Along the river, there are five reservoirs that were analyzed in this study (Fig. 1). The Foz do Areia Reservoir (FA, 26° 00' 60.82" S 51° 65' 68.22" W) has the largest hydropower plant and is the closest to the metropolitan region of Curitiba (MRC). The other reservoirs, toward the river mouth, are Segredo (SE, 25° 79' 13.36" S, 52° 11' 89.36" W), Salto Santiago (SS, 25° 63' 73.08" S, 52° 60' 20.19" W), Salto Osório (SO, 25° 53' 71.06" S, 53° 00' 33.31" W), and Salto Caxias (SC, 25° 53' 64.43" S, 53° 49' 90.70" W).

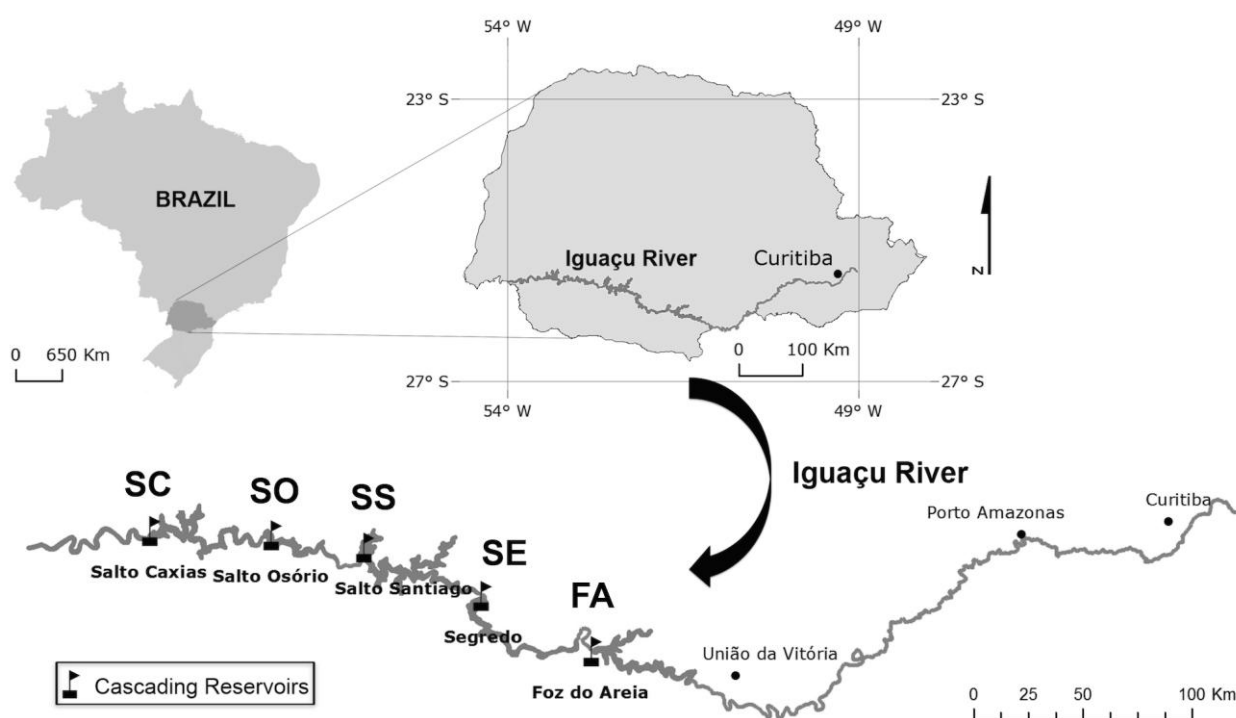


FIGURE 1. Map of Iguaçu River and the major cascading reservoirs, according to the proximity from the metropolitan region of Curitiba city (MRC), the main source of pollutants. FA – Foz do Areia; SE – Segredo; SS – Salto Santiago; SO – Salto Osório; SC – Salto Caxias.

Fish Sampling

A total of 346 adult fish (*A. bifasciatus*, *C. iguassuensis*, and *G. brasiliensis*) were captured in the reservoirs during summer (January–February) and winter (July–August) of 2013 (Table 1). Fish were captured through fishing nets (4–6 cm mesh) and trawl net with sampling every 4 h during a period of 24 to 48 h. Fish were then anesthetized with 25–30 mg.l⁻¹ benzocaine and killed in the field to avoid stress due to fish transportation. The same procedure and capture effort was exerted in all studied reservoirs.

TABLE 1. Fish captured in the studied reservoirs of Iguaçu River.

Species		n		Weight (g)	
		Sum	Win	Sum	Win
FA	<i>A. bifasciatus</i>	20	20	14.31 (± 1.85) a	22.78 (± 3.53) ab
	<i>G. brasiliensis</i>	20	4	13.7 (± 4.71) a	24.19 (± 2.52)
	<i>C. iguassuensis</i>	6	1	15.3 (± 18.85) a	88.8
SE	<i>A. bifasciatus</i>	20	20	31.4 (± 7.07) b	26.48 (± 2.93) ac
	<i>G. brasiliensis</i>	20	4	29.82 (± 4.75) b	23.71 (± 2.98)
	<i>C. iguassuensis</i>	3	0	51.16 (± 87.52)	
SS	<i>A. bifasciatus</i>	20	20	14.45 (± 5.58) a	13.67 (± 2.12) b
	<i>G. brasiliensis</i>	20	4	12.39 (± 12.39) a*	39.58 (± 10.11)
	<i>C. iguassuensis</i>	2	4	79.33 (± 37.14)	26.12 (± 3.94)
SO	<i>A. bifasciatus</i>	9	20	5.32 (± 1.36) a*	25.04 (± 4.48) c
	<i>G. brasiliensis</i>	9	0	17.98 ± 18.08	
	<i>C. iguassuensis</i>	17	0	17.77 (± 8.94) a	
SC	<i>A. bifasciatus</i>	20	20	34.38 (± 5.73) b	33.81 (± 5.92) c
	<i>G. brasiliensis</i>	3	0	260 (± 32.4) b	
	<i>C. iguassuensis</i>	20	20	96.68 (± 31.04) b	96.68 (± 31.04)

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir

Physicochemical parameters, microcystin-LR and metals detection

Temperature, pH, and oxygen dissolved data were provided by the State of Parana Electric Company - COPEL and TRACTEBEL Energy companies (Table 2). Microcystin-LR was extracted according to Matthiensen et al. (1999) with modifications. The intra-cellular toxin was determined in 1 L of water collected per reservoir and filtered in a glass fiber filter (GF/C 45 mm, Millipore), which was frozen and lyophilized for 24 h. Twenty-five milliliters of pure methanol (HPLC grade, J.T. Baker) was added to extract the toxin at room temperature for 1 h. In order to optimize the extraction, this step was repeated. After the second extraction, the toxin was concentrated with a vacuum rotary evaporator at 80 rpm at 40 °C. The extract was solubilized in 1 ml of methanol and stored at -20 °C. On the other hand, the toxins dissolved in the column of water of the reservoirs were determined in 1 L of filtered water (GF/C 45 mm, Millipore) with addition of 10 % trifluoroacetic acid (TFA) and filtered again. A solid phase column (C18) was used to extract the toxin from the filtered water, which was eluted with 3 ml of 0.1 % methanol/TFA (v/v) at a flux of 10 ml min⁻¹. The collected extracts were dried with nitrogen gas at 45 °C and suspended in methanol. Finally, the extracts were analyzed with a LC-MS ion trap (HPLC Shimadzu Prominence) through electrospray source.

Metals (Ag, Co, Cd, Cr, Cu, Mn, Ni, Pb and Zn) were measured in water, sediment, and fish muscle from the five reservoirs. The water was sampled at 30 cm below the surface and preserved with 0.5 % of HNO₃, while the sediment was collected 10 m away from the shore. Samples were protected from light and kept at 4 °C until analysis. For analysis of toxic metals bioaccumulation, muscle samples of all fish were pooled per group and stored at -75 °C until analysis according to Cotta et al. (2006). After acid digestion (Method US EPA 3005A for water; Method US EPA 3050B for sediment and tissue samples), a flame atomic absorption spectrometer was used to detect metals (FAAS, Varian, AA 240FS).

Polycyclic aromatic hydrocarbons detection in bile

The concentration of polycyclic aromatic hydrocarbons (PAHs) in bile was determined following a protocol from Hanson, et al. (2009), with minor modifications. Bile of three individuals was pooled and stored in amber glass vials at -75 °C. Bile

samples were diluted (1:1000) in 48 % methanol and added onto 96-well black microplate for PAHs through different excitation/emission wavelengths (288/330, 334/376, 364/406, and 380/422) in spectrophotometer. For quantification, a PAH mix (Cod. 47930-U, SUPELCO) was utilized to establish a PAHs standard curve.

Biological sampling

After anesthesia, some biometric parameters such as total length (TL) and total weight (TW) were obtained for a condition factor of Fulton (K) determination. Blood samples were obtained through caudal vein (for *C. iguassuensis* and *G. brasiliensis*) or cardiac puncture (for *A. bifasciatus*) with heparinized syringes and stored in fetal bovine serum for Comet assay and smeared in a slide for micronucleus counting. After euthanasia, the spinal cord was severed and liver and gill samples were dissected for light and scanning electron microscopy respectively. For biochemical analyses, the liver, muscle, and brain samples were stored at -75°C . The weight of liver was obtained to determine the hepatosomatic index (HSI) and using the formula $\text{HSI} = \text{liver mass} / (100 \times \text{total fish mass})$.

Biochemical biomarkers

Liver, brain, and muscle samples were thawed and homogenized at 1:10 (w/v) ratio with the following buffers: A Tris–HCl/ EDTA buffer (20 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) was used for the liver samples and a 0.1-M potassium phosphate buffer was used for muscle (pH 7.5) and brain (pH 8.0) samples. Homogenized samples were then centrifuged at 12000g, 4°C for 30 min; the supernatants were collected and stored at -75°C . Total protein concentration was determined (BRADFORD et al., 1976 with minor modifications of volumes for microplates) and utilized to normalize protein concentrations in supernatants. For *A. bifasciatus*, liver samples of two fish were pooled due to the small size and only catalase, glutathione S-transferase, and lipid peroxidation assays were performed.

Acetylcholinesterase (AChE) activity assay (Ellman et al. 1961) was performed with the muscle and brain supernatants. Catalase (Aebi 1984) and glutathione S-transferase (GST, Keen et al. 1976) activity, non-protein thiols (NPT) /

GSH concentration (Sedlak and Lindsay 1968), lipid peroxidation (LPO, Jiang et al. 1991, 1992) and protein carbonylation (PCO, Levine et al. 1994) assays were performed with liver supernatants. Glutathione peroxidase (GPx) activity was analyzed only for *G. brasiliensis* liver supernatants. Previously published protocols (Costa et al. 2010a, 2010b; Brito et al. 2012; Osório et al., 2014) were utilized for analyzing all biochemical biomarkers.

Genotoxicity biomarkers

Comet assay (Speit and Hartmann, 1999) and piscine micronucleus test (Heddle, 1973) were performed with erythrocytes, according to previously published protocols (Cestari et al., 2004; Ferraro et al., 2004; Ramsdorf et al. 2009).

Histopathological biomarkers

Light microscopy Samples of liver were fixed (70 % ethanol, 4 % formaldehyde, 5 % glacial acetic acid) for 16 h, dehydrated in a graded series of ethanol and xylene, and embedded in Paraplast-Plus (Sigma® St Louis, USA). Sections of 5 µm were obtained using a microtome (Leica), stained with Hematoxylin/Eosin, and mounted with Entellan (Merck®). Analysis was performed according to Bernet et al. (1999) with a few modifications.

Scanning electron microscopy The second left gill arch was washed with phosphate buffer, fixed (2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for at least 24 h, dehydrated in ethanol series (Merck®) and liquid CO₂, and metalized with gold for further analysis in scanning electron microscope JEOL KAL-6360LV.

Statistical analysis

All data were checked for outliers (Grubb's test), normality (Shapiro's test), and homoscedasticity (Bartlett's test) before analysis. Either two-way ANOVA test followed by the Tukey- Kramer post-test (parametric data) or Kruskal-Wallis test followed by the Dunn's test (nonparametric data) were utilized, with $p < 0.05$. Correlation analysis (Spearman test) was performed between the variables and age

parameters (weight and total length), in order to evaluate the influence of age on biological responses.

Biological data was integrated by two different methods. First, PERMANOVA was applied on biological responses to assess the differences between the two studied seasons (summer and winter). The second integration method was aimed to highlight associations among the variables measured in this study (both biomarker responses and contaminant levels in water, sediment, and body burden) during each of the two seasons (summer and winter) and for the three studied fish species (*A. bifasciatus*, *G. brasiliensis*, and *C. iguassuensis*). Factor analysis with principal component analysis as the extraction method (FA/PCA) was utilized. Associations between the different biomarkers (Comet assay, micronucleus assay, GST, CAT, GSH, GPx, PCO, AChE, LPO, Histopathological index, K, and HSI), metal loads in water (Ni, Cd, Zn, Cr, and Mn), sediment (Ni, Cd, Zn, Cr, Mn, Ag, Pb, and Cu), muscle body burden (Cd, Zn, Cr, Mn, Pb, Cu, and Co), and PAH metabolites in bile were assessed. Three matrices with original data (one for each fish species) were integrated. Since the matrices presented some missing data, the FA/PCA integration for each of the data matrix was performed in two steps: First, the variables showing missing data were omitted, and the FA/PCA was done with all cases; second, the cases showing missing data were omitted, and the analysis was done with all variables.

The variables were auto-scaled (standardized) and treated with equal importance. The selected variables to be interpreted were those associated with the factors with a loading cutoff of at least 0.50, a value which is more conservative than the loading cutoff recommended by Tabachnic and Fidell (1996). The relevance of the observed associations to each of the six sampling stations (cases) was estimated by calculating the factor score from each case for the centroid of all cases for the original data. All the statistical and multivariate analyses were performed using STATISTICA 12 software (StatSoft Inc. USA).

RESULTS

Condition factor (K) and hepatosomatic index (HSI)

Differences of condition factors between groups were observed only for individuals from the species *A. bifasciatus*. The individuals from SS reservoir presented lower condition factor (1.18) than those of FA (1.31, winter), SE (1.34, summer), and SC (1.33, summer and winter) reservoirs. In general, fish from the five reservoirs had different ages, as estimated by their weight and total length (Table 1). For the three fish species, the weights of the collected fish from SE and SC were higher than those from FA, SS, and SO for the summer samples. Correlation analysis showed a weak negative correlation ($r = 0.3\text{--}0.6$) for parameters of age (weight and total length) only with the response of AChE in the muscle of *G. brasiliensis* and *A. bifasciatus*.

The HSIs of *G. brasiliensis* and *A. bifasciatus* from the FA reservoir were significantly higher in the fish captured in the winter (3.33 and 0.97, respectively) than in the summer (1.36 and 0.60). *G. brasiliensis* from the FA reservoir had higher HSI than those from the other reservoirs (SE: 0.58; SS: 1.97).

Physicochemical parameters

Water temperature, pH, dissolved oxygen (DO), and intracellular Microcystin-LR concentrations are summarized in Table 2. The highest registered temperature was 27.5 °C (average of 24.9 °C) in SC reservoir (summer), and the lowest was 7.13 °C (average of 16.3 °C) in FA reservoir (winter). SE reservoir had the lowest pH value (5.90 - summer and 4.93 - winter). No variation in DO was observed during the seasons for FA and SE reservoirs (around 7 to 8 mg L⁻¹), but remarkable variations were recorded at SS, SO, and SC, with the lowest concentration of DO for the SS reservoir (4.59 mg L⁻¹ summer). Intracellular Microcystin-LR concentrations were higher in FA and SS reservoirs during the summer. In the column of water, Microcystin-LR was only detected in FA (summer) at low concentrations (1.08 ng L⁻¹, data not shown).

TABLE 2. Physicochemical parameters of temperature, pH and dissolved oxygen and microcystin-LR intracellular in the water of five reservoirs of Iguaçu River.

Reservoir	Season	Temperature (°C)	pH	Dissolved oxygen (mg.L ⁻¹)	Mc-LR Intrac. (µg.L ⁻¹)
<i>Conama</i>			6-9	>5	
FA	Summer	26.25	7.71	7.36	65,5
	Winter	7.13	6.73	7.63	<LQ
SE	Summer	23.70	5.90	7.17	0,27
	Winter	19.23	4.93	7.47	<LQ
SS	Summer	22.22	7.47	4.59	18,42
	Winter	17.35	7.13	9.21	<LQ
SO	Summer	24.57	7.30	5.61	-
	Winter	18.06	6.86	9.57	-
SC	Summer	27.50	6.53	7.60	<LQ
	Winter	19.86	6.63	9.13	<LQ

FA = Foz do Areia; SE = Segredo; SS = Salto Santiago; SO = Salto Osório; SC = Salto Caxias. Mc-LR Intrac. = Microcystin-LR intracellular. <LQ = Lower than limit of quantification

Chemical analysis of metals

Water: Chromium was above the levels permitted by Brazilian legislation (CONAMA 2012) in the water from FA, SS and SO (summer), and SS (winter) reservoirs. Cd, Zn and Mn were detected in the water of all reservoirs at both seasons, but at concentrations below the limit established by legislation. The highest concentrations of Ni and Cd were detected in SC reservoir, whereas that of Mn was highest in FA at both studied seasons (Table 3). Other metals such as Ag, Cu, Co and Pb were below the limit of quantification in the water of all reservoirs.

Sediments: Concentrations of Pb and Cu were higher in SO than in the other reservoirs (Table 3).

Fish: From all studied species and reservoirs had high concentrations of Zn in the muscle. Elevated levels of Cr, Cu, Mn, Cd and Pb were also observed in the samples (Table 4). *A. bifasciatus* and *C. iguassuensis* from SO reservoir (summer) had the highest Pb concentration. Relatively high concentrations of Cr were also detected in fish from almost all reservoirs, whereas Cd was mainly bioaccumulated in the muscle of fish from SC reservoir (summer).

Chemical speciation of metals was not distinguished by the method of detection performed.

TABLE 3. Metals concentrations detected in the water and in the sediment of the five studied reservoirs of Iguaçu River.

		WATER ($\mu\text{g.L}^{-1}$)					SEDIMENT (mg.kg^{-1})			
		Ni	Cd	Zn	Cr	Mn	Pb	Cu	Mn	Fe
<i>Conama</i>		25	10	5000	50	500	91,3	197		
SUMMER	FA	<LQ	1.60 ± 1.15	72.56 ± 20.85	75.70 ± 18.66	111.46 ± 5.20	<LQ	5.3 ± 0.2	$39. \pm 2.0$	104.0 ± 3.3
	SE	<LQ	2.23 ± 0.96	11.66 ± 5.20	<LQ	<LQ	<LQ	8.9 ± 0.3	330.7 ± 24.4	1136.0 ± 158.0
	SS	<LQ	0.43 ± 0.25	23.25 ± 3.74	69.63 ± 15.16	55.30 ± 4.57	<LQ	6.6 ± 0.6	67.4 ± 6.5	312.5 ± 11.0
	SO	<LQ	0.27 ± 0.05	42.75 ± 8.98	72.53 ± 26.16	16.83 ± 1.55	30.6 ± 1.8	76.3 ± 1.7	18.3 ± 0.8	1670 ± 80
	SC	5.2 ± 0.56	3.06 ± 0.61	60.50 ± 41.43	<LQ	5.36 ± 1.87	<LQ	4.1 ± 0.5	40.8 ± 4.0	92.9 ± 9.0
WINTER	FA	<LQ	0.56 ± 0.40	43.30 ± 23.72	46.06 ± 14.93	72.23 ± 5.39	<LQ	3.9 ± 0.2	22.2 ± 2.6	23.4 ± 2.1
	SE	4.8 ± 3.82	2.96 ± 0.37	106.66 ± 19.87	<LQ	14.00 ± 2.00	<LQ	3.9 ± 0.2	22.2 ± 2.6	1527.3 ± 95.5
	SS	<LQ	1.00 ± 0.72	37.90 ± 12.58	62.53 ± 18.71	21.66 ± 3.30	<LQ	3.2 ± 0.4	195.5 ± 23.2	138.9 ± 27.2
	SO	<LQ	1.50 ± 0.4	35.00 ± 12.0	<LQ	7.56 ± 1.20	21.1 ± 2.1	51.0 ± 1.6	17.6 ± 0.8	1214.6 ± 71.6
	SC	6.5 ± 2.55	4.03 ± 0.80	2.55 ± 5.72	<LQ	16.9 ± 2.94	<LQ	11.9 ± 0.7	250.8 ± 9.1	2586.1 ± 515

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir

<LQ = Lower than limit of quantification

TABLE 4. Metals concentrations detected in the muscle of fish from the five reservoirs of Iguaçu River.

Muscle (mg.kg ⁻¹)		Cr		Cu		Mn		Cd		Zn		Pb	
		SUM	WIN	SUM	WIN	SUM	WIN	SUM	WIN	SUM	WIN	SUM	WIN
FA	<i>A. bifasciatus</i>	4.10 ±3.0	<LQ	1.23 ±0.8	0.94 ±0.7	5.52	0.48 ±0.6	0.27 ±0.1	1.39 ±0.2	52.93 ±0.3	58.92 ±7.4	15.99 ±0.9	8.75 ±3.1
	<i>G. brasiliensis</i>	5.11 ±1.4	2.76	NA	<LQ	12.50	<LQ	0.79 ±0.1	2.14 ±3.0	25.79 ±3.0	30.10 ±11.0	2.75	<LQ
	<i>C. iguassuensis</i>	NA	12.05	NA	3.48	20.35 ±10.7	<LQ	NA	<LQ	14.77 ±1.9	15.08	51.70	
SE	<i>A. bifasciatus</i>	<LQ	13.17 ±11.9	1.58 ±0.2	0.21 ±0.3	4.82	<LQ	<LQ	2.79 ±0.8	84.77	72.87 ±22.8	8.75 ±1.8	<LQ
	<i>G. brasiliensis</i>	7.23 ±0.7	10.53 ±0	3.71 ±0.4	<LQ	0.90 ±0.3	<LQ	0.90 ±0.3	0.41 ±0.6	30.49 ±3.4	12.05 ±2.2	NA	<LQ
SS	<i>A. bifasciatus</i>	3.76 ±4.1	1.65 ±1.9	1.76 ±0.4	1.07 ±1.3	0.96	2.90 ±0.9	0.79 ±0.2	<LQ	59.68 ±13.3	46.11 ±4.0	13.16 ±0.4	12.79 ±1.2
	<i>G. brasiliensis</i>	7.82 ±0.2	<LQ	3.20 ±0.1	NA	11.84 ±2.3	7.87 ±0.3	0.93 ±0.2	NA	33.21 ±4.4	31.03 ±0	NA	<LQ
SO	<i>A. bifasciatus</i>	2.94	3.55 ±0.3	2.30	1.18 ±0.1	<LQ	21.59 ±15.9	<LQ	<LQ	60.25	37.31 ±0.6	56.06	11.30± 2.0
	<i>G. brasiliensis</i>	7.10 ±0.6	3.76 ±0.4	3.65 ±0.2	0.45 ±0.2	16.77 ±2.2	<LQ	1.12 ±0.3	<LQ	34.83 ±5.4	51.12 ±15.3	NA	9,5 ±0.4
	<i>C. iguassuensis</i>	1.61 ±1.5	5.67	7.74 ±0.2	0.77	NA	8.38 ±5.4	NA	0.86	41.71 ±13.9	47.83	87.94	7.54 ±0.9
SC	<i>A. bifasciatus</i>	7.96 ±0.3	<LQ	2.06 ±0.1	0.94 ±0.7	<LQ	0.48 ±0.6	4.18 ±1.9		28.01 ±3.4		1.27 ±1.4	
	<i>G. brasiliensis</i>	0.63	2.76	<LQ	<LQ	<LQ	<LQ	12.68± 1.6		30.44 ±4.1		>LQ	

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir
 <LQ = Lower than limit of quantification; NA = Not analyzed

PAHs in bile

The 2-ring PAHs were the ones predominantly detected in the bile of the three species from the five reservoirs. The fish from FA reservoir had the highest amounts of the 2-ring PAHs (Table 5). *A. bifasciatus* from SS and *G. brasiliensis* from SO reservoir showed the highest concentrations of larger PAHs.

TABLE 5. Polycyclic Aromatic Hydrocarbons detected in the bile of fishes from the five studied reservoirs of Iguaçu River in summer.

	PAHs ($\mu\text{g.g}^{-1}$)	2 rings	4 rings	5 rings	6 rings
Summer	FA <i>A. bifasciatus</i>	115.95	5.91 $\pm$ 19.77	4.57	0.37 $\pm$ 39.57
	<i>G. brasiliensis</i>	133.52 $\pm$ 29.62	5.76 $\pm$ 2.08	1.23 $\pm$ 0.3	0.21 $\pm$ 0.07
	<i>C. iguassuensis</i>	81.54 $\pm$ 14.27	7.96 $\pm$ 4.00	3.06 $\pm$ 2.19	0.55 $\pm$ 0.23
	SE <i>A. bifasciatus</i>	65.38 $\pm$ 24.20	9.04 $\pm$ 3.95	10.49 $\pm$ 5.69	0.96 $\pm$ 0.54
	<i>G. brasiliensis</i>	65.76 $\pm$ 10.81	9.38 $\pm$ 1.14	3.99 $\pm$ 1.40	0.60 $\pm$ 0.085
	SS <i>A. bifasciatus</i>	86.35 $\pm$ 24.54	11.59 $\pm$ 0.94	18.71 $\pm$ 4.51	2.17 $\pm$ 0.94
	<i>G. brasiliensis</i>	94.35 $\pm$ 15.55	7.20 $\pm$ 4.23	2.26 $\pm$ 0.20	0.38 $\pm$ 0.18
	SO <i>G. brasiliensis</i>	54.16 $\pm$ 25.50	15.16 $\pm$ 9.61	5.8 $\pm$ 0.83	1.18 $\pm$ 0.23
	<i>C. iguassuensis</i>	39.59	8.56	2.42	0.45
	SC <i>A. bifasciatus</i>	42.78 $\pm$ 32.24	16.95 $\pm$ 2.24	17.83 $\pm$ 5.07	0.92 $\pm$ 0.32
	<i>G. brasiliensis</i>	22.70	4.50	1.83 $\pm$ 14.2	0.30
	<i>C. iguassuensis</i>	112.33 $\pm$ 69.93	5.24 $\pm$ 2.15	1.45 $\pm$ 0.33	0.20 $\pm$ 0.03

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir. Letters indicate significant differences between sites in the same season (a, b); * indicate seasonal differences for the same site.

Biochemical biomarkers

A. bifasciatus showed the more conclusive results when analyzing the livers of individuals from both seasons, but no clear effect of contamination gradient was verified. GST activity was higher in samples from the SE reservoir when compared to SS, SO (winter), and SC (summer) (Fig. 2). Elevated lipid peroxidation (LPO) was observed in *A. bifasciatus* captured during the winter from the FA and SO reservoirs compared to SS and SC reservoirs. The liver of *G. brasiliensis* had higher activity of CAT in individuals from the SO reservoir when compared to FA and SS (summer), while for winter it was higher in the individuals from SE (Fig. 2). The GPx activity was enhanced in *G. brasiliensis* individuals from the SS and SO reservoir compared to individuals from the FA site (summer; data not shown). Corroborating with this result, concentrations of non-protein thiols in individuals from FA reservoir were higher than SS individuals. Protein carbonyls were higher in SE than in FA (summer) and SS (winter). No statistical differences were observed between the groups for GST activity and lipid peroxidation in *G. brasiliensis* (data not shown). The CAT activity in *C. iguassuensis* was also higher in individuals from SO than SE and SC (summer), but no significant differences were observed for GST activity, PCO (Fig. 2) and LPO analysis (data not shown).

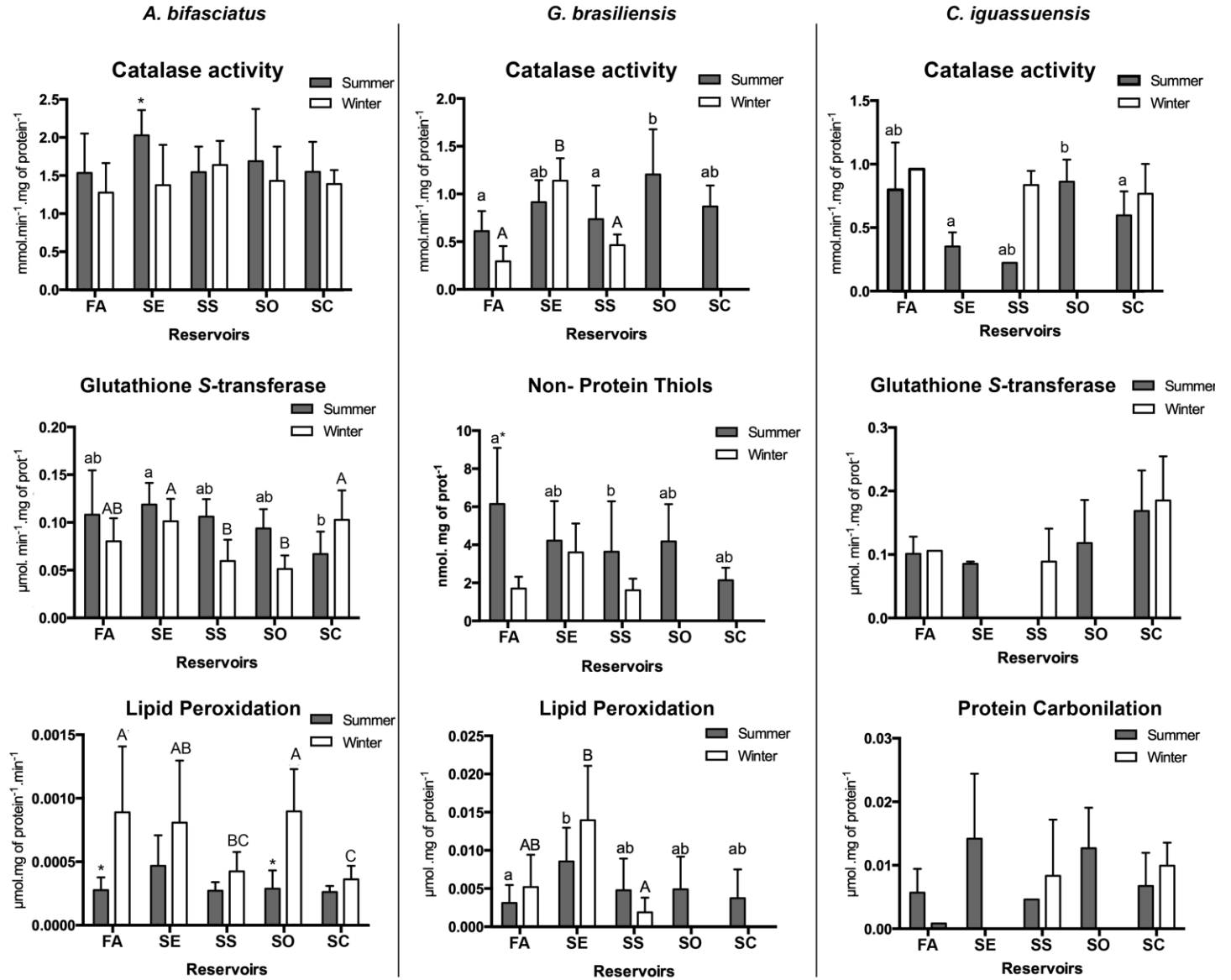


FIGURE 2. Biochemical Biomarkers in the liver. Letters indicate significant differences between sites in the same season (a, b); * indicate seasonal differences for the same site.

In general, the AChE activity was lower in individuals from SC reservoir than from the other reservoirs, particularly in the muscle of *G. brasiliensis* and *C. iguassuensis* (Fig. 3). For *A. bifasciatus*, brain AChE activity was the highest in the fish from SO (summer) and SC (winter). AChE enzymatic activity was typically higher in summer than winter (Fig. 3).

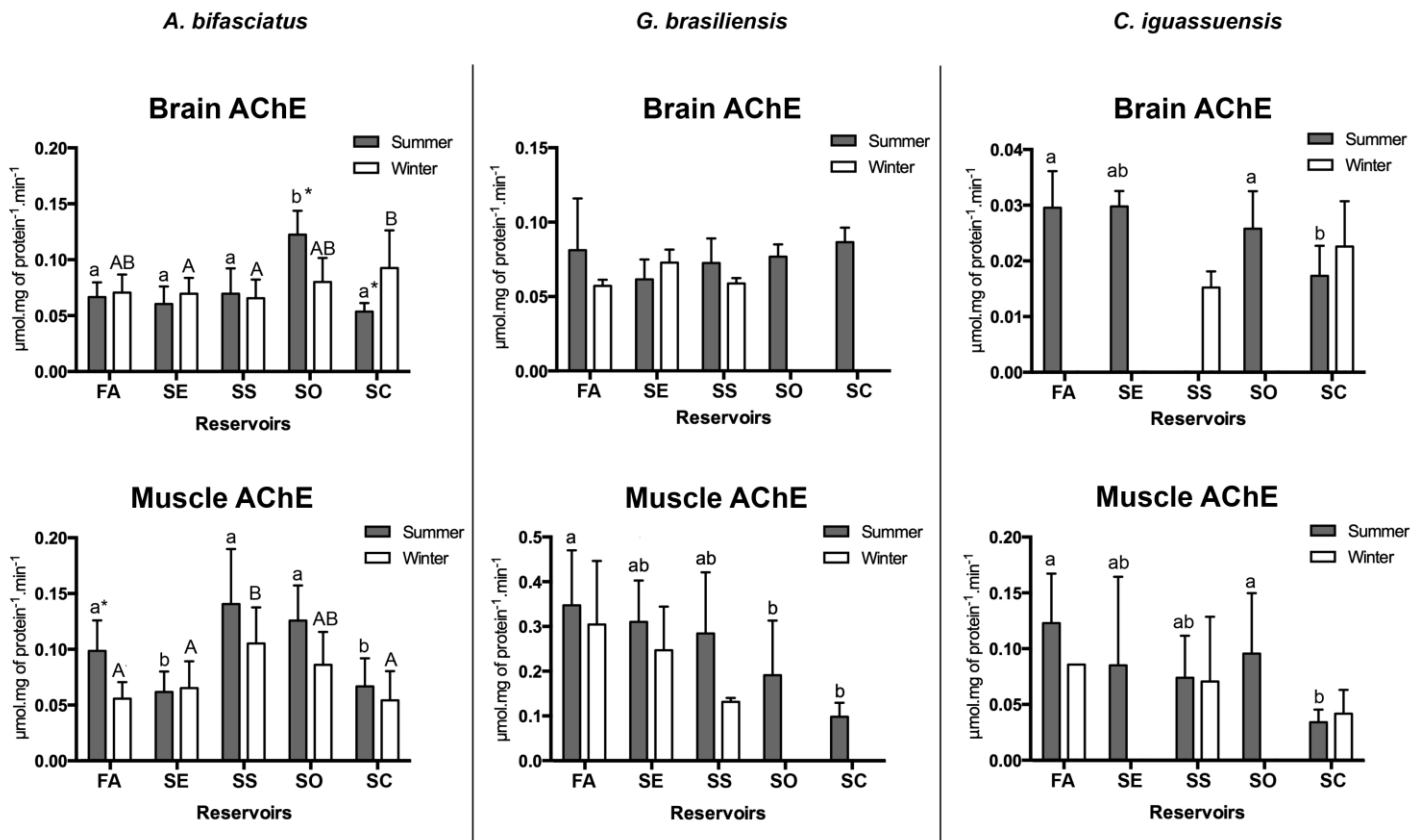


FIGURE 3. Acetylcholinesterase activity (AChE) in the brain and muscle. Letters indicate significant differences between sites in the same season (a, b); * indicate seasonal differences for the same site.

Genotoxicity biomarkers

A. bifasciatus and *C. iguassuensis* from SC reservoir (summer) had the highest scores of DNA damage in red blood cells (Fig. 4). Nuclear morphological alterations (NA), such as notched and micronuclei in erythrocytes, were more frequent in *G. brasiliensis* fish from SC reservoir (summer). For *A. bifasciatus*, NA was more frequent in the fish from SE (summer) reservoir.

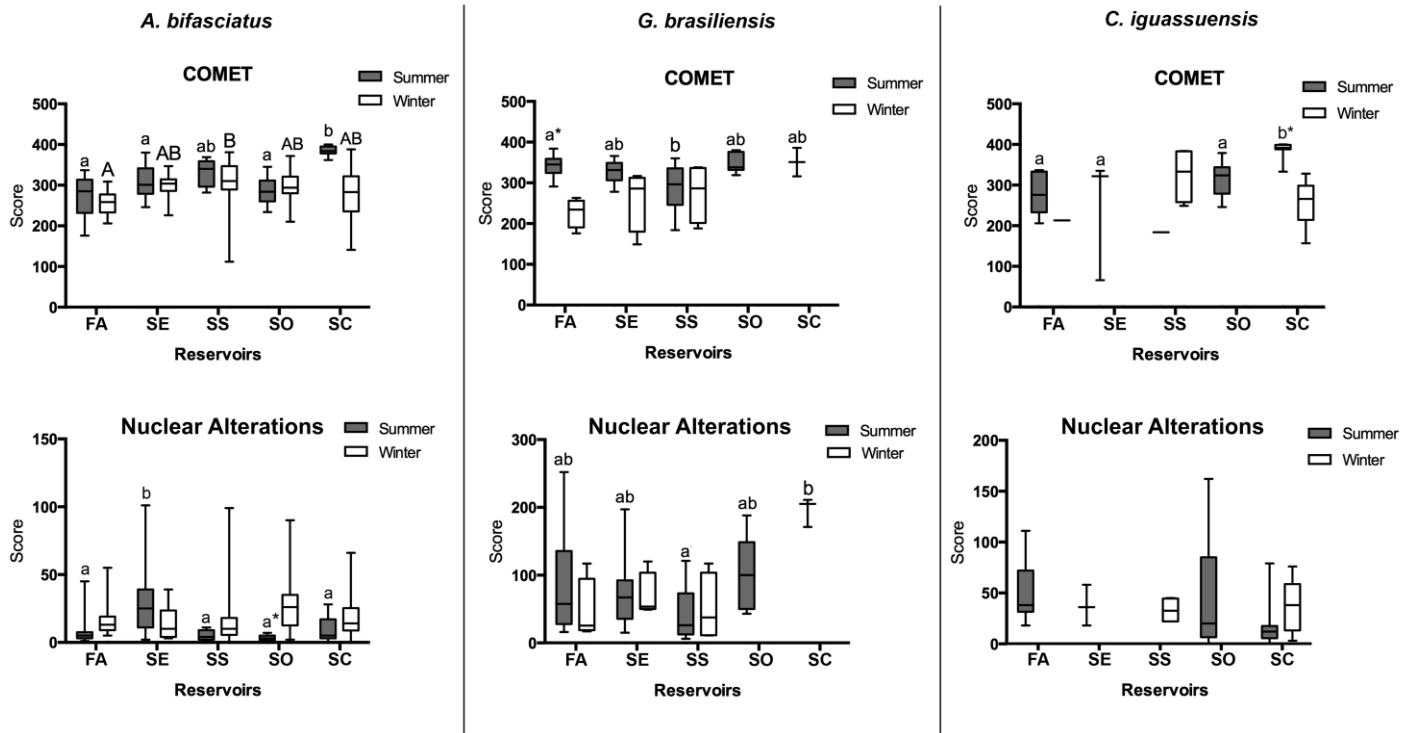


FIGURE 4. Comet assay and nuclear morphological alterations in the blood cells. Letters indicate significant differences between sites in the same season (a b); * indicate seasonal differences for the same site.

Histopathological analysis

According to Bernet's Lesion Index for the liver samples, no differences were observed among individuals captured in the studied sites. However, *C. iguassuensis* fish from SC reservoir had higher index than those from SO reservoir (summer; data not shown). In general, hepatic lesions were more frequent in *G. brasiliensis* than in *A. bifasciatus* and *C. iguassuensis*. However, the alterations were more frequent in summer than winter (Table 6). Despite of this, some important histopathological findings were observed in individuals from all reservoirs, such as necrosis, inflammatory responses (leukocytes infiltration, perivascular and perinuclear granulomatosis) and presence of parasites (Fig. 5). Other alterations, such as intracellular deposits and vacuolization that were also observed, indicated physiological and circulatory disturbances in the liver.

Hyperplasia, lamellar fusion, structural alterations of the lamellae and epithelium, and parasites were frequent in the gills of fish from the studied species (Table 6, Fig. 6). The fish captured in FA reservoir had a high incidence of fused lamellae, indicating an alteration in gills normal function. In general, the occurrence of lesions was homogeneously distributed between the species and the reservoirs in the summer, while in winter the variation was restricted between the groups of the same species.

TABLE 6. Histopathological analysis with frequency (%) of alterations in fish liver and gills from the five reservoirs.

		LIVER												GILLS							
		Necrosis		Parasites		Inflammation		Circulatory Disturbance		Melanomacrophage centers		Vacuolation		Lamellar fusion		Structural alterations 1st lamellae		Structural alterations 2nd lamellae		Epithelium alterations	
		Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win
FA	<i>A. bifasciatus</i>	86.6	80	73.3	66.7	33.3	20	62,5	100	62,5	-	-	-	12,5	40	62,5	100	62,5	100	75	60
	<i>G. brasiliensis</i>	72.2	66	22.2	-	22.2	33.3	20		100	66.6	33.3	100	25		20		100	50		50
	<i>C. iguassuensis</i>	100										60									
SE	<i>A. bifasciatus</i>	85.7	80	78.6	80	14.3	40	30	85,7	10	-	-	-	10	14	30	85,7	10	28,5	40	71
	<i>G. brasiliensis</i>	73.6		21		15.7	25		25	40	50	26.3	25			25		40	50	20	50
	<i>C. iguassuensis</i>	100				50					-		-								
SS	<i>A. bifasciatus</i>	100	93	50	53.3	16.6	46.6	85,7	55	28,5		-	100			85,7	55	28,5		75	55
	<i>G. brasiliensis</i>	75	100	25		25		30		60	-	43.7	-	20		30		60	50	30	25
SO	<i>A. bifasciatus</i>	77.7	53	100	80	-	66.6	55,5	83,3	44,4	-	-	-	22,2	16	55,5	83,3	44,4	16,6	33,3	66
	<i>G. brasiliensis</i>	75		37.5		25				55		-		22				55			
	<i>C. iguassuensis</i>	100				20						20									
SC	<i>A. bifasciatus</i>	87.5	71	62.5	71.4	25	85,7	33,3		33,3		-				33,3		33,3		100	
	<i>G. brasiliensis</i>	100		-		33.3						-		66						66	
	<i>C. iguassuensis</i>	100	100			22	33,3	5,5				83	33,3								

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir

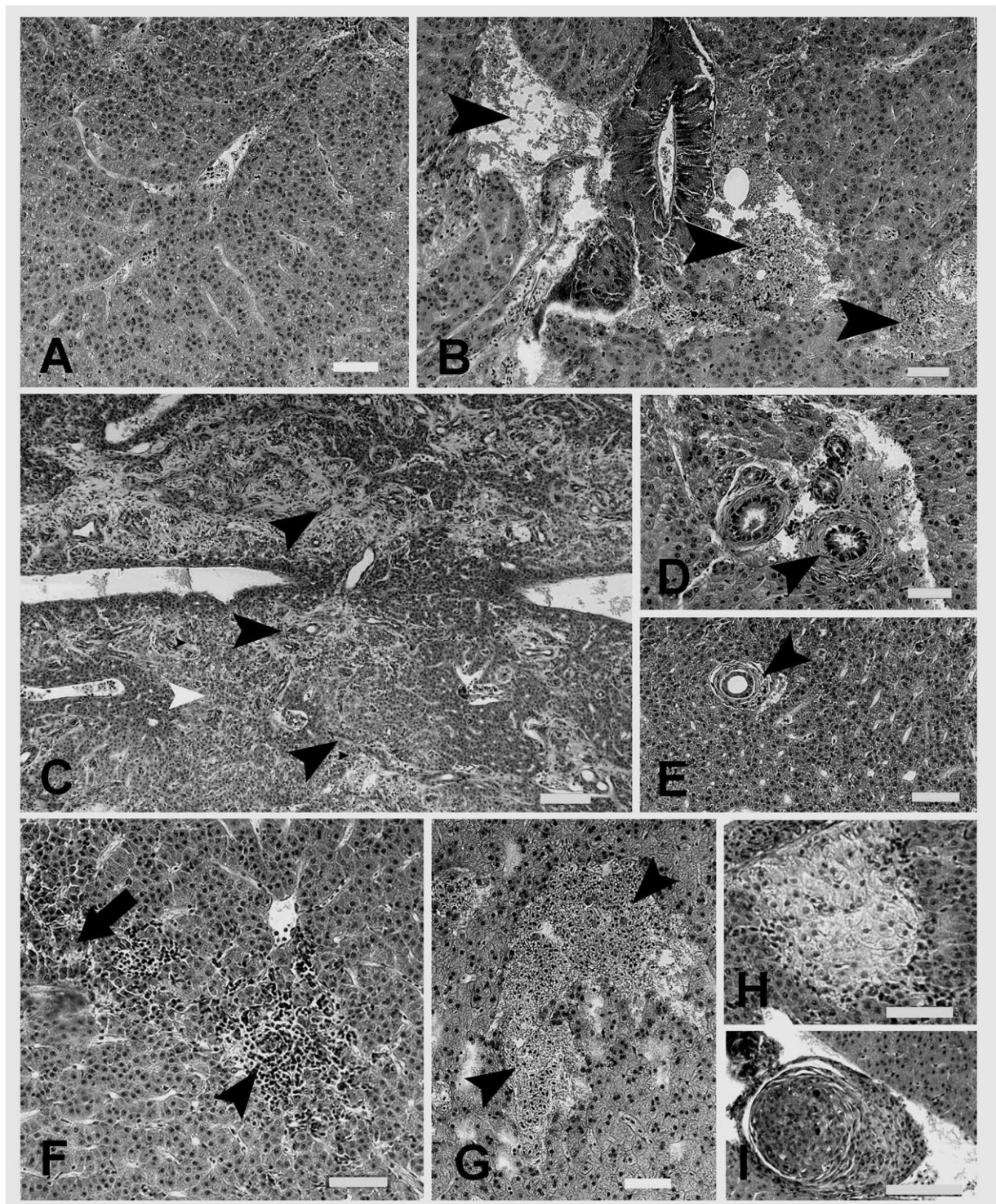


FIGURE 5. Liver sections of *Astyanax bifasciatus* (light microscopy). a Liver without alterations. b Necrosis (arrows head). c Tissue differentiation (arrows head). d Perivascular granulomatosis (arrows head). e Granulomatosis peritubular (arrows head) (scale bar = 50 μ m). f Inflammatory response (arrows head) and pre- necrotic area (arrow). g Hemorrhagic area (arrows head). h and i Invasive and encapsulated neoplastic areas, respectively (arrows head). Stain: Hematoxylin and Eosin. Bars: 100 μ m (c, i), 50 μ m (other images).

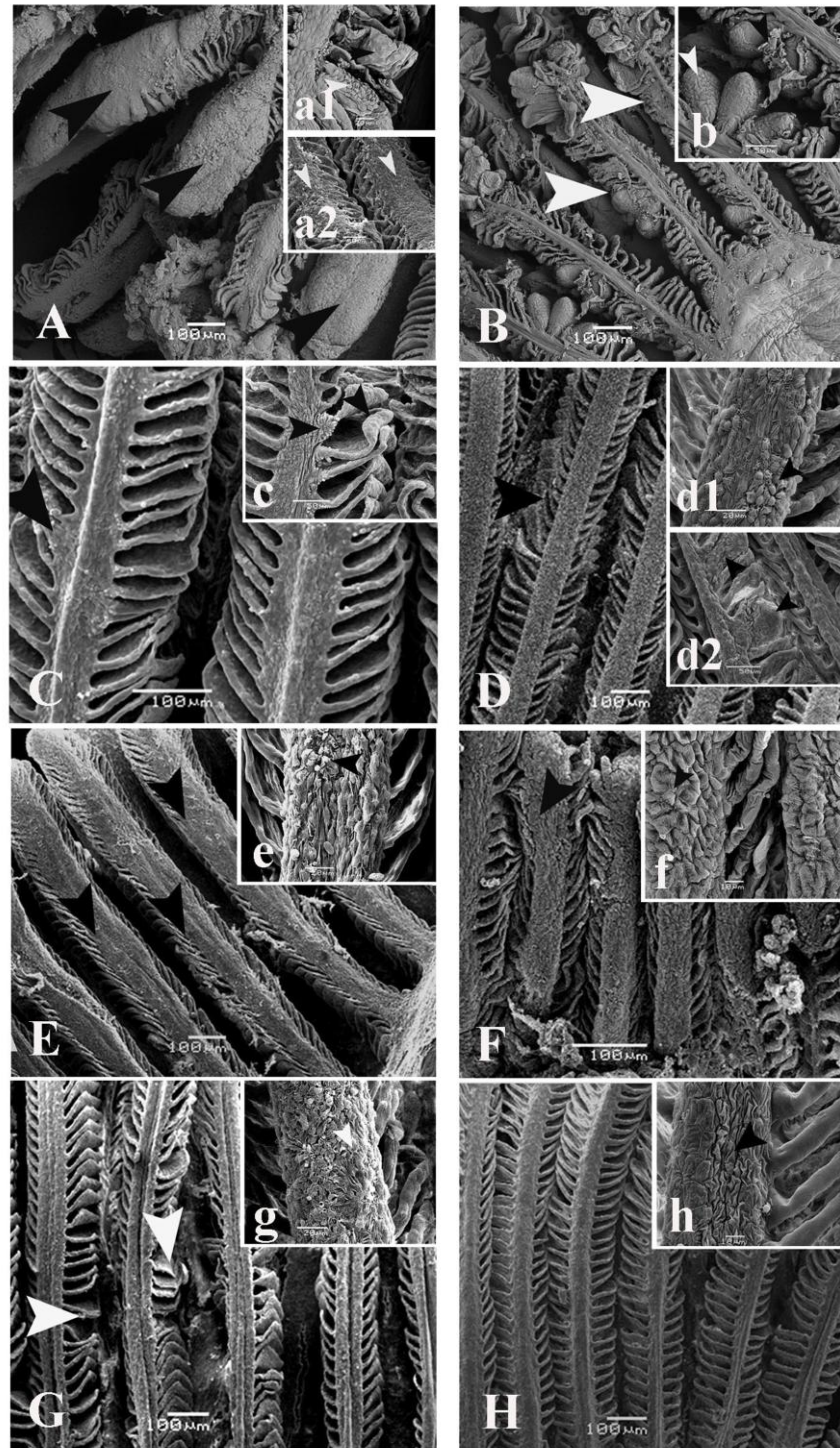


FIGURE 6. Gills of *Astyanax bifasciatus* (SEM). a FA winter: fusion of secondary lamellae (arrowhead) and the damages on lamellae epithelia (respectively a1 and a2). b FA summer: secondary lamellae fusion and aneurisms (arrowhead). c SE winter: fusion (arrowhead) and disarrange on secondary lamellae. d SE summer: damages on secondary lamellae (arrowhead), epithelial cell death (d1) and secondary lamellae fusion (d2). e SO winter: secondary lamellae fusion (arrowhead) and epithelial cell death. f SO summer: intense epithelial damages (arrow) as detailed in (f). g SS winter: damages in primary (arrowhead) and secondary lamellae epithelia. h SS summer: few damages are observed, except for the occurrence of epithelial effects on secondary lamellae.

Multivariate analysis

The PERMANOVA test revealed significant differences between sites and seasons for the interaction of all measured variables for the studied species. The principal component analysis (PCA), based on individual biomarker values, was performed in order to plot a unified view of the responses of all studied species (Figure S1, ESM 1).

Integrative analyses

Three different datasets were used to perform FA/PCA, metals levels in the water column and sediment, biomarker responses, and contaminant levels (metals and PAHs in the bile) in the three different fish species for an integrated description of environmental quality in the different sampling sites. Tables showing the factor loadings and the estimated scores of the factors for each case are presented in detail in the ESM 1 (Tables S1 and S2).

The total explained variance ranged from 69.03 to 100 % in all analyses. In the summer, the FA, SE, and SC sites showed relevant scores which were associated with the effects seen in the blood tissue, antioxidant responses (induction or inhibition), biomarkers of damage (including increased AChE levels), and low condition factor. In the results obtained for summer, the sites SS and SO showed no relevant effects in the blood for the three studied species. In these sites, antioxidant responses were not found in *A. bifasciatus*, although *G. brasiliensis* and *C. iguassuensis* showed catalase activity relatively higher in SO.

The results obtained from FA/PCA and the estimation of the relevance of factors for each studied site showed that the FA reservoir, followed by SC, presented the highest metal concentrations in water (summer sampling). On the other hand, the SE and SO site showed the highest number of metals in the sediment associated with factors of higher relevance. Different patterns of metals body burden were verified between fish species, with higher bioaccumulation of metals in *A. bifasciatus* from FA and SC, and *G. brasiliensis* collected in the SO reservoir.

Regarding the environmental quality in the winter, the results of the FA/PCA also showed a consistency in the damage responses (disturbed AChE activity and higher levels of LPO) and effects in the blood tissue (relatively higher frequencies of

blebbed, notched, and mononuclear erythrocytes) in *A. bifasciatus* and *C. iguassuensis*. These damage responses were associated with levels of metals in water and sediment from the SC site. In turn, few biological responses of relevance were verified for both *A. bifasciatus* and *G. brasiliensis* from SE reservoir, while SS showed a consistency on antioxidant responses during winter regardless the species, suggesting an intermediate environmental quality.

4 DISCUSSION

The results of the current study show that all of the reservoirs along the Iguaçu River, especially FA, SE, and SC reservoirs, are jeopardized by human activities. The Metropolitan region of Curitiba is a primary source of pollutants while other diffuse sources are also found along the river. The levels of metals detected in water, sediments, and fish muscle from the five reservoirs were either similar or higher than concentrations reported for other impacted aquatic environments (Authman et al. (2012), Maceda-Veiga et al. (2013), Noël et al. (2013) and Voigt et al. (2014), This shows that majority of toxic metals in impacted environments are bioavailable. Furthermore, relevant biological responses observed in fish through the multi-biomarker approach used in this study demonstrate an impact on the health of these organisms.

No clear gradient of pollution was verified along the Iguaçu River course. According to the results obtained from chemical and biological analyses, two aspects are relevant to consider the degree of pollution in the reservoirs: (i) the proximity to the MRC and (ii) the presence of diffuse sources of pollutants along the Iguaçu River. The integrated analysis was able to conclude that there was no clear gradient of pollution between the five reservoirs. The conditions of FA and SE reservoirs confirmed that MRC and others small cities were important sources of pollutants from domestic and industrial wastes to the Iguaçu River. Additionally, the SC reservoir had the poorest environmental quality found possibly due to the intense agricultural activity in that region. The FA/PCA analysis result reinforces that SS and SO reservoirs had the better environmental condition compared to the other reservoirs. However, when considering seasonal effects, the best environmental condition was observed for SE and SS reservoirs in the winter. On the other hand, the worst condition in winter showing greater environmental impact was different between the fish species: FA reservoir for *G. brasiliensis*, SO reservoir for *A. bifasciatus*, and SC reservoir *C. iguassuensis*. These findings indicate a difference in the sensitivity of the different species analyzed for the pollutants present in each reservoir.

Seasonal effects and others factors influencing biological responses of the three fish species

Overall, the integrated set of biomarkers and PERMANOVA analysis

confirmed that the biological responses suggest that individuals were more exposed to more pollutants during summer than in winter. The concentration of chemicals could be higher due to the lower water level in the reservoirs during the summer, especially in the FA reservoir. Furthermore, biological activity may also be correlated with the higher temperature during this season. Based on the physicochemical analysis, the five reservoirs presented distinct abiotic factors, such as temperature, that may interfere with chemical bioavailability (Fujita et al. 2001; Malczyk and Branfireun 2015) and affect biological responses (Elahee and Bhagwant 2007; de la Haye et al. 2012; Wäge et al. 2015).

Organic matter content in water, associated with domestic sewage, may also influence these responses, lead to eutrophication, and consequently to cyanobacteria proliferation (Yan et al. 2016; Thevenon et al. 2011). According to a recent report (Ide et al., 2016), organic matter content (dissolved organic carbon, nitrite, nitrate, orthophosphate, total nitrogen, and phosphate) and the density of phytoplankton decreased significantly along the Iguaçu River. This study, therefore, suggests a pollution gradient of domestic waste from the origin toward the river's mouth. However, significant variation in these parameters was observed only for the first 100 km range of the Iguaçu River from the MRC; between the five reservoirs, there was no longer evident variation. Therefore, the gradient of pollution was verified only before the reservoirs.

Physicochemical parameters are not enough to predict the chemicals effects on fish health

Although physicochemical analysis is utilized to evaluate water quality by environmental agencies in Brazil, they are not enough to predict the real risk of aquatic organisms that are exposed to many pollutants. According to the previous report published by the State Agency for Environment Control - IAP (IAP 2008), the reservoir with the best water quality in Iguaçu River was SC. However, SC reservoir had the worst water quality in the present study. FA reservoir was considered by the agency (IAP) as the worst in terms of water quality, particularly due to the high deficit of oxygen, chlorophyll a, and cyanobacteria blooms (IAP 2008). Water quality gradually improved in the subsequent reservoirs, as these parameters decreased (IAP 2008). However, the current study presents a different view of the water quality

in the reservoirs, providing strong evidences that there are other sources of contamination along the river, which refute the hypothesis of a gradient of pollution between the reservoirs.

The five reservoirs are differently impacted by sources of pollution

The FA reservoir is the closest to MRC, the main source of pollutants. Data from this study confirm that the FA site is a highly impacted, showing high levels of metals (Cr, Zn, and Mn) in the water and fish's muscle, high concentrations of microcystin-LR in the water, and 2-ring PAHs in the fish's bile. Biological responses, such as high LPO, HSI, and gill lesions in the three fish species, corroborate this condition. Zn and Mn are both ubiquitous elements, essential for normal physiological function and are indicators of human activity. However, deficiency or excess of these metals can be harmful (Giardina et al. 2009). They are known to inhibit antioxidant responses (Qu et al. 2014) and affect the nervous system, including changes of behavior and altered cognitive function (ATSDR - Public Health Statement: Cadmium. 2999). The concentrations of Cr in water were above the established by Brazilian legislation. Cr can bind to biomolecules, resulting in lipid peroxidation, protein carbonylation, and DNA damage (Mattagajasingh et al. 2008; Nickens et al. 2010). Finally, the highest levels of 2-ring PAHs were measured in the three species of fish from FA reservoir, indicating the influence of industrial activities in the MRC, such as crude oil refinement (Gallotta and Christensen, 2012; Leite et al., 2011).

The SE reservoir is the second furthest from MRC and, according to our data, also presented poor water quality. The reservoir had low pH, contained Cd in the water, Mn in the sediment, and elevated carbonylated proteins in the fish's liver. Acidic condition increases metal solubility in water and can interfere with normal gill osmoregulation, acid–base balance, and uptake/homeostasis of essential elements like Zn, Fe, and Cu in fish (Levit and Bozeman, 2010; Qu et al. 2014).

Metals are ubiquitous elements, but industrial and agricultural activities can increase metal concentration in the environment. Fertilizers can contain Cd (WHO 2011), as well as Co, Cu, Zn, Pb, and Ni as impurities, and herbicides present high levels of Fe, Mn, Pb, and Ni (Gimeno-García et al. 1996). Culture of soybeans, wheat, and corn increase from SE until SC reservoir, representing a potential source of pesticides for these sites.

SS and SO are, respectively, the third and fourth intermediate reservoirs, located in a region of intense agricultural activity. Considering their distances from MRC, other sources of contaminants may be responsible for the low dissolved oxygen, high levels of Cr in the water, low condition factor of the fish in SS reservoir, as well as toxic concentrations of Pb, Cu, and Zn in the sediment, and Pb in the muscle in SO reservoir. These metals may lead to gills dysfunction, oxidative damage (Wei and Yang, 2015), apoptosis (Luzio et al. 2013; Monteiro et al. 2009), and neurotoxicity (White et al. 2007).

SC reservoir is the furthest from MRC, but it has the high concentrations of Cd and Ni in the water, Cd in the muscle, low AChE activity, and high DNA damage in blood cells in all three fish species analyzed. Non-essential metals like Cd can cause deleterious effects on biota even at low concentrations (Satarug et al., 2009). Although the concentration of Cd was below the established level set by the Brazilian legislation, Cd is a human carcinogen, a potent endocrine disruptor, and exerts toxic effects on kidney, skeletal, respiratory, and reproductive systems (Gerbron et al. 2015; Jiménez-Ortega et al. 2012; Wang et al. 2014; WHO 2011). Mn and Pb can inhibit AchE (Santos et al. 2012), and mixtures of Pb + Cd (detected in SC) can induce neuronal degeneration in the brain even at low concentrations (Cobbina et al. 2015). In summary, independent of the distance from MRC, the studied reservoirs are impacted, at distinct levels, by chemicals from distinct sources.

The presence of complex mixtures in aquatic ecosystems complicate environmental risk assessment

Assessing chemical interactions (e.g., additive, synergistic, or antagonistic effects) is a great challenge for ecotoxicologists (Norberg et al. 2015). According to Gauthier et al. (2014), metal-PAH mixtures can have additive effects, e.g., alteration of membrane permeability to metals and mutual inhibition of detoxification by CYP biotransformation pathway. Mixture of the carcinogens such as Cd and benzo(a)pyrene (B[a]P) can impair the activity of antioxidant enzymes, though these chemicals can induce them when separate (Costa et al. 2010a, 2010b). This scenario illustrates the complexity in establishing the interaction between natural exposure to pollutants and biological effects. Biogeochemistry can only partially explain the differences of metal concentrations in water, sediment, and muscle of

fish, whereas different metabolic roles of essential and non-essential elements, environmental factors (Monroy et al. 2014), and food habit (Kalantzi et al. 2013) are related to the distinct bioaccumulation of metals in the three fish species.

A. bifasciatus reflected the environmental quality better than G. brasiliensis and C. iguassuensis

A. bifasciatus represented a good bioindicator of biomonitoring study to the Iguazu River, since its biological responses reflected the environmental quality better than the other two fish species in the current study. The same conclusion had been reported by Freire et al. (2015) for this species. *A. bifasciatus* is widely distributed in the Iguazu River (Daga and Gubiani 2012; Pie et al. 2009), and studies in different regions of Brazil and South America also describe the genus *Astyanax* sp. as a good bioindicator for biomonitoring (Alberto et al. 2005; de Lemos et al. 2008; Paulino et al. 2014; Prado et al. 2011, 2014).

Different biomarkers showed that the health of fish is compromised

Besides the importance of using different species in biomonitoring programs, it is also crucial to evaluate several responses in target organs to better comprehend the health status of the fish. Biochemical and genotoxicity analysis demonstrated alterations of enzymes involved in antioxidant defenses and nervous system, as well as damages in lipids, proteins, and DNA. These responses were correlated to the bioavailability of metals and PAHs, which induce several lesions and alterations in target tissues, as described by Liu et al. (2010, 2011).

Despite of the similar indexes of lesion among the groups, important pathological changes were observed in the liver and gills of the three fish species. Histopathological findings in target organs represent a strong evidence of chemical exposure in the field. The liver is described as a key organ for biotransformation of chemicals in vertebrates and a potential target for chemical-induced injury. Some chemicals as well as metals can lead to necrosis of hepatocyte cells (Oliveira Ribeiro et al. 2002; Sánchez-ChaRDI et al. 2009), DNA fragmentation, and redox unbalance (Proskuryakov et al. 2003). In the gills, some pathological alterations such as primary and secondary lamellae damage, fusion of adjacent lamellae, neoplasia,

and aneurisms are characteristic of waterborne exposure to chemicals (Liu et al. 2010; Srivastava et al. 2009). Incidence of lesions in target organs, evidences of neurotoxicity, and macromolecules damage associated with bioaccumulation of toxic metals and PAHs indicated the impact on fish health related to low water quality of the five reservoirs.

Integration of multiple biomarkers and chemical analysis is an efficient strategy for evaluation of water quality

The integration of biomarkers in fish and metal detection represents an efficient strategy for evaluation of water quality and risk assessment of human exposure (Carvalho et al. 2012; Omar et al. 2014; Sakuragui et al. 2013; Gusso-Choueri et al. 2015). An integrated analysis was performed through FA/PCA in order to improve the environmental diagnosis, since it can show the cases when relevant responses are significantly associated with contaminants present in the reservoirs to show the environmental quality of a site.

Final considerations

The results of this study provide strong evidence that human activities greatly impact the five reservoirs of the Iguaçu River. Domestic and industrial wastes from MRC and other small cities were considered as the main responsible to impact FA reservoir even with a considerable distance from this source of pollutants. Water quality of the second reservoir, SE, is impacted by what compounds from the MRC and small cities and agricultural activities. SS and SO reservoirs are primarily subject to pesticides inputs, with less impact from MRC. Finally, SC reservoir had the poorest water quality, indicating that there is no gradient of pollution between the five reservoirs from the origin of the river toward the mouth.

The effects of the chemicals described here demand a risk assessment for the biota and human populations in this area since the Iguaçu basin has a high environmental and socioeconomical importance. These sets of results represent the first study performed in the reservoirs of Iguaçu River showing the impacts in fish health and can be used for its management or to establish conservation politics.

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SUPPLEMENTARY MATERIAL

PCA data (Figure 1) for *A. bifasciatus* were explained until the fourth component based on the standardized data. PC1 accounted for 18.93% of the variability. This PC was negatively loaded by NA, GST, CAT, and K and positively loaded by LPO, AChEM, AChEB, and HSI. PC2 accounted for 14.58% of the variability in data being most negatively loaded by COMET, NA, PCE, LPO, HSI and K and positively loaded by GST, CAT, and AChEM. PC3 accounted for 13.25% of the variability in data being most negatively loaded by COMET, CAT, AChEM, and K and positively loaded by PCE, LPO, GST, and AChEB. PC4 accounted for 12.17% of the variability in data being most negatively loaded by PCE, GST, and K and positively loaded by NA, LPO, CAT, AChEM, and HSI.

For *G. brasiliensis*, three components were extracted from the standardized dataset. Principal component 1 accounted for 19.98% of the variability in data. This PC was negatively loaded by NA, CAT, AChEB, PCO, GST, GPx and positively loaded by AChEM, GSH, HSI, and K. PC2 accounted for less variability in data (19.68%) being negatively loaded by COMET, PCE, CAT, AChEM, LPO, PCO and GSH and positively loaded by GST, GPx, HSI, and K. PC3 accounted for 12.51% of the variability in data, being negatively loaded by COMET, NA, GST, and GSH and positively loaded for CAT, LPO, PCO and K.

For *C. iguassuensis*, data was explained until the third component based on the standardized data. PC1 accounted for 22.94% of the variability. This PC was negatively loaded by NA, PCE, AChEM, AChEB, and LPO and positively loaded by COMET, GST, HIS, and K. PC2 accounted for 15.39% of the variability in data being most negatively loaded by AChEM, AChEB, GST, HSI, and K and positively loaded by COMET, NA, and LPO. PC3 accounted for 13.06% of the variability in data being most negatively loaded by PCE, AChEB, LPO, and K and positively loaded by COMET, NA, CAT, AChEM, and GST.

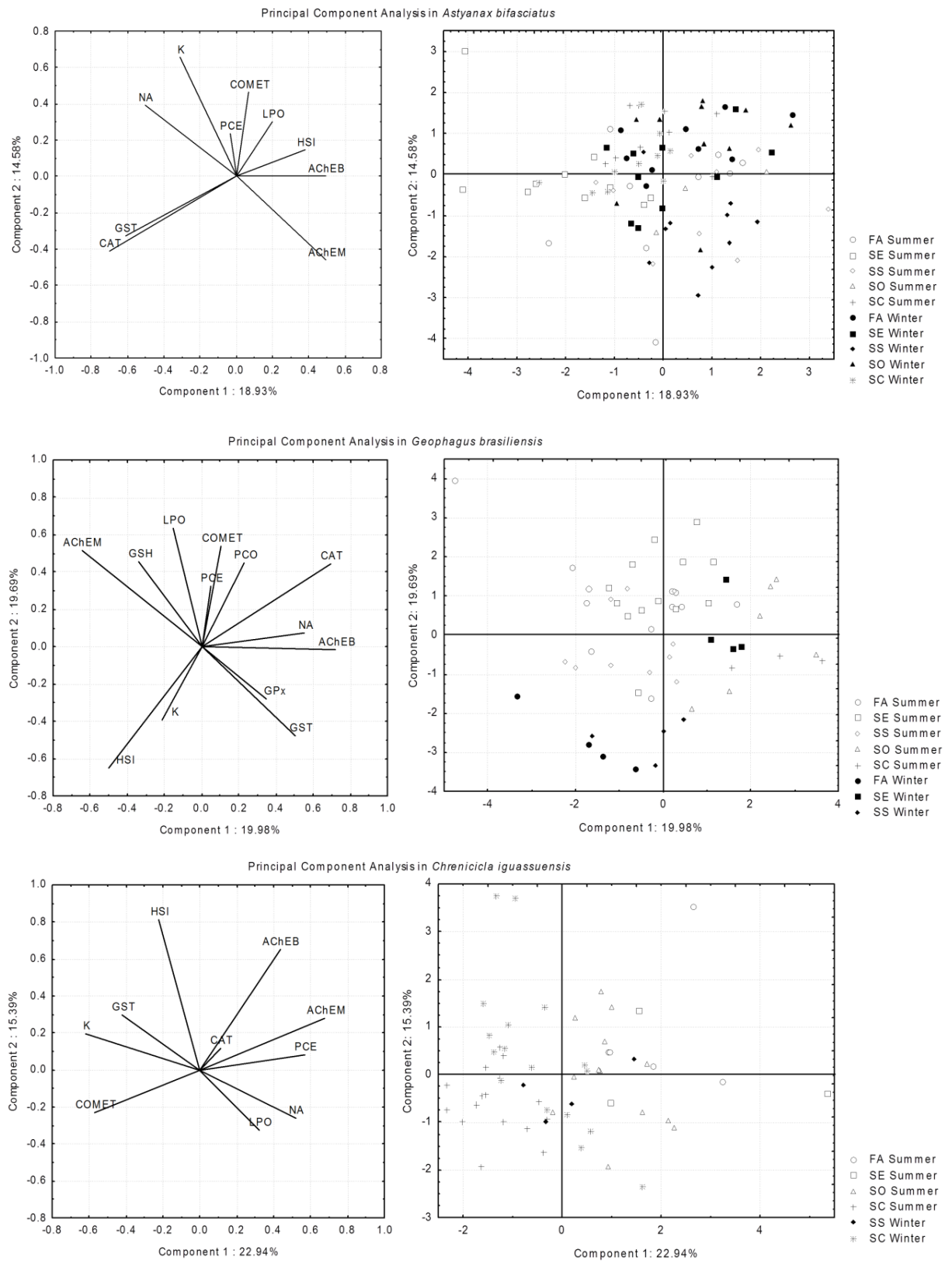


FIGURE 1. The principal component analysis involving biomarkers in *Astyanax bifasciatus*, *Geophagus brasiliensis* and *Chrenicicla iguassuensis* from the five reservoirs of Iguaçu River.

TABLE 1. Factor scores estimated for each of the sampling stations for *A. bifasciatus*, *G. Brasiliensis* and *C. iguassuensis* (A) accounted for all cases; (B) accounted for all variables.

		(A)								(B)							
		F1		F2		F3		F4		F1		F2		F3		F4	
		Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win	Sum	Win
FA	<i>A. bifasciatus</i>	-0.487	-0.709	-1.310	-0.038	0.169	-1.692	0.126	0.244	-1.373	-	0.534	-	0.281	-		
	<i>G. Brasiliensis</i>	-0.200	-0.944	1.638	-0.951	-0.179	-0.424	-1.417	-0.739	-0.161	-0.931	-0.468	-0.602	1.637	-1.323	1.102	0.691
	<i>C. iguassuensis</i>	1.294		-1.045		-0.760		0.307		0.899		0.319		-1.158			
SE	<i>A. bifasciatus</i>	-0.538	-0.319	1.029	0.357	1.595	-0.030	1.623	-1.936	0.088	-	-1.423	-	0.466	-		
	<i>G. Brasiliensis</i>	-0.241	0.656	1.011	0.212	-0.558	-0.808	2.052	-0.356	-0.234		-0.429		0.486		-1.796	
	<i>C. iguassuensis</i>	0.630		1.597		-0.610		-0.900		0.608		0.529		1.265			
SS	<i>A. bifasciatus</i>	-0.524	-0.445	-1.225	-0.068	-0.269	-0.836	0.535	0.683	0.268	-	0.060	-	-1.475	-		
	<i>G. Brasiliensis</i>	-0.733	-0.935	0.068	-1.279	0.008	0.052	0.031	0.306	-0.737	-	0.094	-	-0.211	-	-0.232	-
	<i>C. iguassuensis</i>		0.076		-0.589		0.016		0.342		-		-		-		
SO	<i>A. bifasciatus</i>	2.463	1.086	-0.972	1.764	0.317	-1.032	0.269	0.057	-		-		-			
	<i>G. Brasiliensis</i>	0.394		0.131		2.369		0.117		0.199		1.975		-0.054		0.124	
	<i>C. iguassuensis</i>	0.354		0.181		1.956		0.078		-0.167		-1.487		0.108			
SC	<i>A. bifasciatus</i>	-0.186	-0.340	-0.230	0.693	1.045	0.734	-1.264	-0.337	1.017		0.828		0.727			
	<i>G. Brasiliensis</i>	2.003		-0.830		-0.459		0.005		1.863		-0.569		-0.535		0.110	
	<i>C. iguassuensis</i>	-1.291	-1.063	-0.768	0.624	-0.151	-0.451	-1.320	1.494	-1.340		0.639		-0.216			

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir

TABLE 2. Table of decision with score calculated for relevant effects of variables (reservoir vs species vs season) according to integrated analysis.

Reserv / sp.	Summer 2013					Winter 2013				
	FA	SE	SS	SO	SC	FA	SE	SS	SO	SC
<i>A. bifasciatus</i>	8	9	6	2	9	5	2	3	8	5
<i>G. brasiliensis</i>	9	9	4	3	14	7	1	6		
<i>C. iguassuensis</i>	10	11		2	7			4		11

REFERENCES

- Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, 105, 121–6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/6727660>
- Alberto A, Camargo AFM, Verani JR, Costa OFT, Fernandes MN (2005). Health variables and gill morphology in the tropical fish *Astyanax fasciatus* from a sewage-contaminated river. *Ecotoxicol Environ Saf* 61 (2):247–55, doi:10.1016/j.ecoenv.2004.08.009
- Araldi RP, de Melo TC, Mendes TB, de Sá Júnior PL, Nozima BHN, Ito ET, de Cassia Stocco R (2015) Using the comet and micronucleus assays for genotoxicity studies: a review. *Biomed Pharmacother* 72:74–82, doi:10.1016/j.biopha.2015.04.004
- ATSDR - Public Health Statement: Cadmium. (n.d.). Retrieved from <http://www.atsdr.cdc.gov/phs/phs.asp?id=46&tid=15>. Accessed Sept 2012
- Authman MMN, Abbas WT, Gaafar AY (2012) Metals concentrations in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) from illegal fish farm in Al-Minufiya Province, Egypt, and their effects on some tissues structures. *Ecotoxicol Environ Saf* 84:163–172, doi:10.1016/j.ecoenv.2012.07.005
- Baumgartner, G., Pavanelli, S. C., Baumgartner, D., Bifi, A. G., Debona, T., & Frana, V. A. (2012). *Peixes do baixo rio Iguaçu*, 1st edn. Eduem, Maringá (City), p 203
- Bernet D, Schmidt H, Meier W, Burkhardt-Holm P, Wahli T (1999) Histopathology in fish: proposal for a protocol to assess aquatic pollution. *J Fish Dis* 22(1):25–34, doi:10.1046/j.1365-2761.1999.00134.x
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1-2):248–54. doi:10.1016/0003-2697(76)90527-3
- Brito I, Arruda Freire C, Yamamoto FY, Silva de Assis HC, Rodrigues Souza-Bastos L, Cestari MM, de Oliveira Ribeiro CA (2012) Monitoring water quality in reservoirs for human supply through multi-biomarker evaluation in tropical fish. *J Environ Monit* 14(2):615, doi:10.1039/c2em10461j
- Carneiro, C., Andreoli, C. V., Cunha, C. de L. da N., & Gobbi, E. F. (2014). *Reservoir Eutrophication: preventive management: an applied example of Integrated Basin Management Interdisciplinary Research*. IWA Publishing. Retrieved from <https://www.books.google.com/books?id=aflrBQAAQBAJ&pgis=1>
- Carvalho CDS, Bernusso VA, De Araújo HSS, Espíndola ELG, Fernandes MN (2012) Biomarker responses as indication of contaminant effects in *Oreochromis niloticus*. *Chemosphere* 89(1):60–69, doi:10.1016/j.chemosphere.2012.04.013
- Cestari, M. M., Lemos, P. M. M., Ribeiro, C. A. de O., Costa, J. R. M. A., Pelletier, E., Ferraro, M. V. M., ... Fenocchio, A. S. (2004). Genetic damage induced by trophic doses of lead in the neotropical fish *Hoplias malabaricus* (Characiformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations. *Genetics and Molecular Biology*, 27(2), 270–274. doi:10.1590/S1415-47572004000200023
- Cobbina SJ, Chen Y, Zhou Z, Wu X, Feng W, Wang W, Yang L (2015) Low concentration toxic metal mixture interactions: effects on essential and non-

essential metals in brain, liver, and kidneys of mice on sub-chronic exposure. *Chemosphere* 132:79–86, doi:10.1016/j.chemosphere.2015.03.013

Collins A, Koppen G, Valdiglesias V, Dusinska M, Kruszewski M, Møller P, Bonassi S (2014) The comet assay as a tool for human biomonitoring studies: the ComNet project. *Mutat Res Rev Mutat Res* 759:27–39, doi:10.1016/j.mrrev.2013.10.001

Conselho Nacional do Meio Ambiente (CONAMA) (2012) Resoluções do Conama: Resoluções vigentes publicadas entre setembro de 1984 e janeiro de 2012. / Ministério do Meio Ambiente. Brasília:MMA, 2012. p 1126

Contreras López MC (2003) Determination of potentially bioaccumulating complex mixtures of organochlorine compounds in wastewater: a review. *Environ Int* 28(8):751–9, doi:10.1016/S0160-4120(02)00120-4

Costa DDM, Neto FF, Costa MDM, Morais RN, Garcia JRE, Esquivel BM, Ribeiro C a O (2010a) Vitellogenesis and other physiological responses induced by 17- β -estradiol in males of freshwater fish *Rhamdia quelen*. *Comp Biochem Physiol Part C: Toxicol Pharmacol* 151(2):248–257, doi:10.1016/j.cbpc.2009.11.002

Costa PM, Chicano-Gálvez E, López Barea J, DelValls TA, Costa MH (2010b) Alterations to proteome and tissue recovery responses in fish liver caused by a short-term combination treatment with cadmi- um and benzo[a]pyrene. *Environ Pollut* (Barking, Essex : 1987) 158(10):3338–46, doi:10.1016/j.envpol.2010.07.030

Cotta JAO, Rezende MOO, Piovani MR (2006) Evaluation of metal content in sediments of the Betari River in the Parque Estadual Turístico do Alto Ribeira - PETAR-, São Paulo, Brazil. *Quimica Nova*, doi:10.1590/S0100-40422006000100009

Daga VS, Gubiani É a (2012) Variations in the endemic fish assemblage of a global freshwater ecoregion: associations with introduced spe- cies in cascading reservoirs. *Acta Oecologica* 41:95–105, doi:10. 1016/j.actao.2012.04.005

de la Haye KL, Spicer JI, Widdicombe S, Briffa M (2012) Reduced pH sea water disrupts chemo-responsive behaviour in an intertidal crus- tacean. *J Exp Mar Biol Ecol* 412:134–140, doi:10.1016/j.jembe. 2011.11.013

de Lemos CT, Iranço FDA, de Oliveira NCDÁ, de Souza GD, Fachel JMG (2008) Biomonitoring of genotoxicity using micronuclei assay in native population of *Astyanax jacuhiensis* (Characiformes: Characidae) at sites under petrochemical influence. *Sci Total Environ* 406(1–2):337–343, doi:10.1016/j.scitotenv.2008.07.006

Elahee KB, Bhagwant S (2007) Hematological and gill histopathological parameters of three tropical fish species from a polluted lagoon on the west coast of Mauritius. *Ecotoxicol Environ Saf* 68(3):361–71, doi:10.1016/j.ecoenv.2006.06.003

ELLMAN, G. L., COURTNEY, K. D., ANDRES, V., & FEATHER-STONE, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7, 88– 95. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/ 13726518>

Ferraro, M. V. M., Fenocchio, A. S., Mantovani, M. S., Ribeiro, C. de O., & Cestari, M. M. (2004). Mutagenic effects of tributyltin and inor- ganic lead (Pb II) on the fish *H. malabaricus* as evaluated using the comet assay and the piscine micronucleus and chromosome aberration tests. *Genetics and Molecular Biology*, 27(1), 103–107. doi:10. 1590/S1415-47572004000100017

Freire CA, Souza-bastos LR, Chiesse J, Tincani FH, Piancini LDS, Randi MAF, De

- Oliveira-ribeiro CA (2015) RESEARCH ARTICLE A multibiomarker evaluation of urban, industrial, and agricultural exposure of small characins in a large freshwater basin in southern Brazil, 13263–13277., doi:10.1007/s11356-015-4585-5
- Fujita, T., Nishimura, K., Takayama, C., Yoshida, M., & Uchida, M. (2001). Handbook of Pesticide Toxicology. Handbook of Pesticide Toxicology. Elsevier. doi:10.1016/B978-012426260-7.50032-X
- Gallotta FDC, Christensen JH (2012) Source identification of petroleum hydrocarbons in soil and sediments from Iguaçu River Watershed, Paraná Brazil using the CHEMSIC method (CHEMometric analysis of Selected Ion Chromatograms). J Chromatogr A 1235:149–158, doi:10.1016/j.chroma.2012.02.041
- Gauthier PT, Norwood WP, Prepas EE, Pyle GG (2014) Metal-PAH mixtures in the aquatic environment: a review of co-toxic mechanisms leading to more-than-additive outcomes. Aquat Toxicol 154:253–269, doi:10.1016/j.aquatox.2014.05.026
- Gerbron M, Geraudie P, Xuereb B, Marie S, Minier C (2015) In vitro and in vivo studies of the endocrine disrupting potency of cadmium in roach (*Rutilus rutilus*) liver., Marine Pollution Bulletin, 10.1016/j.marpolbul.2015.03.043
- Giardina A, Larson SE, Wisner B, Wheeler J, Chao M (2009) Long-term and acute effects of zinc contamination of a stream on fish mortality and physiology. Environ Toxicol Chem 28(2):287–95, doi:10.1897/07-461.1
- Gimeno-García E, Andreu V, Boluda R (1996) Heavy metals incidence in the application of inorganic fertilizers and pesticides to rice farming soils. Environ Pollut 92(1):19–25, doi:10.1016/0269-7491(95)00090-9
- Gusso-Choueri, P. K., Choueri, R. B., de Araújo, G. S., Cruz, A. C. F., Stremel, T., Campos, S., ... Ribeiro, C. A. O. (2015). Assessing pollution in marine protected areas: the role of a multi-biomarker and multi-organ approach. Environmental Science and Pollution Research International. doi:10.1007/s11356-015-4911-y
- Hanson N, Persson S, Larsson A (2009) Analyses of perch (*Perca fluviatilis*) bile suggest increasing exposure to PAHs and other pollutants in a reference area on the Swedish Baltic coast. J Environ Monit : JEM 11(2):389–93, doi:10.1039/b817703a
- Heddle, J. A. (1973). A rapid in vivo test for chromosomal damage. Mutation Research, 18(2), 187–90. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/4351282>
- IAP, I. A. do P. (2008). Qualidade das águas dos Reservatórios do Estado do Paraná 2005 a 2008.
- IBGE, I. B. de G. e E. (2010). IBGE | Censo 2010. Retrieved September 29, 2015, from <http://www.censo2010.ibge.gov.br/>
- Ide, A., Osawa, R., Marcante, L., Pereira, J., & Azevedo, J. C. (2016). Occurrence of pharmaceutical products, female sex hormones and caffeine in a subtropical region in Brazil. Clean Soil Air Water, 1st edn. Eduem, Maringá (City), p 203
- Jiang, Z. Y., Hunt, J. V., & Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Analytical Biochemistry, 202(2), 384–9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1519766>

- Jiang, Z. Y., Woollard, A. C., & Wolff, S. P. (1991). Lipid hydroperoxide measurement by oxidation of Fe²⁺ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. *Lipids*, 26(10), 853–6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1795606>
- Jiménez-Ortega V, Cano Barquilla P, Fernández-Mateos P, Cardinali DP, Esquifino AI (2012) Cadmium as an endocrine disruptor: correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin. *Free Radic Biol Med* 53(12):2287–97, doi: 10.1016/j.freeradbiomed.2012.10.533
- Kalantzi I, Black KD, Pergantis SA, Shimmield TM, Papageorgiou N, Sevastou K, Karakassis I (2013) Metals and other elements in tissues of wild fish from fish farms and comparison with farmed species in sites with oxic and anoxic sediments. *Food Chem* 141(2):680–94, doi:10.1016/j.foodchem.2013.04.049
- Keen JH, Habig WH, Jakoby WB (1976) Mechanism for the several activities of the glutathione S transferases. *J Biol Chem* 251(20): 6183–6188
- Leite, N. F., Peralta-Zamora, P., & Grassi, M. T. (2011). Distribution and origin of polycyclic aromatic hydrocarbons in surface sediments from an urban river basin at the Metropolitan region of Curitiba, Brazil. *Journal of Environmental Sciences (China)*, 23(6), 904–11. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/22066212>
- Levine, R. L., Williams, J. A., Stadtman, E. R., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology*, 233, 346–57. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8015469>
- Levit, S. M., & Bozeman, M. (2010). A Literature Review of Effects of Cadmium on Fish, (November), 16. Retrieved from http://www.nature.org/wherewework/northamerica/states/alaska/files/cadmium_literature_review_dec_2010.pdf
- Linde-Arias AR, Inácio AF, Novo L a, de Alburquerque C, Moreira JC (2008) Multibiomarker approach in fish to assess the impact of pollution in a large Brazilian river, Paraíba do Sul. *Environ Pollut* 156(3):974–979, doi:10.1016/j.envpol.2008.05.006
- Liu XJ, Luo Z, Li CH, Xiong BX, Zhao YH, Li XD (2011) Antioxidant responses, hepatic intermediary metabolism, histology and ultra- structure in *Synechogobius hasta* exposed to waterborne cadmium. *Ecotoxicol Environ Saf* 74(5):1156–1163, doi:10.1016/j.ecoenv. 2011.02.015
- Liu XJ, Luo Z, Xiong BX, Liu X, Zhao YH, Hu GF, Lv GJ (2010) Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in *Synechogobius hasta*. *Ecotoxicol Environ Saf* 73(6):1286–1291, doi:10.1016/j.ecoenv.2010.06.019
- Luzio, A., Monteiro, S. M., Fontaínhas-Fernandes, A. A., Pinto-Carnide, O., Matos, M., & Coimbra, A. M. (2013). Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. *Aquatic Toxicology (Amsterdam, Netherlands)*, 128–129, 183–9. doi:10.1016/j.aquatox.2012.12.018
- Maceda-Veiga A, Monroy M, Navarro E, Viscor G, de Sostoa A (2013) Metal concentrations and pathological responses of wild native fish exposed to sewage discharge in a Mediterranean river. *Sci Total Environ* 449:9–19,

doi:10.1016/j.scitotenv.2013.01.012

Malczyk, E. A., & Branfireun, B. A. (2015). Mercury in sediment, water, and fish in a managed tropical wetland-lake ecosystem. *The Science of the Total Environment*, 524-525C, 260–268. doi:10.1016/j.scitotenv.2015.04.015

Mattagajasingh SN, Misra BR, Misra HP (2008) Carcinogenic chromium(VI)-induced protein oxidation and lipid peroxidation: Implications in DNA-protein crosslinking., *Journal of Applied Toxicology*, doi:10.1002/jat.1364

MATTHIENSEN A, YUNES JS, CODD GA (1999) Ocorrência, distribuição e toxicidade de cianobactérias no estuário da Lagoa dos Patos, RS. *Rev Bras Biol* 59(3):361–376, doi:10.1590/S0034-71081999000300002

Mela M, Randi MAF, Ventura DF, Carvalho CEV, Pelletier E, Oliveira Ribeiro CA (2007) Effects of dietary methylmercury on liver and kidney histology in the neotropical fish *Hoplias malabaricus*. *Ecotoxicol Environ Saf* 68(3):426–35, doi:10.1016/j.ecoenv.2006.11.013

Mills LJ, Chichester C (2005) Review of evidence: are endocrine- disrupting chemicals in the aquatic environment impacting fish populations? *Sci Total Environ* 343(1–3):1–34, doi:10.1016/j.scitotenv.2004.12.070

Monroy M, Maceda-Veiga A, de Sostoa A (2014) Metal concentration in water, sediment and four fish species from Lake Titicaca reveals a large-scale environmental concern. *Sci Total Environ* 487:233–44, doi:10.1016/j.scitotenv.2014.03.134

Monteiro SM, dos Santos NMS, Calejo M, Fontainhas-Fernandes A, Sousa M (2009) Copper toxicity in gills of the teleost fish, *Oreochromis niloticus*: effects in apoptosis induction and cell proliferation. *Aquat Toxicol (Amsterdam, Netherlands)* 94(3):219–28, doi:10.1016/j.aquatox.2009.07.008

Nickens KP, Patierno SR, Ceryak S (2010) Chromium genotoxicity: A double-edged sword. *Chem Biol Interact* 188(2):276–88, doi:10.1016/j.cbi.2010.04.018

Noël L, Chekri R, Millour S, Merlo M, Leblanc J-C, Guérin T (2013) Distribution and relationships of As, Cd, Pb and Hg in freshwater fish from five French fishing areas. *Chemosphere* 90(6):1900–10, doi:10.1016/j.chemosphere.2012.10.015

Norberg, G. F., Gerhadsson, L., Mumtaz, M., Ruiz, P., & Fowler, B. A. (2015). *Handbook on the toxicology of metals*. Chemosphere (Fourth Edi, Vol. 9). Elsevier. doi:10.1016/0045-6535(80)90137-X

Oliveira Ribeiro, C. A., Belger, L., Pelletier, E., & Rouleau, C. (2002). Histopathological evidence of inorganic mercury and methyl mercury toxicity in the arctic charr (*Salvelinus alpinus*). *Environmental Research*, 90(3), 217–25. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12477467>

Oliveira Ribeiro CA, Filipak Neto F, Mela M, Silva PH, Randi MAF, Rabitto IS, Pelletier E (2006) Hematological findings in neotropical fish *Hoplias malabaricus* exposed to subchronic and dietary doses of methylmercury, inorganic lead, and tributyltin chloride. *Environ Res* 101(1):74–80, doi:10.1016/j.envres.2005.11.005

Omar W a, Saleh YS, Marie M-AS (2014) Integrating multiple fish bio- markers and risk assessment as indicators of metal pollution along the Red Sea coast of Hodeida, Yemen Republic. *Ecotoxicol Environ Saf* 110:221–231, doi:10.1016/j.ecoenv.2014.09.004

- Osório FHT, Silva LFO, Piancini LDS, Azevedo ACB, Liebel S, Yamamoto FY, de Oliveira Ribeiro CA (2014) Water quality assessment of the Tubarão River through chemical analysis and bio-markers in the Neotropical fish *Geophagus brasiliensis*. *Environ Sci Pollut Res* 21(15):9145–9160, doi:10.1007/s11356-013-1512-5
- Paulino, M. G., Benze, T. P., Sadauskas-Henrique, H., Sakuragui, M. M., Fernandes, J. B., & Fernandes, M. N. (2014). The impact of organochlorines and metals on wild fish living in a tropical hydroelectric reservoir: bioaccumulation and histopathological biomarkers. *The Science of the Total Environment*, 497-498C, 293–306. doi:10.1016/j.scitotenv.2014.07.122
- Pie MR, Baggio RA, Boeger WA, Patella LA, Ostrensky A, Vitule JRS, Abilhoa V (2009) Molecular data reveal a diverse *Astyanax* species complex in the upper Iguaçu River. *J Fish Biol* 75(9):2357–62, doi: 10.1111/j.1095-8649.2009.02438.x
- Prado PS, Pinheiro APB, Bazzoli N, Rizzo E (2014) Reproductive bio-markers responses induced by xenoestrogens in the characid fish *Astyanax fasciatus* inhabiting a South American reservoir: an integrated field and laboratory approach. *Environ Res* 131:165–73, doi: 10.1016/j.envres.2014.03.002
- Prado PS, Souza CC, Bazzoli N, Rizzo E (2011) Reproductive disruption in lambari *Astyanax fasciatus* from a Southeastern Brazilian reservoir. *Ecotoxicol Environ Saf* 74(7):1879–1887, doi:10.1016/j.ecoenv.2011.07.017
- Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? *Exp Cell Res* 283(1):1–16, doi:10.1016/S0014-4827(02)00027-7
- Qu, R., Feng, M., Wang, X., Qin, L., Wang, C., Wang, Z., & Wang, L. (2014). Metal accumulation and oxidative stress biomarkers in liver of freshwater fish *Carassius auratus* following in vivo exposure to waterborne zinc under different pH values. *Aquatic Toxicology (Amsterdam, Netherlands)*, 150, 9–16. doi:10.1016/j.aquatox.2014. 02.008
- Rabitto IS, Alves Costa JRM, Silva de Assis HC, Pelletier EE, Akaishi FM, Anjos A, Oliveira Ribeiro CA (2005) Effects of dietary Pb(II) and tributyltin on neotropical fish, *Hoplias malabaricus*: histopathological and biochemical findings. *Ecotoxicol Environ Saf* 60(2): 147–56, doi:10.1016/j.ecoenv.2004.03.002
- Ramsdorf, W. A., Guimarães, F. de S. F., Ferraro, M. V. M., Gabardo, J., Trindade, E. da S., & Cestari, M. M. (2009). Establishment of experimental conditions for preserving samples of fish blood for analysis with both comet assay and flow cytometry. *Mutation Research*, 673(1), 78–81. doi:10.1016/j.mrgentox.2008.11.010
- Sakuragui MM, Paulino MG, Pereira CDS, Carvalho CS, Sadauskas-Henrique H, Fernandes MN (2013) Integrated use of antioxidant enzymes and oxidative damage in two fish species to assess pollution in man-made hydroelectric reservoirs. *Environ Pollut* 178:41–51, doi:10.1016/j.envpol.2013.02.032
- Sánchez-Chardi A, Ribeiro CAO, Nadal J (2009) Metals in liver and kidneys and the effects of chronic exposure to pyrite mine pollution in the shrew *Crocidura russula* inhabiting the protected wetland of Doñana. *Chemosphere* 76(3):387–94, doi:10.1016/j.chemosphere. 2009.03.036
- Santos DR, Yamamoto FY, Filipak Neto F, Randi MAF, Garcia JE, Costa DDM, de Oliveira Ribeiro CA (2016) The applied indicators of water quality may underestimate

the risk of chemical exposure to human population in reservoirs utilized for human supply-Southern Brazil., *Environmental Science and Pollution Research International*, doi:10.1007/s11356-015-5995-0

Santos D, Milatovic D, Andrade V, Batoreu MC, Aschner M, Marreilha dos Santos AP (2012) The inhibitory effect of manganese on acetylcholinesterase activity enhances oxidative stress and neuroinflammation in the rat brain. *Toxicology* 292(2–3):90–8, doi:10.1016/j.tox.2011.11.017

Satarug S, Garrett SH, Sens MA, Sens DA (2009) Cadmium, environmental exposure, and health outcomes. *Environ Health Perspect* 118(2):182–190, doi:10.1289/ehp.0901234

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25(1):192–205, doi:10.1016/0003-2697(68)90092-4

Speit, G., & Hartmann, A. (1999). The comet assay (single-cell gel test). A sensitive genotoxicity test for the detection of DNA damage and repair. *Methods in Molecular Biology* (Clifton, N.J.), 113, 203–12. doi:10.1385/1-59259-675-4:203

Srivastava R, Srivastava SM, Abidi R, Das MK (2009) Cellular level effects of metal toxicity on gills and liver of fishes. *Comp Biochem Physiol A Mol Integr Physiol* 154(1):S19–S20, doi:10.1016/j.cbpa.2009.05.071

Tabachnick BG, Fidell LS (1996) Using multivariate statistics. Harper Collins College Publishers, New York

Thevenon F, Graham ND, Herbez A, Wildi W, Poté J (2011) Spatio- temporal distribution of organic and inorganic pollutants from Lake Geneva (Switzerland) reveals strong interacting effects of sewage treatment plant and eutrophication on microbial abundance. *Chemosphere* 84(5):609–17, doi:10.1016/j.chemosphere.2011.03.051

Tian D, Zheng W, He G, Zheng Y, Andersen ME, Tan H, Qu W (2015) Predicting cytotoxicity of complex mixtures in high cancer incidence regions of the Huai River Basin based on GC-MS spectrum with partial least squares regression. *Environ Res* 137:391–7, doi:10.1016/j.envres.2014.12.027

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ Toxicol Pharmacol* 13(2):57–149, doi:10.1016/S1382-6689(02)00126-6

Voigt, C. L., da Silva, C. P., Doria, H. B., Randi, M. A. F., de Oliveira Ribeiro, C. A., & de Campos, S. X. (2014). Bioconcentration and bioaccumulation of metal in freshwater Neotropical fish *Geophagus brasiliensis*. *Environmental Science and Pollution Research International*. doi:10.1007/s11356-014-3967-4

Wäge, J., Hardege, J. D., Larsson, T. A., Simakov, O., Chapman, E. C., Arendt, D., & Rotchell, J. M. (2015). Effects of low seawater pH on the marine polychaete *Platynereis dumerilii*. *Marine Pollution Bulletin*. doi:10.1016/j.marpolbul.2015.04.027

Wang HJ, Liu ZP, Jia XD, Chen H, Tan YJ (2014) Endocrine disruption of cadmium in rats using the OECD enhanced TG 407 test system. *Biomed Environ Sci : BES* 27(12):950–9, doi:10.3967/bes2014.135

Wei K, Yang J (2015) Oxidative damage induced by copper and beta- cypermethrin in gill of the freshwater crayfish *Procambarus clarkii*. *Ecotoxicol Environ Saf*

113:446–53, doi:10.1016/j.ecoenv.2014.12. 032

White LD, Cory-Slechta DA, Gilbert ME, Tiffany-Castiglioni E, Zawia NH, Virgolini M, Basha MR (2007) New and evolving concepts in the neurotoxicology of lead. *Toxicol Appl Pharmacol* 225(1):1–27, doi:10.1016/j.taap.2007.08.001

WHO. (2011). Cadmium in Drinking-water, 16. Retrieved from http://www.who.int/water_sanitation_health/dwq/chemicals/cadmium.pdf Yan HY, Zhang XR, Dong JH, Shang MS, Shan K, Wu D, Wang GY (2016) Spatial and temporal relation rule acquisition of eutrophication in Da'ning River based on rough set theory. *Ecol Indic* 66:180–189, doi:10.1016/j.ecolind.2016.01.032

CAPÍTULO II

Vitellogenin expression and others biomarkers show evidences of endocrine disruption in fish species from Iguaçu River - Southern Brazil

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ABSTRACT

The adverse effects of endocrine disrupting chemicals (EDCs) on aquatic wildlife and human health represent a current issue of high public concern. The presence of some compounds able to interfere with endocrine and reproductive system is poorly studied in aquatic environments of South America. The aim of the present study was to investigate the occurrence of EDCs in five reservoirs from the Iguaçu River, the second most polluted river in Brazil, through evaluation of reproductive biomarkers in three native fish species (*Astyanax bifasciatus*, *Chrenicicla iguassuensis* and *Geophagus brasiliensis*). Additionally, a polyclonal antiserum anti-vitellogenin from *G. brasiliensis* and a capture ELISA assay were developed for detection of estrogenic or anti-estrogenic activities in male and female fish, respectively. The specificity and sensitivity of ELISA assay was determinant to observe the vitellogenin (vtg) expression in male fish and lower levels of vitellogenin and estradiol in the plasma of female fish. These findings were associated to immature gonads and lower gonadosomatic index in adult females, representing strong evidences of endocrine disruption in *G. brasiliensis*, mainly from the Foz do Areia reservoir. Additionally, histological analysis in gonads of both endemic species (*Astyanax bifasciatus* and *Chrenicicla iguassuensis*) confirmed the impact of chemicals in others studied reservoirs. The current results show that endocrine disruptors chemicals significantly affect reproductive aspects of fishes from Iguaçu River. This environmental condition is due to human activities, especially by sewage and industrial release at the source of the river (metropolitan region of Curitiba) and agriculture along the river. In this sense the FA reservoir was the most affected by chemicals with endocrine properties.

Keywords: Endocrine disrupting chemicals, Iguaçu River, reproductive biomarkers, native fish, vitellogenin

1 INTRODUCTION

An increasing list of substances known as endocrine disrupting chemicals (EDCs), which cause disturbs in endocrine system of vertebrates, represents a current issue of high public concern. In this sense, the environmental exposure may affect the reproductive system leading to adverse effects on aquatic wildlife or even human health (Schug et al., 2011; Weiss, 2011).

According to Bars et al. (2012) and Frische et al. (2013) several chemicals are grouped as endocrine disruptors: (i) natural compounds (steroid hormones, estrogenic isoflavones and fungal estrogens), (ii) chloride pesticides (iii) pharmaceuticals, (iv) phtalates, bisphenol A, (v) alkylphenols, (vi) trace metals, (vii) polycyclic aromatic hydrocarbons and (viii) polychlorinated biphenils (PCBs). The establishment of risk assessment of EDCs exposure is a great challenge for scientific community since these contaminants are present as complex mixtures triggering several mechanisms of estrogenic/anti-estrogenic activity (Kiyama and Wada-kiyama, 2015). In order to access the effects of EDCs in aquatic organisms, the biomarkers are very useful to the risk assessment of exposed wildlife populations or even human populations. Thus, multiple endpoints should be considered, including the altered steroidogenesis process as the functional and pathological parameters in target tissues (Barber et al 2011; Thorpe et al 2001).

Vitellogenin (vtg) is a lipophosphoglycoprotein expressed in oviparous females in response to estrogen signaling via binding to nuclear estrogen receptors (ER) present in hepatocytes (Nagler et al., 2010). This protein is expected in higher concentrations only in mature females during reproductive season, due to its role as an egg-yolk precursor protein (Hiramatsu et al., 2015). However the exposure of exogenous estrogens or estrogen-mimics compounds can also induce higher vtg synthesis in males or even in juveniles fishes (Nikoleris et al., 2016). According to Hiramatsu et al. (2006) and Matozzo et al. (2008) the evaluation of its expression in male fish represents a very well established molecular biomarker for estrogenic activity in aquatic environments, under relevant concentrations. Additionally, the absence or reduced vtg levels in females during reproductive season is an indicative of anti-estrogenicity, being a potential cause of great impacts over reproduction (Bugel et al., 2011).

Despite of different assays have been developed for vtg quantification in fish plasma (Luna and Coady, 2016), the Western Blot, Enzyme-linked and radio-immunosorbent assays are the most currently utilized (Hara et al., 2007). Additionally, others parameters as histopathological analysis of gonads consist in a complementary study of the adversity of effects due the exposure to endocrine disruptors (Leino et al 2005; Louiz et al 2009).

Considering the scarcity of biomonitoring programs detecting EDCs in freshwater ecosystems from South America, it is necessary to include more studies investigating the sensitivity of native fish species to the impacts of human activities. The aim of the current study was to investigate the adverse effects of endocrine disruptors chemicals in three species of native fish from Iguaçu River, the second most polluted river in Brazil (IBGE, 2008). The species considered in the current study were: *Astyanax bifasciatus* (Characidae), an herbivorous endemic species with reproductive activity during the whole year, *Geophagus brasiliensis* (Cichlidae) a native omnivorous species and *Crenicichla iguassuenssis* (Cichlidae) a piscivorous endemic species both with reproductive season during summer. In the present study were considered the analysis of vitellogenin and steroid hormone levels in plasma as molecular biomarkers, including gonad histology and gonadosomatic index in both male and female fish as complementary investigation.

2 MATERIAL AND METHODS

Chemicals

Paraplast®, Bradford reagent, bovine serum albumin (BSA), 17 α -Ethinyl estradiol, sodium dodecyl sulfate, glycine, tris-HCl, Tris, o-phenylenediamine dihydrochloride, phenyl methyl sulphonyl fluoride (PSMF), β -Mercaptoethanol, Freund adjuvants, Benzocaine were purchased from Sigma-Aldrich (MO,USA). Ethanol, formaldehyde, acetic acid and hematoxylin and eosin were purchased from VETEC (Duque Caxias, RJ, BR). Coomassie brilliant blue, nitrocellulose membrane and goat anti-rabbit IgG antibody conjugated with horseradish peroxidase were obtained from Bio-Rad Laboratories (CA, USA). Pierce chemiluminescent kit was purchased from Pierce (IL,USA). Sensitive x-ray film was purchased from Amersham Bioscience.

Studied Area

The bioavailability of chemicals with endocrine disruptor properties was investigated in five cascading reservoirs of Iguaçu River located at Paraná State - Southern Brazil (Figure 1). Iguaçu River is the longest river in Paraná state with the origin at the Metropolitan Region of Curitiba (MRC) (approximately 3.5 million of citizens) receiving a large amount of urban and industrial wastes without primary treatment. Due to the Iguaçu Falls, the basin of Iguaçu River presents a natural geographical barrier that contributes with the high rate of endemic fish species found along the river, including species described as in risk of extinction. The first studied site is Foz do Areia reservoir (FA) located at 200 km from the MRC (26°00'60.82"S 51°65'68.22"W), impacted mainly by urban and industrial wastes. The following studied sites are Segredo (SE, 25°79'13.36"S, 52°11'89.36"W), Salto Santiago (SS, 25°63'73.08"S, 52° 60'20.19"W), Salto Osório (SO, 25°53'71.06"S, 53°00'33.31"W) and Salto Caxias reservoirs (SC, 25°53'64.43"S, 53°49'90.70"W) which are more impacted by agricultural and livestock activities.

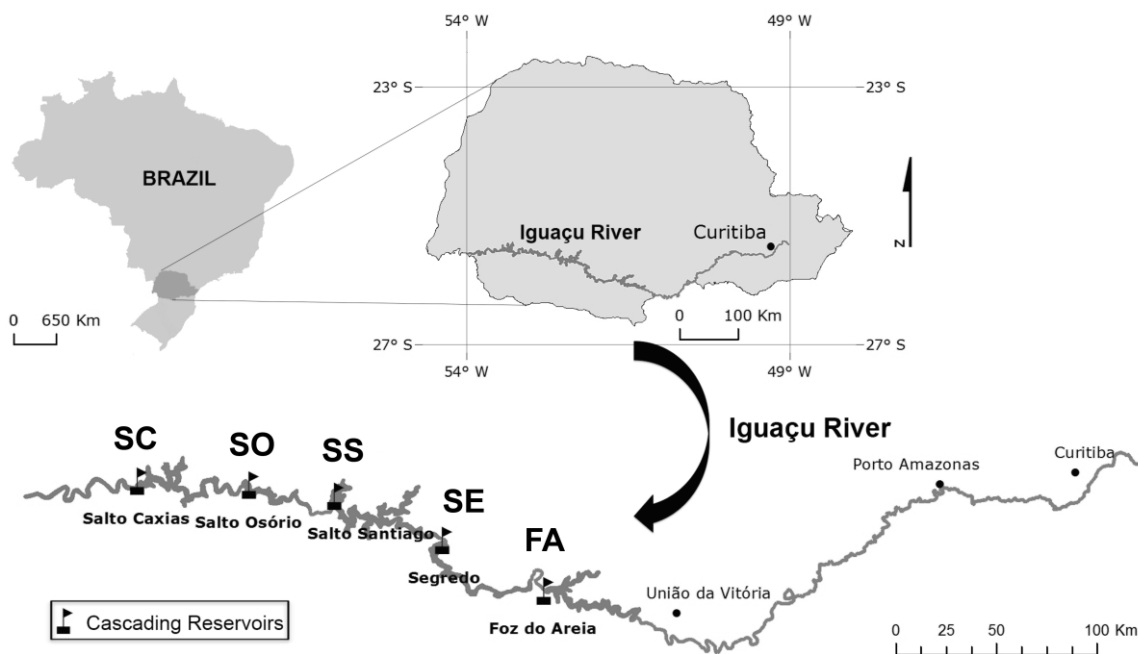


FIGURE 1. Map of Iguaçu River and the major cascading reservoirs.

Fish sampling

Two hundred and nine adult individuals of *Astyanax bifasciatus*, *Chrenicicla iguassuensis* and *Geophagus brasiliensis*, both male and female, were captured in the studied reservoirs during summer (January-February) of 2013 in order to evaluate endocrine disrupting responses (Table 1). The animals were captured through fishing nets (4-6 cm mesh). After anesthetic administration diluted in water (25-30 mg.l⁻¹ of benzocaine) the biometric parameters were measured and blood was sampled from individuals still alive (*G. brasiliensis* and *C. iguassuensis*) as described by Silversand et al. (1993). Blood samples were mixed with the anti-proteolytic PMSF (phenyl-methyl-sulfonylfluoride-10 mM) diluted in saline solution (NaCl at 0.9%); centrifuged at 4,000g for 2 min at room temperature to obtain plasma samples (stored at -75 °C). In the sequence the animals were killed by spinal cord section and the gonads were dissected and chemically preserved for histopathological analysis. Fish sampling was performed in the field to avoid stress during transportation and the same procedure and capture effort was exerted in all studied reservoirs. The weight of gonad was measured to determination of gonadosomatic index, (GSI) calculated by the formula $GSI = (\text{gonad mass} / \text{total fish mass}) \times 100$.

TABLE 1. Biometric parameters, vitellogenin occurrence by western blot and gonad histology of fish from the studied reservoirs of Iguaçu River

				WESTERN BLOT		GONAD HISTOLOGY						
Fish Species		N		Weight (g)	Vitellogenin occurence (%)		Imature		Degeneration		Parasites	
		Male	Female	Average	Male	Female	Male	Female	Male	Female	Male	Female
FA	<i>A. bifasciatus</i>	5	7	14,31 (± 1,85)			0	1 (14%)	3 (60%)	3 (42%)	4 (80%)	1 (14%)
	<i>G. brasiliensis</i>	11	12	13,7 (± 4,71)	2 (16%)	7 (87%)	1 (9%)	7 (58%)	2 (18%)	2 (16%)	5 (45%)	
	<i>C. iguassuensis</i>	2	2	15,3 (± 18,85)	0	0	00		0		0	
SE	<i>A. bifasciatus</i>	6	10	31,4 (± 7,07)			1 (16%)	4(40%)	4 (66%)	5 (50%)	4 (66%)	1 (10%)
	<i>G. brasiliensis</i>	15	7	29,82 (± 4,75)	0	6 (100%)	0	2 (28%)	8 (53%)		9 (60%)	
	<i>C. iguassuensis</i>	2	1	51,16 (±87,52)	0	1 (100%)	0	1	0		1 (33%)	1
SS	<i>A. bifasciatus</i>	5	14	14,45 (± 5,58)			0	0	2 (40%)	3 (21%)	2 (40%)	2 (14%)
	<i>G. brasiliensis</i>	6	10	12,39 (± 12,39)	0	2 (66%)	0	1 (10%)	4 (66%)	5 (50%)	1(16%)	
	<i>C. iguassuensis</i>	0	2	79,33 (± 37,14)	0	0		1 (50%)		0		0
SO	<i>A. bifasciatus</i>	3	2	5,32 (± 1,36)			0	0	2 (66%)	1 (50%)	1 (33%)	
	<i>G. brasiliensis</i>	3	4	17,98 ± 18,08	0	1 (100%)	0	0	2 (66%)	2 (50%)	1 (33%)	
	<i>C. iguassuensis</i>	2	7	17,77 (± 8,94)	2 (40%)	0	0	4 (57%)	1 (50%)	1(14%)	0	1(14%)
SC	<i>A. bifasciatus</i>	6	11	34,38 (± 5,73)			3 (50%)	3 (27%)	5 (83%)	4 (36%)	2 (33%)	
	<i>G. brasiliensis</i>	2	0	260 (± 32,4)		0	0		1 (50%)		2 (100%)	
	<i>C. iguassuensis</i>	8	5	96,68 (± 31,04)		5 (100%)	0	2 (40%)	3 (37%)	3 (60%)	2 (25%)	

FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir.

Production of polyclonal vitellogenin antiserum anti Geophagus brasiliensis

In order to verify the highest expression of vitellogenin induced in the plasma of male *Geophagus brasiliensis*, four different concentrations of 17 α -Ethinyl estradiol (EE2): 0,1; 1,0; 10 and 20 mg EE2/Kg, were selected (Costa et al., 2010). Adult fish from a fish farm were maintained in cages (Piscicultura Panama, Paulo Lopes, SC) and fed every two days with commercial food pellets (Nicoluzzi® ration) during the experimental period. For each dose, 15 male individuals received a single intraperitoneal injection containing the EE2 diluted in canola oil. A control group of 10 male individuals received an injection of canola oil (vehicle control) and another group of 10 male individuals did not receive the canola oil (negative control). A group of 10 female fish were used as a positive control without EE2 injection. After 15 days of exposure fish were anesthetized with benzocaine (2%) and blood was sampled as previously described. The induction of vtg was verified by SDS-PAGE gel stained with Coomassie blue and confirmed with Western Blott analysis.

Vitellogenin Expression Detection by Western Blot Assay

Plasma proteins concentration was determined through Bradford method (1976). About 50 μ g of protein was firstly reduced and denatured with sample buffer (1 M Tris-HCl pH 6.8, SDS, glycerol, bromophenol blue and β mercaptoethanol) and separated with 8% SDS-PAGE (Mini Protean II, BioRad®). Proteins in the gel were transferred to a nitrocellulose membrane (pore of 0.45 μ m) in buffer (5 mM Tris-base, 192 mM glycine, 0.037% SDS, 20% methanol) at 100 V, during 60 min and 4°C. The membrane was blocked in 5% non-fat-dry milk (Molico®) diluted in TBST buffer (1 M Tris-HCl pH 7.4, 4 M NaCl and 0.05% Tween-20) for 1h. The membranes were washed three times of 5 minutes between every step, and after incubated with anti-*R. quelen* vitellogenin polyclonal antiserum (produced in Cellular Toxicology Laboratory, Federal University of Parana), diluted 1:4000 in blocking solution for 16h at 4°C. After washing step with TBST buffer, the bound antiserum was detected in the membrane with goat anti-rabbit IgG conjugated with horseradish peroxidase (bio-rad®, 1:4000). The reaction was developed through a Pierce chemiluminescent kit (Thermo Scientific®, São Paulo) on the membrane exposed to a sensitive x-ray film (GE Life Science®, São Paulo) in a range of 1 to 30 min.

Purification of Vitellogenin and Rabbit Immunization

Samples that showed the highest vtg expression were selected for purification through Miniprep cell (BioRad®), separating the proteins according to the molecular weight. For each run, about 1 mg of proteins in SDS-reducing buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, bromophenol blue and 5% β mercaptoethanol) were added to a 5% resolving polyacrylamide gel (4% polyacrylamide stacking gel). The separation was performed using a running buffer (25mM TrisHCl, 192mM glycine, 0,1%SDS) and conditions of 1W constant power. The eluted proteins were collected through a fraction collector in a rate of 100 μ L/min. The purified vtg was observed in a SDS-PAGE gel stained with silver nitrate. Fractions containing the isolated protein were pooled and concentrated with desalinization column (PD-10, GE) for posterior lyophilization.

The rabbit immunization process was developed according to Harlow and Lane (1988). To produce the polyclonal antiserum a total of 3 immunizations were performed in a New Zealand Rabbit via intramuscular and subcutaneous: 200 μ g of vtg and Freund Adjuvant Complete (1:1) was used for the first immunization and for the second and third was administered 100 μ g of vtg and Freund Adjuvant Incomplete (1:1) in intervals of 21 days. The hyper immune serum of the rabbit was collected after 54 days of immunization by cardiac puncture under anesthesia (Ketamine-Xylazine 40:5mg/kg) and, posterior kill by anesthetic overdose. The blood was maintained at 37° C for 1 hour and overnight at 4° C and centrifuged at 3000xg for 7 minutes. The polyclonal antiserum anti vitellogenin from *G. brasiliensis* was maintained at -20° C.

The produced antiserum was tested by western blot assay using untreated male individuals of *G. brasiliensis* samples and individuals exposed to 20 mg EE2 to test the specificity and optimal assay conditions (antiserum dilution and protein amount). Samples from *G. brasiliensis* and *C. iguassuensis* from the five reservoirs of Iguaçu River were also tested for detection of estrogenicity after sensibility tests for *C. iguassuensis*.

Capture ELISA assay

The method of capture by ELISA was developed according to Lomax et al., (1998), with modifications. The vtg in the plasma samples was determined by competition between purified antigen coated in the microtiter plates and the free antigen in the sample for the binding of anti-vtg antiserum.

The 96-well microtiter plates (NUNC[®], MaxiSorb) were coated with 100 µl per well of 20 ng of purified vtg diluted in sodium carbonate buffer (0,05M, pH 9,6) incubated in a shaker overnight at 4° C. The non-specific binding of the antiserum was determined with a blank containing only the sodium carbonate buffer. Before each step the plates were inverted and washed three times of 5 minutes with PBST (0,02 M sodium phosphate buffer, 0,15 M sodium chloride, 0,05% Tween-20, pH 7,5) to remove all unbound contents. After coating, the free binding sites were blocked with 200 µl of 1% BSA (Sigma[®]) in PBST for 2 hours at room temperature (RT). To optimize the protocol, previously, different dilutions of antiserum (1:1000; 1:5000; 1:10000; 1:50000) and samples (1:10; 1:50; 1:100) were tested. Diluted samples and standards (15 - 1000 ng of vtg per well) were preincubated overnight (1:1) at 4° C with the vtg antiserum diluted 1:5000, in a 1,5 ml microtube. Then, 100 µl of the preincubated mix was pipetted in duplicate into the coated wells and was incubated for 2h at RT. Following the washing step, 100 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase (bio-rad[®], diluted 1:4000 in TBST) was added and incubated for 2h at RT. At last, 100 µl of o-phenylenediamine dihydrochloride (Sigma[®]) substrate solution (0,5 mg.ml⁻¹, 0,05M citrate buffer pH 5.0, 0,015% H₂O₂) was incubated in the dark for 30 min. The enzyme reaction was stopped with 30 µl of 2 M H₂SO₄ and optical density was measured at 490 nm with a TECAN microplate reader. The concentration of vtg in the sample was determined according to the percentage of antiserum binding calculation, where absorbance values were inversely proportional to the amount of vtg in the sample.

The reliability and precision of the assay was assessed through intra-assay variation, to determine variation within assays, and inter-assay variation to verify variation between assays. The coefficient of variation was calculated (%CV=standard deviation/mean x 100) considering the values of standard curve in triplicates, from six different assays.

Steroid Hormones dosage

The steroids hormones 17- β Estradiol and 11-keto Testosterone were quantified in the plasma of female and male *G. brasiliensis* fish with an Enzyme Immunoassay kit (Cayman[®], US). Previously the steroids were extracted from the samples with ethyl ether according to the methodology proposed by Brown et al., (2003) and procedures were performed according to the manufacture instructions.

Histological procedures

Gonads of male and female fish were fixed in ALFAC fixative solution (ethanol 70%, formaldehyde 4% and glacial acetic acid 5%) for 16 h, dehydrated in graded series of ethanol and xylene and embedded in Paraplast Plus resin (Sigma[®]). Sections of 5 μ m thick were obtained through a semi-automatic microtome (Leica[®]) and stained with hematoxylin and eosin. The slides were observed under light photomicroscope Leica[®] and analyzed according to Leino et al. (2005) and Blazer (2002). Male germ cells were classified in 4 different stages of maturation: I – Primary Spermatogonias; II - Secondary Spermatogonias; III – Spermatocytes; IV – Spermatids and Spermatozoa. For females 5 different stages were considered: I – Primary growth; II – Cortical alveolus; III – Early vitellogenic; IV – Late vitellogenic; V – Mature/spawning oocyte. Sexually immature gonads were considered in males with predominance of Spermatogonias (>80% stage I) and only containing pre-vitellogenic follicles (Stage I, II and III) for females. Additionally, alterations such as degeneration of germ cells, atresia (degeneration of follicles), and presence of parasites or centers of melanomacrophages (CMMs) were also considered.

Statistical Analysis

All data were checked in GraphPad Prism [®] software for outliers (Grubb's test), normality (Shapiro's test) and homoscedasticity (Bartlett's test) before performing the analysis. Either *one-way* ANOVA test followed by the *Tukey-Kramer* post-test (parametric data) or *Kruskal-Wallis* test followed by the *Dunn's* test (nonparametric data) were performed, with $p < 0.05$. Correlations test between variables were verified with Pearson's test.

3 RESULTS

Gonadosomatic index

Higher gonadosomatic index was found for male *A. bifasciatus* from Salto Santiago reservoir (SS) than Foz do Areia (FA), Segredo (SE) and Salto Caxias (SC) reservoirs, whereas for *C. iguassuensis* male individuals from Salto Osorio reservoir (SO) presented a higher index than SC. In female statistical differences were observed only for *G. brasiliensis* from SE reservoir, showing higher index than FA reservoir (Figure 2).

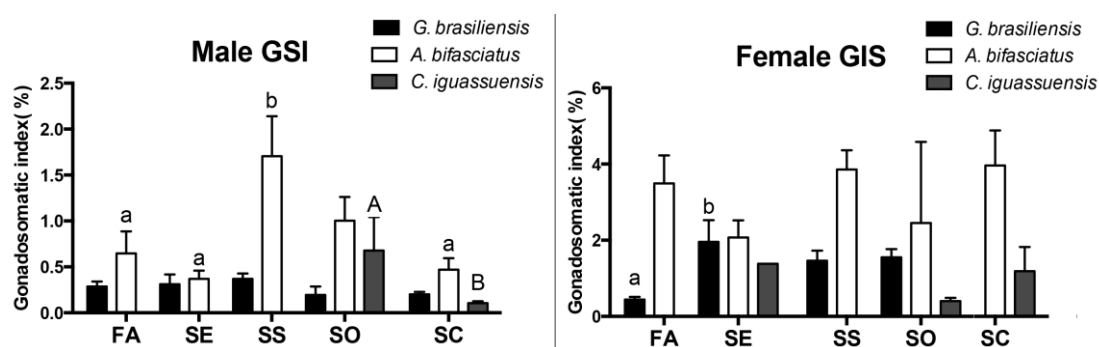


FIGURE 2. Gonadosomatic index from male and female fishes from the five reservoirs of Iguaçu River. Letters indicate significant differences between sites in the same season (a, b).

Vitellogenin Expression

The SDS Polyacrilamide gel stained with Coomassie Blue showed that vtg consisted of a single band of approximately 150 kDa (Figure 3A). Western blott analysis confirmed the presence of the protein in the plasma of females and males exposed to EE2 in a dose-dependent manner, whereas in males from control group was not detected (Figure 3A). The individuals exposed to 20mg EE2/kg of EE2, showed the highest expression of vitellogenin present in all exposed individuals. Protein separation according to the molecular weight with the Miniprep Cell equipment was demonstrated through SDS PAGE gel, stained with silver nitrate (Figure 3B).

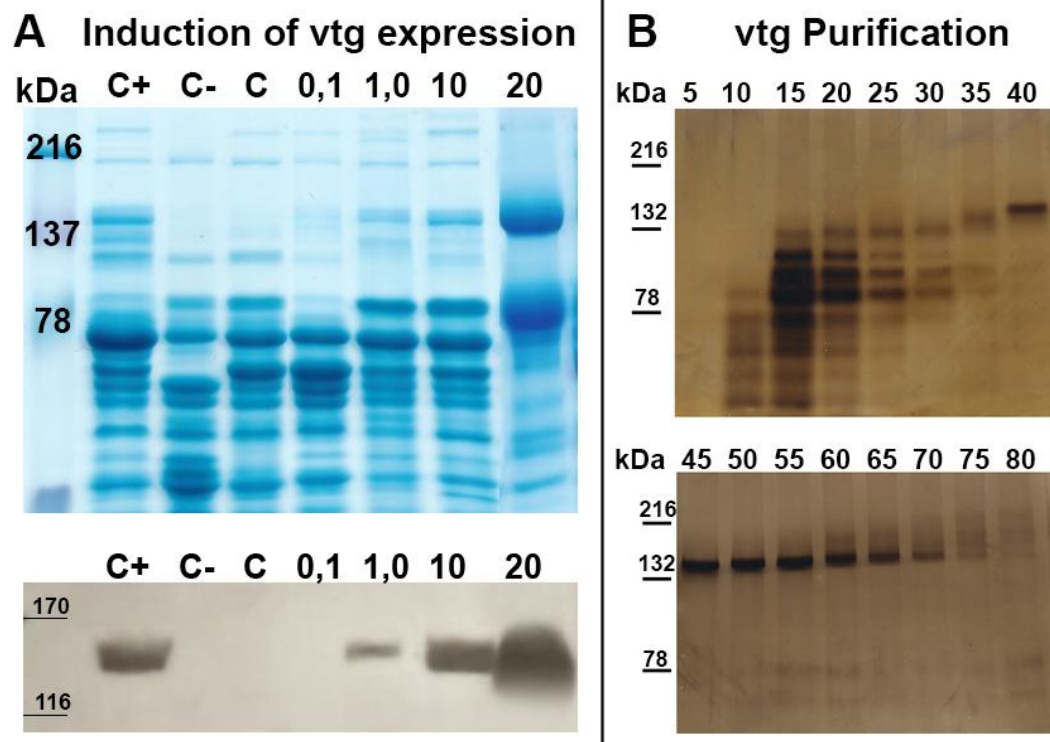


FIGURE 3. SDS Polyacrilamide gel and Western blot showing vitellogenin expression. A- Presence of vitellogenin in *G. brasiliensis* blood samples from female (C+) and male exposed to EE2 (1,0; 10; 20), being absent in male exposed to 0,1 mg of EE2, untreated males (C-) and control (C, injected with canola oil), verified by SDS PAGE gel stained with Coomassie blue and confirmed by Western blot. B – Vitellogenin purification, showing the profile of proteins collected in different fractions (5-80) and the isolation of vitellogenin from the 40th fraction (gel stained with Silver nitrate).

The produced antiserum was specific to the vtg present in plasma of female and exposed male individuals of *G. brasiliensis*, while for unexposed male no cross-reaction was observed. In this case, the optimal conditions for the western blot assay was a dilution of 1:200.000, using 20 µg of protein and a detection limit of 1 µg of total proteins (Figure 4).

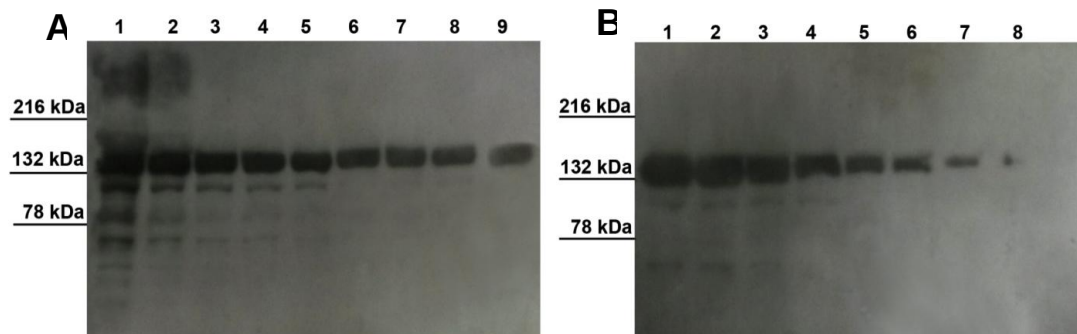


FIGURE 4. Titration of antibody for optimal conditions of western blot assay, with different dilutions of the antibody (A) and different amounts of protein (B) from sample of male exposed to 20 mg of EE2/kg.

The western blotting analyses showed the expression of vtg in two male individuals (16%) of *G. brasiliensis* from the FA reservoir. In females the protein was detected in the majority of individuals from the five studied reservoirs (Table 1, Figure 5). In general, the expression was higher and lower in female individuals from SE and FA reservoirs, respectively (Figure 5).

For *C. iguassuensis* the produced antiserum against *G. brasiliensis* vtg showed cross-reaction to the protein in plasma. Apparently there are two isoforms of approximately 150 kDa and 60 kDa in females, but two male individuals from SO reservoir present reaction only for the 150 kDa isoform.

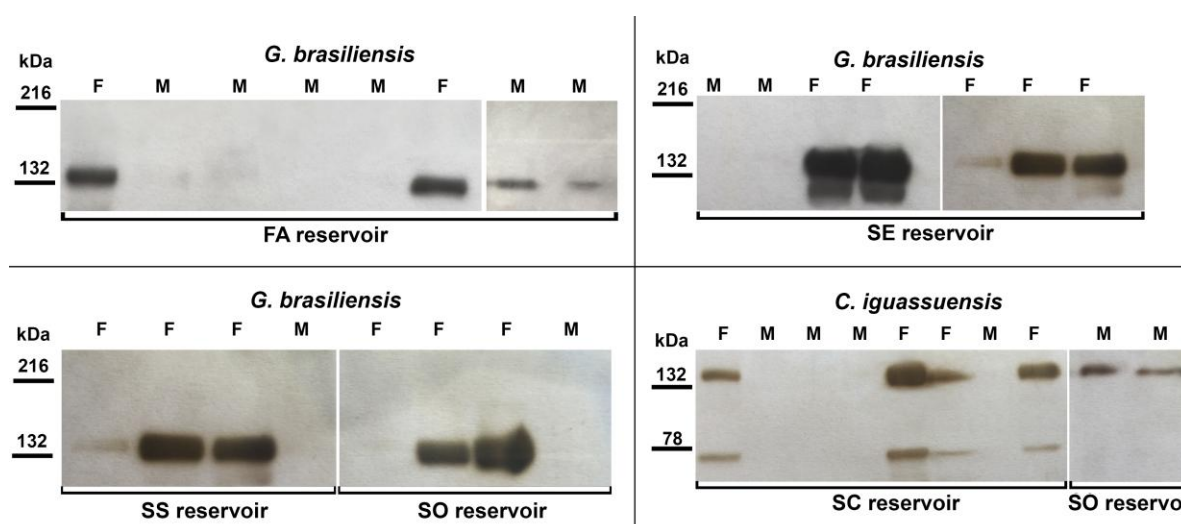


FIGURE 5. Detection of vitellogenin expression in *G. brasiliensis* and *C. iguassuensis* from the five reservoirs of Iguaçu River, by western blot assay. F = Female samples; M = Male samples.

The ELISA assay was more sensitive than western blot for detection of vtg in plasma. The expression of vtg in male samples of *G. brasiliensis* from the studied sites ranged from 95 ng/ml to 38 µg/ml (Figure 6) and only one individual presented values not detected. Untreated males showed lower levels of vtg (approximately 40 ng.ml⁻¹) with a half of individuals presenting values under the limit of detection. Fish exposed to 20 mg of EE2.kg⁻¹ showed an average of 1.48 mg of vtg.ml⁻¹ of plasma.

The values described for females of *G. brasiliensis* from Iguaçu River revealed lower concentration of vtg than EE2-treated males (1.3 µg.ml⁻¹ to 161.6 µg.ml⁻¹ respectively). In females from SE reservoir the levels of vtg were significantly

higher than individuals from FA reservoir, however in males *G. brasiliensis* no differences were observed. The calculation of Intra-assay and inter-assay coefficients were respectively 5.45% and 9.07%, considered as an acceptable range for the monitoring quality control.

Steroid hormones

In females, the concentrations of Estradiol and 11-keto testosterone were higher in *G. brasiliensis* from SE reservoir than other individuals from the studied sites (Figure 6). The results showed that the estradiol levels were positively correlated with vitellogenin concentrations. Despite the concentrations of 11-keto testosterone and estradiol in the plasma of male *G. brasiliensis* were detectable, no different profiles were found among individuals from the studied reservoirs (Figure 6).

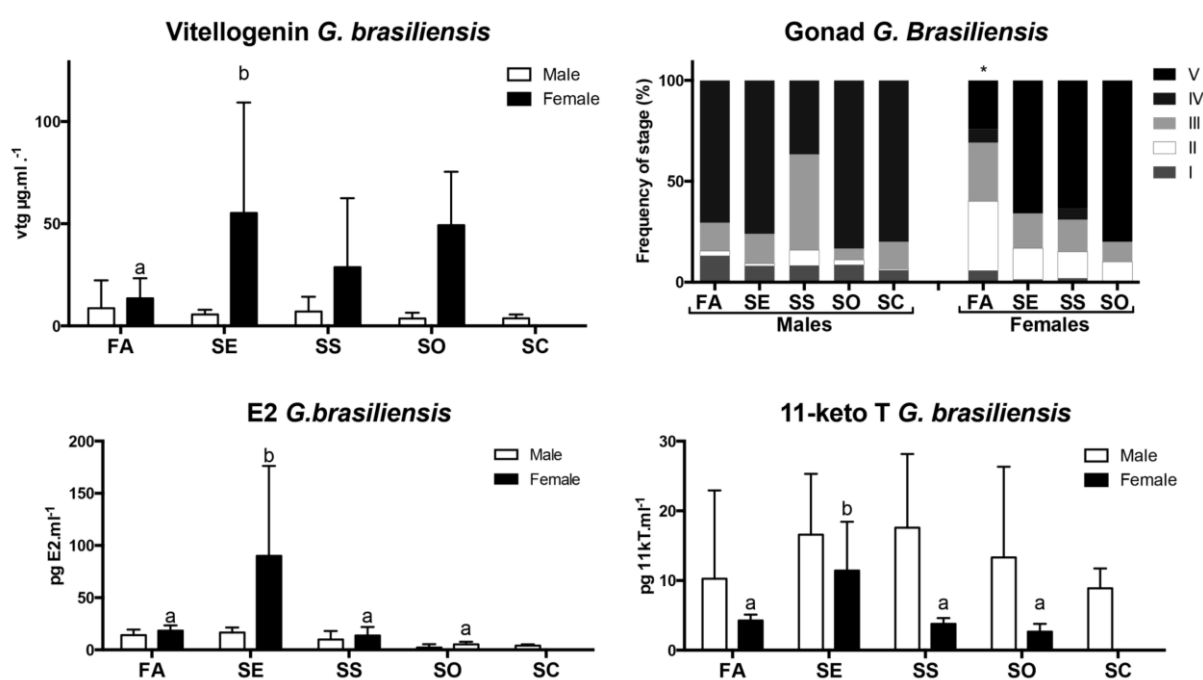


FIGURE 6. Different biomarkers in *G. brasiliensis* from the five reservoirs of Iguaçu River. Detection of vitellogenin expression by ELISA assay in both Male and Female blood samples. Gonad histology with the different stages of spermatogenesis (Males) and oogenesis (Females) observed. Estradiol (E2) levels and 11-keto Testosterone levels. FA = Foz do Areia Reservoir; SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir. Letters indicate significant differences between sites in the same season (a, b); * indicate seasonal differences for the same site.

Gonad histology

In general, the incidence of immature gonads, especially for female gonad, was the main change observed (Table 1). In male *A. bifasciatus* was observed the occurrence of testes-ova only in two individuals from FA reservoir (Figure 7). Higher frequency of immature gonads and degeneration of germ cells was verified in male of *A. bifasciatus* from SC reservoir (Figure 7). The degeneration of germ cells, including syncytic cells, and necrosis, was found in male individuals from all studied sites presenting different levels of alteration. Parasites were more frequent in males than females, mainly from FA and SE for both *A. bifasciatus* and *G. brasiliensis* species.

The pattern of immature ovaries showed differences according to the studied species. For *A. bifasciatus* more than 40% of gonads in female was immature in SE reservoir, while for *G. brasiliensis* more than 50% were sexually immature in FA reservoir (Table 1). The occurrence of immature gonad in *G. brasiliensis* from this reservoir was associated with lower levels of vitellogenin and lower GSI in females. Finally *C. iguassuensis* from SO reservoir presented more than 50% of the individuals with immature gonads. Atresic follicles or degeneration of follicles and eosinophils were observed in all studied groups (Figure 8). The occurrence of CMMs was more frequent in *G. brasiliensis* than other studied species, with the highest incidence of 75% in females from FA reservoir.

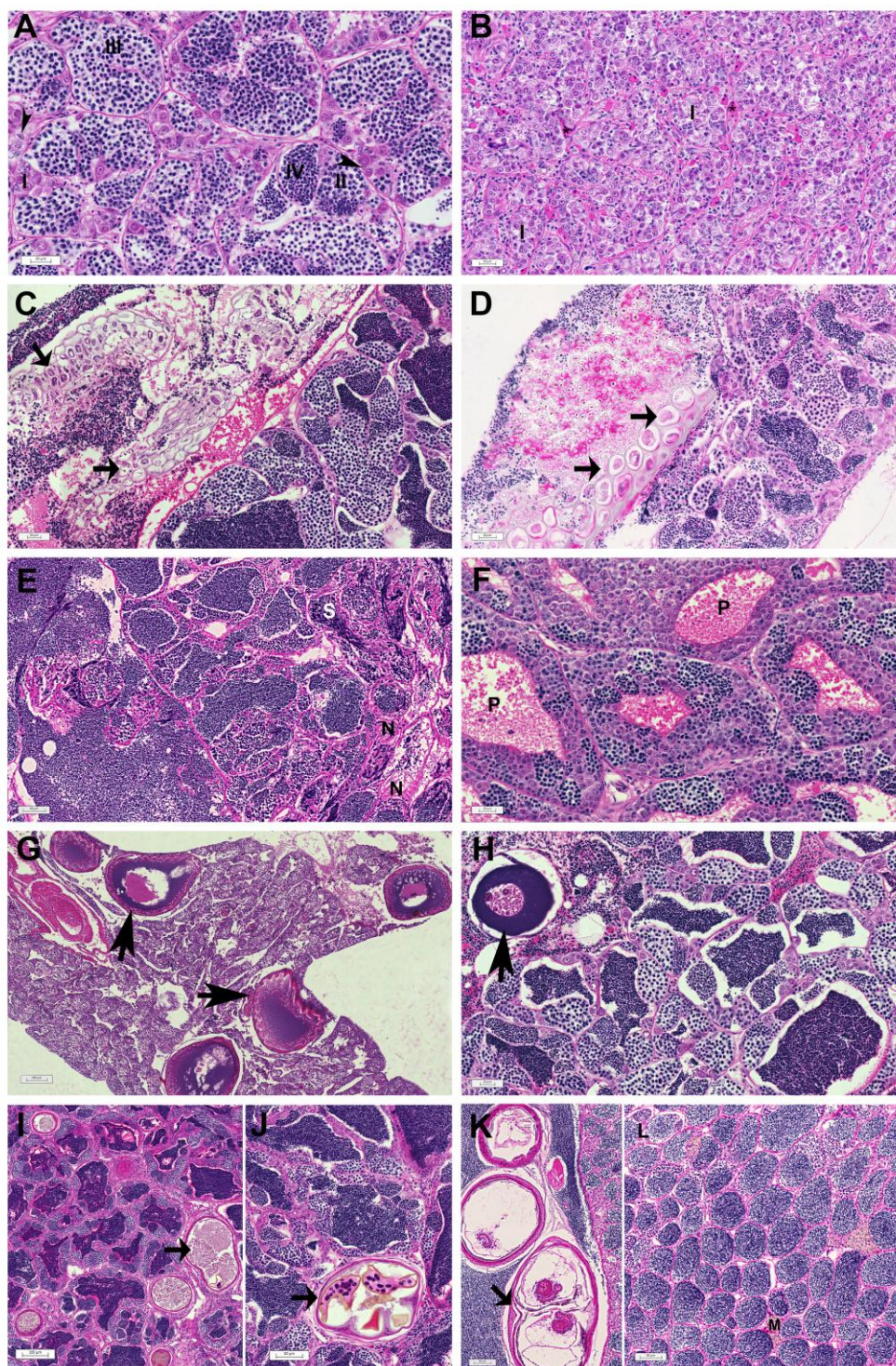


FIGURE 7. Histological section of testes from male fish sampled in the five reservoirs of Iguaçu River. A – Normal structure of testes of *A. bifasciatus* (SS) with germ cells in all stages (I, II, III, IV) of development and Sertoli cells (arrow point) within the cysts of seminiferous tubules. B – Immature gonads of *A. bifasciatus* (SC) with primary spermatogonias (I) and absent spermatocytes, high concentration of unicellular eosinophilic parasites. C, D- High proliferation of parasites occupying a large area. E - Degenerative findings of germ cells, including necrosis (N) and syncyatic cells (S). F – Elevated deposition of eosinophilic proteins (P) within the lumen of seminiferous tubules. G, H – Occurrence of testes-ova (arrow) in *A. bifasciatus* from FA reservoir, with oocytes in stage III of development and spermatogonias, and a cortical alveolus oocyte within a testes presenting all stages of development. Different species of pluricellular parasites within the testes. Melanomacrophage center (M) in testes of *G. brasiliensis* from FA reservoir.

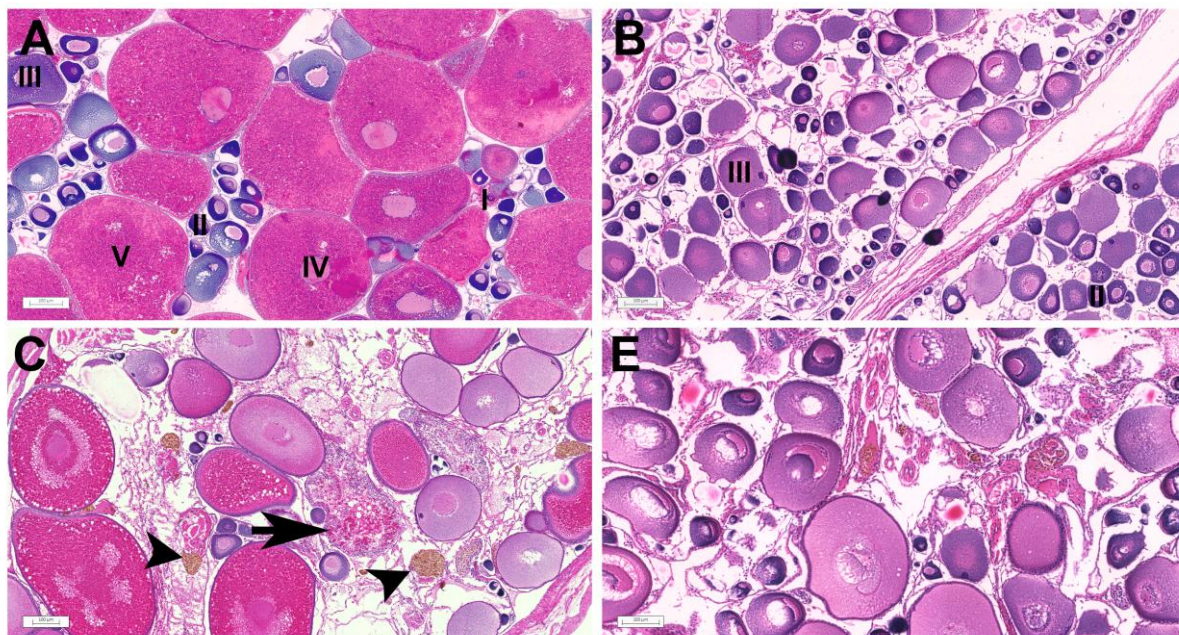


FIGURE 8. Histological section of ovaries from female fishes sampled in the five reservoirs of Iguaçu River. A – Mature *A. bifasciatus* ovary showing high frequency of late vitellogenic (IV) and mature oocyte (V), as others stages of development (I, II, III). B – Sexually immature female with oocytes from I – III stages of development. C – Ovary from *C. iguassuensis* (SC reservoir) with atresia follicle (arrow) and high incidence of melanomacrophage centers (point of arrow). D Female without late vitellogenic follicles.

DISCUSSION

The evidences of endocrine disruption in native and endemics fish species of Iguaçu River, especially in individuals from the FA reservoir, represent an important highlight of chemical impact to human and wildlife populations. As this studied site is the closer to the Metropolitan Region of Curitiba (MRC), it is acceptable that these findings are related to the urban and industrial activities found in the origin of the river. Additionally, both the higher expression of vtg in males and lower levels of vitellogenin, including E2, associated to low GSI and immature gonads of females, are strong indicative of endocrine disrupting effects in *G. brasiliensis* from FA reservoir. Furthermore the production of the anti-vtg antiserum and the development of an ELISA assay was an important strategy for the detection of endocrine disruptive effects, as described in the present study.

Vitellogenin induction occurred in a dose-dependent manner by EE2 exposure in male of *G. brasiliensis*. This data is corroborating with other studies where a monotonic response was also described (Madsen et al., 2013; Zhong et al., 2014). The same was found in males exposed to other kinds of EDCs such as nonylphenol and bisphenol-A, as reported by Pait and Nelson (2003). A single injection of 1 mg.kg^{-1} of EE2 was enough to induce vtg expression in males of *G. brasiliensis* after a period of 15 days of exposure, whereas the same response was not observed after exposure to 0.1 mg.kg^{-1} of EE2. This finding shows that male individuals of this species need higher doses of EE2 to activate the vtg expression, since in other fish species this same dose or lower is enough to induce vtg synthesis in males (Flick et al., 2014; Park et al., 2010; Park and Gye, 2014). This is an interesting point as the presence of vtg in plasma of male *G. brasiliensis* from Iguaçu River, as found in the present study, may suggest relevant concentrations of EDCs specially in FA reservoir. Additionally, the data support the evidence of a more resistance attributed to *G. brasiliensis* species as high concentrations of estrogenic chemicals are necessary to induce some response in this fish species.

For the last two decades the production of a polyclonal antibody anti-vitellogenin and the development of ELISA assays, represent together an efficient strategy for detection of estrogenic activity in different fish species worldwide (Selcer and Verbanic, 2014; Wang et al., 2015a). However only recently this tool was available to native Brazilian fish species (Costa et al., 2016). The main advantages

for the production of an anti-vtg antiserum and application in aquatic environment studies are: a) vitellogenin structure allows an easier purification process and higher immunogenicity in rabbits due to its high molecular weight and chemical complexity; b) as vtg is a very conserved protein, the produced antiserum may possess cross-reactivity between different species; c) good cost benefit, once produced, the polyclonal antiserum can be used for many years.

Distinct protocols have been developed for each step of vitellogenin antiserum production process, especially for the purification methods. Despite of two-step chromatography is the most common strategy applied for vitellogenin purification (Shao et al., 2004), the present study showed that the electrophoresis is an alternative approach with an easy protocol ensuring efficient results.

The method of ELISA assay applied in the present study, demonstrated specificity and sensitivity that allows to detect the presence of estrogenic activity even at low concentrations. Therefore represents an adequate tool for both biomonitoring and fish screening assays, as recommended by OECD framework (OECD, 2012). On the other hand, Western blot assay may be more suitable for biomonitoring studies, where the presence of environmental estrogens is detected only in relevant concentrations. Both immunoassays validated the applicability of the antibody produced to *G. brasiliensis* and were efficient to detect vitellogenin levels in fish from Iguaçu River, indicating bioavailability of EDCs for two species.

In male fish from the five studied reservoirs of Iguaçu River, the vtg levels were higher than untreated male *G. brasiliensis*. Determination of basal levels of vitellogenin is required for an appropriate use of this biomarker (Jubeaux et al., 2012). This aspect is very important since regular low levels of endogenous estrogen is synthesized in males, necessary for testicular development and sperm formation (Almeida et al., 2016). The levels found in the current study were also similar to other fish species from impacted environments as described by Madsen et al. (2013). According to the authors concentrations above $0.1 \mu\text{g} \cdot \text{ml}^{-1}$ in male flounders indicate relevant estrogenic inputs. Other studies from different regions of the globe reported the average concentrations of vtg in 13 different fish species from 43 rivers impacted by wastewater effluent, ranging values from $0.1 \mu\text{g} \cdot \text{ml}^{-1}$ to $52 \text{ mg} \cdot \text{ml}^{-1}$ (Desforges et al. 2010). In Brazil the studies reporting the vtg induction in male fish are very recent and scarce (Osorio et al., 2014, Santos et al., 2016 in press), highlighting the importance of the current study.

The lower levels of vitellogenin in female of *G. brasiliensis* from FA reservoir suggest the effects of antiestrogenic compounds. The majority of the studies investigating EDCs exposure in fish through vtg measurements have focused on male estrogenic effects. However vitellogenin production may also be impaired in females exposed to EDCs, interfering directly in the quality and quantity of eggs, and ultimately the viability of embryos produced (Yilmaz et al., 2015). Therefore, the lower levels of plasma vtg detected in females from FA reservoir represented a relevant reproductive endpoint, indicating the influence of EDCs originated from the MRC.

One of the tested hypotheses of the current study was the relevancy of human activities at the MRC, which are introducing many potential EDCs in Iguaçu River, despite of the distance longer than 200 km upstream. Our previous study (manuscript in revision) showed through multi-biomarkers approach that all reservoirs from Iguaçu River are jeopardized by human activities, especially the FA, SE and SC reservoirs. The environmental impact found in FA and SE reservoirs added to the current results corroborates the hypothesis that MRC represent the most important source of pollutants to Iguaçu River. According to Ide et al. (2016) emerging contaminants (caffeine, pharmaceutical products, female sex hormones, UV filters) were detected in Iguaçu River closer to MRC, demonstrating the role of domestic and industrial sewage in the pollution of the river. The same study reported that only caffeine was found in the reservoir 200 km downstream.

Wastewater treatment plants are considered as one of the main sources of EDCs in freshwater ecosystems (Huerta et al., 2015). Many compounds originated from domestic wastewater effluents, such as EE2, nonylphenol, octylphenol are known to have affinity for estrogen receptors and induce vtg expression in oviparous male or sexually immature fish (Andersen et al 2003; Shappell et al 2010; Vázquez et al 2009; Genovese et al., 2014). These findings showed that even in non-detectable levels of known EDCs chemicals, the fish from the studied reservoirs are affected by endocrine disruptors, despite of distance and high dilution or even biological events along the river. The explanation can be the presence of other estrogenic and antiestrogenic chemicals not chemically investigated.

In our previous study, high concentrations of toxic metals and PAHs were detected, respectively, in muscle and bile of individuals from the studied reservoirs. These findings showed association with different responses in target organs, such as induced oxidative stress and liver damage in the same fish species. Thus, these

endpoints are in accordance that hazard chemicals such as toxic metals and PAHs are also related to adverse effects, including endocrine disturbances, as described by Huang et al. (2012). Different metals like Zn, Cd and Pb detected in the five studied reservoirs can also increase or decrease vitellogenin expression in female fish in a dose/time-dependent manner (Huang et al., 2014). Additionally, the presence of an oil refinery company (REPAR) in the MRC might be the most important source of PAHs detected in the bile of the three fish species as previously reported (manuscript under revision). According to Kennedy and Smyth, (2015) and Fernandes and Porte (2013), PAHs, such as Benzo[a]pyrene and hydroxylated PAHs exposure are also related to induce endocrine disturbances, interfering in steroids levels.

Despite of the impact in Iguazu River due to the human activities in MRC, other sources of pollutants are present along the river as intense agricultural (e.g. soybeans, corn and wheat farms) and livestock activities affecting mainly SS, SO and SC reservoirs. The constant use of pesticides intensifies the release of toxic metals and organic chemicals in the reservoirs enhancing the bioavailability of these pollutants. Mixture of endocrine disrupting pesticides is reported to induce adverse effects in rat offspring (Jacobsen et al., 2012; Hass et al., 2012) affecting steroidogenesis in H295R cells (Taxvig et al., 2013), but not observed in a single chemical exposure.

The diversity of environmental chemicals in the studied reservoirs makes it difficult to identify what kind of pollutants is related with vtg expression and other reproductive endpoints. Furthermore, exposure of biota to EDCs is likely not constant, ranging temporally and spatially, including the influence of abiotic factors such as temperature that may influence biological responses (Madeira et al., 2013; Vinagre et al., 2012). There are few available studies showing the mechanisms and effects of chemical mixtures inducing or inhibiting vtg expression (Manikkam et al 2013; Li et al., 2012). Vitellogenin levels may not only depend on EDCs that bind directly to estrogen receptors, but also on chemicals able to interfere with hormone levels, or even induce liver injury. Additionally, the Estradiol and 11-keto testosterone concentrations in female *G. brasiliensis* from SE reservoir were higher than other groups, suggesting that EDCs could either increase the synthesis of these steroid hormones or even inhibit in individuals from the other reservoirs.

The morphological changes in gonads as described in the present study are also consequence of adverse effects of endocrine disruption agents in fish and

represent an additional biomarker to investigate the presence of EDCs in Iguaçu River. Abnormal vtg and steroid hormones levels have been associated with delayed gonadal maturation development, (Duffy et al., 2014), a reduced number of ovulated eggs and gonadosomatic index (Park et al., 2010), affecting the reproductive success of fish populations. Several biomonitoring studies reported the use of gonads histology to explain effects of EDCs on both ovaries and testes (Carballo et al., 2005; Hinck et al., 2007; Solé et al., 2003). More recently both vtg and histopathological studies were used together to evaluate the presence of EDCs in reservoirs used to human supply at RMC (Santos et al., 2016 in press), using encaged *Oreochromis niloticus* as bioindicator. The main changes associated to estrogen agonists' chemical exposure include fewer mature and more atretic follicles in females, as well as testes-ova, degeneration of germ and Sertoli cells in males (Leino et al., 2005). Other studies reported testicular changes such as germ cell syncytium, Sertoli and Leydig cell hyperplasia or hypertrophy, changes in testicular interstitial volume, decreased sperm production and consequent reduction of seminiferous tubules diameter (Miles-Richardson et al., 1999). Although all these findings were not noticed in the present study, the degeneration of germ cells and atresia found in fish from all reservoirs could suggest the adverse effects of EDCs present in the river. Additionally, the occurrence of parasites and center of melanomacrophages in the gonads may reinforce the conditions of low health as briefly reported above.

The incidence of females sexually immature during reproductive season was higher in fish from FA, SE and SC reservoirs, which are the same most impacted sites according to our previous study. These findings in female support again the hypothesis that MRC is the most important source of EDCs to Iguaçu River, and also that agricultural activity represent another important source of EDCs mainly to SC reservoir. The evaluation of gonadal staging is an expressive biomarker since can reveal the presence of EDCs' exposure, leading to disturbs in testicular and ovary maturation (Leino et al., 2005).

The three studied fish species in the present study showed different responses for the same environmental exposure condition. The histopathology of gonads indicated different patterns of alterations, especially the frequency of immature gonads, when comparing the three species of fish from the same reservoir. These findings could be explained by the difference in food habit, reproductive strategy, behavior and metabolism of the three fish species. *A. bifasciatus* was the

most abundant of the three studied species in the five reservoirs, probably due to the success of reproductive strategy that is active during all period of the year. Despite of that, this species present some limitations due to the small size that difficult the collect of enough biological material to analysis. In this sense, the use of vtg expression is difficult to access in the mentioned species. In contrast, the two other species were a quite well bioindicators for molecular biomarkers in the present investigation, since different reproductive endpoints obtained were enough to elucidate the present study including the gonad histopathology.

Final considerations

Overall the present results permitted to conclude that Iguaçu River, classified as the second most polluted river of Brazil, have the MRC as an important source of EDCs followed by agricultural activities. These sources are relevant factors that affect reproductive health of fishes from the five reservoirs of Iguaçu River. Considering the diversity of chemicals present along the Iguaçu River it is difficult to access the mechanisms of estrogenicity/anti-estrogenicity triggered in the exposed fish. Due to the occurrence of many endemic fish species and the use of Iguaçu water for human supply the present study is a top of concern. Additionally, the data analysis suggests strongly that further studies are necessary to investigate other endocrine endpoints to better understand the risk of exposure to wildlife and human population

REFERENCES

- Almeida, F.L., Lopes, J.S., Crescencio, R., Izel, A.C.U., Chagas, E.C., Boijink, C., 2016. Early puberty of farmed tambaqui (*Colossoma macropomum*): Possible influence of male sexual maturation on harvest weight. *Aquaculture* 452, 224–232. doi:10.1016/j.aquaculture.2015.10.031
- Bars, R., Fegert, I., Gross, M., Lewis, D., Weltje, L., Weyers, A., Wheeler, J.R., Galay-Burgos, M., 2012. Risk assessment of endocrine active chemicals: Identifying chemicals of regulatory concern. *Regul. Toxicol. Pharmacol.* 64, 143–154. doi:10.1016/j.yrtph.2012.06.013
- Blazer, V.S., 2002. Histopathological assessment of gonadal tissue in wild fishes. *Fish Physiol. Biochem.* 26, 85–101. doi:10.1023/A:1023332216713
- Borgert, C.J., Mihaich, E.M., Quill, T.F., Marty, M.S., Levine, S.L., Becker, R. a., 2011. Evaluation of EPA's Tier 1 Endocrine Screening Battery and recommendations for improving the interpretation of screening results. *Regul. Toxicol. Pharmacol.* 59, 397–411. doi:10.1016/j.yrtph.2011.01.003
- Brown, J., Ph, D., Walker, S., Steinman, K., Royal, F., 2003. ENDOCRINE MANUAL FOR REPRODUCTIVE NON-DOMESTIC SPECIES. *Smithson. Natl. Zool. Park. Conserv. Res. Cent.* (internal Publ.
- Bugel, S.M., White, L. a., Cooper, K.R., 2011. Decreased vitellogenin inducibility and 17 β - estradiol levels correlated with reduced egg production in killifish (*Fundulus heteroclitus*) from Newark Bay, NJ. *Aquat. Toxicol.* 105, 1–12. doi:10.1016/j.aquatox.2011.03.013
- Carballo, M., Aguayo, S., De La Torre, A., Muñoz, M.J., 2005. Plasma vitellogenin levels and gonadal morphology of wild carp (*Cyprinus carpio*) in a receiving rivers downstream of Sewage Treatment Plants. *Sci. Total Environ.* 341, 71–79. doi:10.1016/j.scitotenv.2004.08.021
- Costa, D.D.M., Neto, F.F., Costa, M.D.M., Morais, R.N., Garcia, J.R.E., Esquivel, B.M., Ribeiro, C. a O., 2010. Vitellogenesis and other physiological responses induced by 17- β -estradiol in males of freshwater fish *Rhamdia quelen*. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 151, 248–257. doi:10.1016/j.cbpc.2009.11.002
- Duffy, T.A., Iwanowicz, L.R., McCormick, S.D., 2014. Comparative responses to endocrine disrupting compounds in early life stages of Atlantic salmon , *Salmo salar*. *Aquat. Toxicol.* 152, 1– 10. doi:10.1016/j.aquatox.2014.03.015
- Fernandes, D., Porte, C., 2013. Hydroxylated PAHs alter the synthesis of androgens and estrogens in subcellular fractions of carp gonads. *Sci. Total Environ.* 447, 152–159. doi:10.1016/j.scitotenv.2012.12.068
- Flick, R.W., Bencic, D.C., See, M.J., Jensen, K.M., Biales, A.D., 2014. Sensitivity of the vitellogenin assay to diagnose exposure of fathead minnows to 17 α -ethynylestradiol. *Aquat. Toxicol.* 152, 353–360. doi:10.1016/j.aquatox.2014.04.026

- Frische, T., Bachmann, J., Frein, D., Juffernholz, T., Kehrer, A., Klein, A., Maack, G., Stock, F., Stolzenberg, H.C., Thierbach, C., Walter-Rohde, S., 2013. Identification, assessment and management of “endocrine disruptors” in wildlife in the EU substance legislation-Discussion paper from the German Federal Environment Agency (UBA). *Toxicol. Lett.* 223, 306–309. doi:10.1016/j.toxlet.2013.03.004
- Genovese, G., Regueira, M., Da Cunha, R.H., Ferreira, M.F., Varela, M.L., Lo Nostro, F.L., 2014. Nonmonotonic response of vitellogenin and estrogen receptor gene expression after octylphenol exposure of *Cichlasoma dimerus* (Perciformes, Cichlidae). *Aquat. Toxicol.* 156, 30–40. doi:10.1016/j.aquatox.2014.07.019
- Hara, A., Hirano, K., Shimizu, M., Fukada, H., 2007. Characterization of yolk proteins, development of immunoassays and use as biomarker of exposure to environmental estrogens. *Environ. Sci.* 14, 95–108.
- Hinck, J.E., Blazer, V.S., Denslow, N.D., Echols, K.R., Gross, T.S., May, T.W., Anderson, P.J., Coyle, J.J., Tillitt, D.E., 2007. Chemical contaminants, health indicators, and reproductive biomarker responses in fish from the Colorado River and its tributaries. *Sci. Total Environ.* 378, 376–402. doi:10.1016/j.scitotenv.2007.02.032
- Hiramatsu, N., Cheek, A.O., Sullivan, C., Matsubara, T., Hara, a, 2005. Vitellogenesis and endocrine disruption. *Biochem. Mol. Biol. Fishes* 6.
- Hiramatsu, N., Todo, T., Sullivan, C. V, Schilling, J., Reading, B.J., 2015. General and Comparative Endocrinology Ovarian yolk formation in fishes : Molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins. *Gen. Comp. Endocrinol.* 221, 9–15. doi:10.1016/j.ygcen.2015.01.025
- Huang, G.Y., Ying, G.G., Liang, Y.Q., Liu, S.S., Liu, Y.S., 2014. Expression patterns of metallothionein, cytochrome P450 1A and vitellogenin genes in western mosquitofish (*Gambusia affinis*) in response to heavy metals. *Ecotoxicol. Environ. Saf.* 105, 97–102. doi:10.1016/j.ecoenv.2014.04.012
- Huang, Y.Q., Wong, C.K.C., Zheng, J.S., Bouwman, H., Barra, R., Wahlström, B., Neretin, L., Wong, M.H., 2012. Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts. *Environ. Int.* 42, 91–99. doi:10.1016/j.envint.2011.04.010
- Huerta, B., Rodriguez-Mozaz, S., Nannou, C., Nakis, L., Ruha., Acua, V., Sabater, S., Barcelo, D., 2015. Determination of a broad spectrum of pharmaceuticals and endocrine disruptors in biofilm from a waste water treatment plant-impacted river. *Sci. Total Environ.* 540, 241–249. doi:10.1016/j.scitotenv.2015.05.049
- Jiménez-Ortega, V., Cano Barquilla, P., Fernández-Mateos, P., Cardinali, D.P., Esquifino, A.I., 2012. Cadmium as an endocrine disruptor: correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin. *Free Radic. Biol. Med.* 53, 2287–97. doi:10.1016/j.freeradbiomed.2012.10.533
- Jubeaux, G., Simon, R., Salvador, A., Lopes, C., Lacaze, E., Quéau, H., Chaumot, A., Geffard, O., 2012. Vitellogenin-like protein measurement in caged *Gammarus fossarum* males as a biomarker of endocrine disruptor exposure: Inconclusive experience. *Aquat. Toxicol.* 122-123, 9–18. doi:10.1016/j.aquatox.2012.05.007

- Kennedy, C.J., Smyth, K.R., 2015. Disruption of the rainbow trout reproductive endocrine axis by the polycyclic aromatic hydrocarbon benzo[a]pyrene. *Gen. Comp. Endocrinol.* 219, 102–111. doi:10.1016/j.ygcen.2015.03.013
- Kiyama, R., Wada-kiyama, Y., 2015. Estrogenic endocrine disruptors : Molecular mechanisms of action. *Environ. Int.* 83, 11–40. doi:10.1016/j.envint.2015.05.012
- Kolle, S.N., Ramirez, T., Kamp, H.G., Buesen, R., Flick, B., Strauss, V., van Ravenzwaay, B., 2012. A testing strategy for the identification of mammalian, systemic endocrine disruptors with particular focus on steroids. *Regul. Toxicol. Pharmacol.* 63, 259–278. doi:10.1016/j.yrtph.2012.04.004
- Leino, R.L., Jensen, K.M., Ankley, G.T., 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Pharmacol.* 19, 85–98. doi:10.1016/j.etap.2004.05.010
- Lomax, D.P., Roubal, W.T., Moore, J.D., Johnson, L.L., 1998. An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes etulus*): development , validation and cross-reactivity with other pleuronectids. *Comp. Biochem. Physiol. Part B* 121, 425–436. doi:10.1016/S0305-0491(98)10125-6
- Louiz, I., Ben-Attia, M., Ben-Hassine, O.K., 2009. Gonadosomatic index and gonad histopathology of *Gobius niger* (Gobiidea, Teleost) from Bizerta lagoon (Tunisia): Evidence of reproduction disturbance. *Fish. Res.* 100, 266–273. doi:10.1016/j.fishres.2009.08.009
- Luna, L.G., Coady, K., 2016. Ecotoxicology and environmental safety quantification of *X. laevis* vitellogenin by liquid chromatography tandem mass spectrometry. *Ecotoxicol. Environ. Saf.* 124, 296–302. doi:10.1016/j.ecoenv.2015.10.032
- Madeira, D., Narciso, L., Cabral, H.N., Vinagre, C., Diniz, M.S., 2013. Influence of temperature in thermal and oxidative stress responses in estuarine fish. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 166, 237–43. doi:10.1016/j.cbpa.2013.06.008
- Madsen, L.L., Korsgaard, B., Pedersen, K.L., Bjerregaard, L.B., Aagaard, T., Bjerregaard, P., 2013. Vitellogenin as biomarker for estrogenicity in flounder *Platichthys flesus* in the field and exposed to 17 α -ethinylestradiol via food and water in the laboratory. *Mar. Environ. Res.* 92, 79– 86. doi:10.1016/j.marenvres.2013.09.002
- Maffini, M. V., Rubin, B.S., Sonnenschein, C., Soto, A.M., 2006. Endocrine disruptors and reproductive health: The case of bisphenol-A. *Mol. Cell. Endocrinol.* 254-255, 179–186. doi:10.1016/j.mce.2006.04.033
- Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J. a., Yamini, B., Barbee, S.J., Giesy, J.P., 1999. Effects of waterborne exposure of 17 ??-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat. Toxicol.* 47, 129– 145. doi:10.1016/S0166-445X(99)00009-0

- Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O., Roig, B., 2011. Effect of endocrine disruptor pesticides: A review. *Int. J. Environ. Res. Public Health* 8, 2265–2303. doi:10.3390/ijerph8062265
- Nagler, J.J., Davis, T.L., Modi, N., Vijayan, M.M., Schultz, I., 2010. Intracellular, not membrane, estrogen receptors control vitellogenin synthesis in the rainbow trout. *Gen. Comp. Endocrinol.* 167, 326–330. doi:10.1016/j.ygcen.2010.03.022
- Nicolas, J.M., 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. *Aquat. Toxicol.* 45, 77–90. doi:10.1016/S0166-445X(98)00095-2
- OECD, O.F.E.C.-O.A.D., 2012. Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption.
- Pait, A.S., Nelson, J.O., 2003. Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds. *Aquat. Toxicol.* 64, 331–342. doi:10.1016/S0166-445X(03)00060-2
- Park, C., Aoki, J., Lee, J., Nagae, M., Lee, Y., Sakakura, Y., Hagiwara, A., Soyano, K., 2010. The effects of 17 α -estradiol on various reproductive parameters in the hermaphrodite fish *Kryptolebias marmoratus*. *Aquat. Toxicol.* 96, 273–279. doi:10.1016/j.aquatox.2009.11.006
- Park, C.J., Gye, M.C., 2014. Sensitization of vitellogenin gene expression by low doses of octylphenol is mediated by estrogen receptor autoregulation in the *Bombina orientalis* (Boulenger) male liver. *Aquat. Toxicol.* 156, 191–200. doi:10.1016/j.aquatox.2014.08.013
- Ross, G., 2004. The public health implications of polychlorinated biphenyls (PCBs) in the environment. *Ecotoxicol. Environ. Saf.* 59, 275–291. doi:10.1016/j.ecoenv.2004.06.003
- Rubin, B.S., 2011. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* 127, 27–34. doi:10.1016/j.jsbmb.2011.05.002
- Schug, T.T., Janesick, A., Blumberg, B., Heindel, J.J., 2011. Endocrine disrupting chemicals and disease susceptibility. *J. Steroid Biochem. Mol. Biol.* 127, 204–215. doi:10.1016/j.jsbmb.2011.08.007
- Selcer, K.W., Verbanic, J.D., 2014. Vitellogenin of the northern leopard frog (*Rana pipiens*): Development of an ELISA assay and evaluation of induction after immersion in xenobiotic estrogens. *Chemosphere* 112, 348–354. doi:10.1016/j.chemosphere.2014.04.073
- Shao, J., Shi, G., Liu, J., Jiang, G., 2004. A rapid two-step chromatographic method for the quantitative determination of vitellogenin in fish plasma. *Anal. Bioanal. Chem.* 378, 615–620. doi:10.1007/s00216-003-2235-0
- Solé, M., Raldua, D., Piferrer, F., Barceló, D., Porte, C., 2003. Feminization of wild carp, *Cyprinus carpio*, in a polluted environment: Plasma steroid hormones, gonadal

morphology and xenobiotic metabolizing system. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 136, 145–156. doi:10.1016/S1532-

Soverchia, L., Ruggeri, B., Palermo, F., Mosconi, G., Cardinaletti, G., Scortichini, G., Gatti, G., Polzonetti-Magni, a. M., 2005. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17- β estradiol and nonylphenol. *Toxicol. Appl. Pharmacol.* 209, 236–243. doi:10.1016/j.taap.2005.04.013

Syasina, I.G., Shved, N.A., 2015. Hexestrol- and nonylphenol-induced differential vitellogenin synthesis in female and male barfin plaice *Liopsetta pinnifasciata*. *Environ. Toxicol. Pharmacol.* 39, 597–605. doi:10.1016/j.etap.2015.01.014

Taxvig, C., Hadrup, N., Boberg, J., Axelstad, M., Bossi, R., Bonefeld-Jorgensen, E.C., Vinggaard, A.M., 2013. In vitro - in vivo correlations for endocrine activity of a mixture of currently used pesticides. *Toxicol. Appl. Pharmacol.* 272, 757–766. doi:10.1016/j.taap.2013.07.028

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. *Ecol. Indic.* 23, 274–279. doi:10.1016/j.ecolind.2012.04.009

Wang, J., Bing, X., Yu, K., Tian, H., Wang, W., Ru, S., 2015. Preparation of a polyclonal antibody against goldfish (*Carassius auratus*) vitellogenin and its application to detect the estrogenic effects of monocrotophos pesticide. *Ecotoxicol. Environ. Saf.* 111, 109–116. doi:10.1016/j.ecoenv.2014.10.007

Weiss, B., 2011. Endocrine disruptors as a threat to neurological function. *J. Neurol. Sci.* 305, 11–21. doi:10.1016/j.jns.2011.03.014

Yilmaz, O., Prat, F., José, A., Amano, H., Koksoy, S., Sullivan, C. V, 2015. General and Comparative Endocrinology Estrogen-induced yolk precursors in European sea bass, *Dicentrarchus labrax* : Status and perspectives on multiplicity and functioning of vitellogenins. *Gen. Comp. Endocrinol.* 221, 16–22. doi:10.1016/j.ygcen.2015.01.018

Zhong, L., Yuan, L., Rao, Y., Li, Z., Zhang, X., Liao, T., Xu, Y., Dai, H., 2014. Distribution of vitellogenin in zebrafish (*Danio rerio*) tissues for biomarker analysis. *Aquat. Toxicol.* 149, 1–7. doi:10.1016/j.aquatox.2014.01.022

CAPÍTULO III

RELATIONSHIPS BETWEEN CYTOCHROME P450 SEX STEROID CONCENTRATIONS AND ENDOCRINE DISRUPTION IN TILAPIAS CAGED IN FOUR RESERVOIRS OF THE IGUAÇU RIVER

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ABSTRACT

Environmental chemicals originating from human activities may enter aquatic environments and interfere with the endocrine system of the exposed biota. The risk assessment of these chemicals with high public concern is poorly studied in aquatic ecosystems in South America. The aim of this work was to investigate endocrine effects in male tilapia (*Oreochromis niloticus*) caged in four cascading reservoirs of the Iguaçu River. The occurrence of persistent organic pollutants was evaluated both in water and tissue samples. Endocrine effects were also evaluated through alterations in the synthesis and clearance of androgens and estrogens as well as impacts on gonadal development. Chemical analysis showed the bioaccumulation of persistent organic pollutants in tilapia and levels of PCBs 14 times above the permitted amount in the water of SS reservoir. Cyclodiane organochlorine pesticides such as Aldrin and Isodrin were also above the permitted amount in the water of SO reservoir. RT-qPCR analysis revealed different transcript levels of cytochrome P450s (*cyp1a* and *cyp3a*) in the liver between the four groups, with induced activity in tilapia from SS reservoir. Significant correlations for CYP3A mRNA and activity of CYP3A with steroid levels confirmed the role of this enzyme in the metabolism and clearance of steroid hormones in tilapia. Additionally, brain aromatase activity may play a role in gonad development since it was strongly correlated to gonadosomatic index, as well as steroid levels. Since persistent organic pollutants were also observed in fish with unaltered endocrine parameters, it is unclear whether they might be responsible for the observed effects. Future studies are needed to identify the chemical(s) that are altering CYP in fish exposed to water from the SS reservoir.

Key words: Endocrine effects, male tilapia, Iguaçu River, persistent organic pollutants, cytochrome P450s

1 INTRODUCTION

The Iguaçu River of southern Brazil has significant socioeconomic and environmental relevance, not only as a primary water supply for human consumption but also possessing high potential for hydroelectric power generation, aquaculture activity, and diverse populations of endemic fish and fauna (Baumgartner et al., 2012). However, the river is also the second most polluted waterway in Brazil (IBGE, 2010) due to the large amounts of urban and industrial wastes continuously released into the origin of the river without the proper treatment. Among the several chemicals that are introduced into this aquatic environment due to anthropogenic activities, some are known to interfere in the normal function of the endocrine system and cause adverse effects in living organisms (Hu et al., 2012; Porte et al., 2006; Schug et al., 2011).

Although there are greater evidences of the negative impacts of the environmental endocrine disruptors in human populations and the biota, the risk of exposure to these chemicals is still poorly investigated in aquatic ecosystems from developing countries, as Brazil. The few studies that performed chemical analysis in the Iguaçu River detected potential endocrine disrupting chemicals (EDCs), including human products from domestic sewage in the origin of the river, such as female steroids hormones (E1, E2, EE2), pharmaceuticals, caffeine, UV filters (Ide et al., 2016) and from industrial wastes, as transition metals and, polycyclic aromatic hydrocarbons (Leite et al., 2011; Yamamoto et al., 2016). On the other hand, the impact of the intense agricultural activity (soybeans, corn and wheat farms) performed close to the reservoirs of the Iguaçu River was not investigated yet.

Therefore, complex mixture of chemicals both from urban effluents and agricultural wastes, including pesticides with potential endocrine disrupting effects, may be present in the reservoirs of the Iguaçu River and should be investigated. In general, organochlorine pesticides (OCP) and other persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) are still widespread in the environment and are able to trigger endocrine responses (Hardell et al., 2010; Peck et al., 2016; Rigét et al., 2015). The high stability and lipophilic characteristics of these chemicals, contributes to their potential for bioaccumulation through the food chain which may ultimately affect human health and wildlife (Suib et al., 2013). Considering the diversity of aquatic pollutants, besides the identification of these chemicals, it is helpful to assess the mechanisms and the effects in the exposed organisms to better comprehend the risks of exposure.

Regarding to agonistic activities, several EDCs are able to interact with estrogenic receptors and induce vitellogenin expression in male oviparous animals, making vitellogenin a good and widely used molecular biomarker to detect estrogenic activity in fish (Gillannejad et al., 2016; Hultman et al., 2015; Sumpter and Jobling, 1995; Wang et al., 2015). In normal

sexually mature females the synthesis of vitellogenin depends on the production of the steroid hormone, 17 β -estradiol, which is regulated by the hypothalamus-pituitary-gonadal axis (Hiramatsu et al., 2005), a broad target for EDCs action (Hampl et al., 2014).

EDCs may also interfere in the biosynthesis and metabolism of steroid hormones (Hampl et al., 2014; Tournier et al., 2015), particularly the steps catalyzed by cytochrome P450-dependent enzymes (Kiyama and Wada-kiyama, 2015; Vitku et al., 2014). Cytochrome P450 is the major superfamily of enzymes, generally incorporated into the endoplasmic reticulum and are involved in the metabolism of many endogenous and exogenous compounds (Guengerich, 2008). They are mono-oxygenase heme-containing proteins, mainly expressed in liver that incorporates oxygen into xenobiotics, converting the substrates to more hydrophilic products (Ortiz de Montellano, 2005), which may increase or decrease generation of potentially toxic compounds. Transcriptional activation of different CYPs is regulated by distinct mechanisms: CYP1A1, CYP1A2, and CYP1B1 are mainly regulated via binding to the aryl hydrocarbon receptor (AHR). In contrast, CYP3A depends on pregnane X receptor (PXR) binding (Boelsterli, 2003). Both responses of induction or inhibition of CYPs activities in fish are well established as useful tools for environmental contamination biomonitoring (Burkina et al., 2013; Jasinska et al., 2015). Lipophilic compounds, such as PCBs, Polybrominated diphenyl ethers (PBDEs) and some OCPs, are widely reported as CYP inducers in different organisms.

The CYP3A enzyme is considered the most highly expressed subfamily in the liver of vertebrates and is responsible for the metabolism of at least 50% of all drugs (van Waterschoot and Schinkel, 2011). Additionally, endogenous steroid hormones serve as CYP3A substrates. For example, hydroxylation of Testosterone has been shown to be catalyzed by the CYP3A activity in fish as well as in mammals (Huang et al., 2015; Li and Letcher, 2002). Hydroxylation at either the C6 or C16 position are the major locations of testosterone oxidative metabolism, whereas 1 β -, 2 β , and 15 β -hydroxytestosterone are produced as minor metabolites (Maenpaa et al., 1993).

Another potential CYP target for environmental chemicals is aromatase (CYP19A), which is expressed primarily in the ovary and brain (Biegon, 2015; Fester et al., 2015). This subfamily is responsible for catalyzing the final steps of steroidogenesis in the conversion of androgens into estrogens (Lephart, 1996), and is essential for gonadal differentiation in fish (Guiguen et al., 2010). In the brain, aromatase activity in teleostean fish is much higher than in mammals (Hinfray et al., 2006), possibly due to the capability of fish brain to grow during adulthood and to the role in reproductive strategies (Breton et al., 2015). Many studies have reported alterations of brain and/or ovarian P450 aromatase activities in fish exposed to environmental substances (Cheshenko et al., 2008; Hinfray et al., 2006; Mills et al., 2014), which may influence androgen-estrogen balance and be associated with the development of

various diseases, such as breast cancer (Brodie, 2002; Brodie et al., 1999).

For studying reproductive biology endpoints, the Nile tilapia (*Oreochromis niloticus*), one of the most cultivated fish species worldwide, represents an appropriate animal model (Correia et al., 2010), particularly for molecular level responses (Baroiller et al., 2014). Also, due to its reproduction rates, sexual dimorphism, versatility and easy adaptation in different environments (Costa-pierce, 2003; FAO, 1983) it is a good species for studies with caged fish experiments in aquatic environments. Considering the importance of the Iguaçu River and the gap in field studies investigating the bioavailability of endocrine disruptors in aquatic ecosystems in South America, the aim of this work was: i) to detect persistent organic pollutants both in water and tilapias; ii) to evaluate the endocrine endpoints in tilapia caged in four reservoirs of the Iguaçu River, analyzing alterations of estradiol biosynthesis (CYP19) and metabolism (CYP3A) of other steroid hormones, as well as effects on gonadal development

2 METHODS

Studied Area

The Iguaçu River extends of 1.060 km, draining a basin of approximately 72.000 km² (Baumgartner et al., 2012). Four reservoirs of hydropower plants of the Iguaçu River, located at Paraná State in Southern Brazil, were analyzed in this study (Figure 1). The Segredo Reservoir (SE, 25°79'13.36"S, 52°11'89.36"W) is the closest to the metropolitan region of Curitiba (MRC). The other reservoirs are Salto Santiago Reservoir (SS, 25°63'73.08"S, 52° 60'20.19"W), Salto Osório Reservoir (SO, 25°53'71.06"S, 53°00'33.31"W) and Salto Caxias Reservoir (SC, 25°53'64.43"S, 53°49'90.70"W), which are adjacent sites downstream of each other.

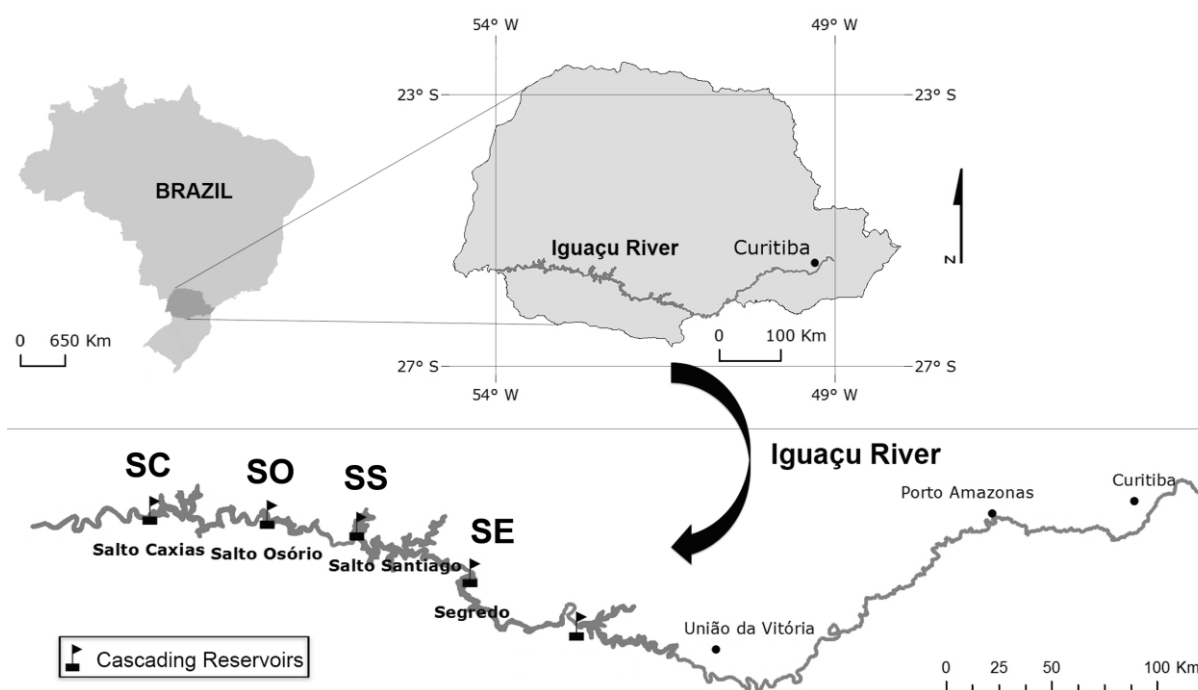


FIGURE 1. Map of the four reservoirs of the Iguaçu River used for the study.

Chemical Analyses

The following compounds were investigated: (i) polychlorinated biphenyls (PCBs) congeners: PCB 8, PCB 18, PCB 31, PCB 28, PCB 33, PCB 52, PCB 49, PCB 44, PCB 74, PCB 70, PCB 66, PCB 95, PCB 56/60, PCB 101, PCB 99, PCB 97, PCB 81, PCB 87, PCB 110, PCB 77, PCB 151, PCB 123, PCB 149, PCB 118, PCB 114, PCB 153, PCB 132, PCB 105, PCB

141, PCB 138, PCB 158, PCB 126, PCB 187, PCB 183, PCB 128, PCB 167, PCB 174, PCB 177, PCB 156, PCB 157, PCB 180, PCB 169, PCB 170, PCB 201, PCB 203, PCB 189, PCB 195, PCB 194, PCB 206, PCB 209, and; (ii) organochlorine pesticides: Dichlorodiphenyltrichloroethane (o,p'- DDT, p,p'-DDT) and DDT metabolites (o,p'-DDE , p,p'-DDE, o,p'- DDD, p,p'- DDD), hexachlorocyclohexane (α -HCH, β -HCH, γ -HCH, δ -HCH, HCB), α -Chlordane, γ -Chlordane, Oxychlordane, Aldrin, Isodrin, Dieldrin, Endrin, HCB, Heptachlor, Heptachlor Epoxide A, Heptachlor Epoxide B, Endossulfan I, Endossulfan II, Metoxichlor and Mirex (iii) Polybrominated Diphenyl Ethers (PBDEs) congeners: BDE 47, BDE 99, BDE 100, BDE 153, BDE 154 and BDE 183;

Two liters of water per reservoir were sampled close to the cages, at 30 cm below the surface, and were stored in glass bottles protected from light, at 4° C until analysis. At the same day, fish tissue (liver, muscle and gills) were sampled and stored at -75 °C until analysis. The organic chemicals were extracted from the water by solid-phase extraction, with Oasis HLB cartridge according to Belenguer et al. (2014), and from the tissue samples (3 g of pool per group) according to Rissato et al. (2004).

The identification and quantification of the organic contaminants were performed by analyzing 2 μ L of the final extracts in a gas chromatograph (Agilent 7890A GC) coupled to a mass spectrometer (Agilent 5975C inert MSD with Triple-Axis Detector). A capillary column of fused silica coated with 5% diphenyl-dimethyl siloxane (30 m length, 0.25 mm internal diameter and 0.25 μ m thick film) was used. Helium was used as the carrier gas, and the oven temperature was programmed to heat from 75° C to 150° C at 15° C min⁻¹, then to 260°C at 2°C min⁻¹, and finally to 300°C at 20°C min⁻¹ and remained constant for 10 min. The injector was conditioned at 280°C, the interface with the detector at 300°C and the ion source at 230°C.

The data were acquired in the SIM (Selected Ion Monitoring) mode. The organic contaminants were identified by matching the retention times and the mass/charge of ion fragments with those obtained from a mixture of external standards (C-WNN and C-WCFS – PCBs, AE-00010 – Pesticides, and Individual BDEs, all of them from AccuStandard, New Haven, CT, USA). For the quantification of organohalogenated compounds, calibration curves were prepared at different concentrations: 1, 5, 10, 20, 80, 100, 150, and 200 pg μ L⁻¹. The individual organic compounds concentrations were based on the integration of the main fragment peak area for each compound using the HP Chemstation program (G2070 BA).

The detection limits (DL) were <0.5 ng g⁻¹ tissue and <0.5 ng L⁻¹ of water for each individual organohalogenated compounds. These data are based on the lowest compound concentration sensitive (0.5 pg μ L⁻¹) multiplied by the final extracted

volume (1000 μL) and divided by the weight tissue (1 g) or volume of water samples (1 L).

Experimental design

A total of two hundred and forty male juvenile (approximately containing 30-50g) specimens of tilapia (*O. niloticus*) from the same lot were obtained from the Research Center in Environmental Aquaculture (Western University of Parana, Toledo). Fish were transported under constant oxygen aeration in a fish transport tank and randomly distributed in four fish cages of 2m³ (60 fish per cage and per reservoir). The cages were placed upstream the dams of the four reservoirs of Iguaçu River. Fish were fed three times per week with commercial food pellets (Nicoluzzi® ration) and maintained encaged for two months (October-December, 2014). After the experimental period, fish were collected from the four cages, anesthetized with MS-222 (0.02% in water), and measured for biometric parameters (total length of 21,7 $\pm$ 2,9 cm and weight average of 201 $\pm$ 73 g). Blood samples were obtained through the caudal vein with a heparinized syringe and mixed with the anti-proteolytic PMSF (phenyl-methyl-sulfonylfluoride–10 mM). Plasma was obtained after centrifugation at 4,000 g, 4°C and stored at –75 °C. The animals were then killed by severing the spinal cord and the liver, gonads and brain were dissected. Gonads were weighted for gonadosomatic index, and divided in three parts: for qPCR analysis, CYP19a activity and histology (fixed chemically). Liver, brain and gonad were stored at -75 °C until activity assays (n=20) and for qPCR analysis, aliquots of the same tissues (10-50 mg per sample, n=15) were stored in 1:10 (w/v) RNeasy Lysis Solution (Ambion, USA) at -75 °C.

Quantitative real-time PCR analysis.

RNA Extraction and Reverse transcription

Total RNA was extracted from tilapia liver and gonads samples using SV Total RNA Isolation System (Promega Corporation, Madison, WI). For the brain samples, RNA extraction was done using TRIzol® reagent (Invitrogen, USA). Concentrations and quality ratios (280/260 and 260/230 >1.8) of RNA were determined spectrophotometrically using ND-1000 (NanoDrop, Wilmington, DE). RNA integrity was measured with 1% Agarose gels stained with ethidium bromide. First strand cDNA was synthesized from 1 μg of total RNA (20 μL reaction mixture) using a Reverse Transcription System (Promega Corporation, Madison, WI), and stored at -20° C.

Primer design

Sequences of genes from *Oreochromis niloticus* were analyzed according to nucleotide databases in GenBank. Primers were designed using IDT DNA PrimerQuest software and optimized using PCR Miner (Table 1).

TABLE 1. Primers sequences of genes of interest from *Oreochromis niloticus*.

Gene name	Sequence	Amplicon Length
<i>vtg</i>	Forward GACACCGGTCAAGTTTGAGTA Reverse CAGACTCTTGCATCTCGTAGAC	158
<i>cyp 19A gonads</i>	Forward: GCAACCAGAACGCTGATACT Reverse: GCCTATGCCAGTCCAGATAAAT	144
<i>cyp 19A brain</i>	Forward ACACATACCAGGTCCTTTCTTC Reverse AGGGTCTCCTCTCCGTTTAT	150
<i>cyp 1A liver</i>	Forward GACGATGAGCTGGTTAGCTTAG Reverse TCCTTGTCAAAGGTGGCATAG	197
<i>GnRH1</i>	Forward CTGCTGTCAACACTGGTCATA Reverse GAAGTTGTCCAGATCCCTCTTC	61
<i>cyp 3A</i>	Forward CACCTCTGGGAGATTGAAAGAG Reverse TGCTGGTTACCACATCCATAC	149
<i>EF a1</i>	Forward AGGCTCGTTTCGAGGAAATC Reverse CCCTTGAACCAGCTCATCTT	148

qPCR amplification

The qPCR amplifications were performed using an iTaq Universal SYBR Green One-Step kit in a total volume of 20 μ l (100 ng of cDNA; primers 0,5 μ M). Each kit was used according to the manufacturer's instructions. The cDNA was denatured and polymerase activated at 95° C for 5 min, then 40 cycles of 10s at 95° C and 30 s of 55° C. Samples were subject to melting curve analysis from 65 to 85° C in 0.5° C increments with continuous fluorescence measurement. The qPCR products were analyzed by agarose gel to confirm sequence identities. Gene expression values were obtained using LightCycler Relative Quantification software. Relative gene copy numbers were derived considering the formula $2^{-\Delta CT}$, where ΔCT is the difference in amplification cycles required to detect amplification product of the gene of interest from the housekeeping gene (EF a1).

Cytochromes P450s Activities

Microsome isolation

Liver, gonads and brain samples were thawed and homogenized at 1:10 (w/v) in Phosphate/EDTA buffer (100 mM Phosphate, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 100 mM KCL, pH 7.4). After a 20 min centrifugation at 12,000g, the cytosol underwent ultracentrifugation for 60 min at 100,000g, the pellet was re-suspended in Phosphate buffer with 20% Glycerol. Total protein concentration was determined (Bradford *et al.*, 1976) and utilized to normalize protein concentrations in microsomal fractions for other assays.

Ovarian and Brain P450 aromatase

Aromatase activity in gonads and brain was determined by the tritiated-water release method according to Lavado *et al.* (2004). For the assay, 10 μ L of 1 β -[³H]androstenedione (40 pmol, 1 mCi in ethanol), was evaporated to dryness in polypropylene tubes under nitrogen gas. Microsomal proteins (0.2 mg) were incubated in the tubes for 45 min at 25 °C in a final volume of 1 mL of 100 mM Tris-HCl, pH 7.6, 0.2 mM NADPH. For blanks, buffer was added instead of microsomal proteins. The reaction was stopped by placing the tube on ice. Organic metabolites and the excess of substrate were eliminated from the aqueous phase by extraction with 100% methylene chloride. Following this, a suspension containing 2.5% (w/v) activated charcoal and 0.25% dextran in Milli-Q water was added and incubated overnight at room temperature. The mixture was centrifuged, and then two aliquots of 1mL of the supernatant and 10 mL of Scintillation cocktail was added in scintillation vials and counted for ³H radioactivity for 5 minutes per sample.

Testosterone Hydroxylation

Testosterone oxidation was assayed by incubating 0.4 mg hepatic microsomal proteins with [¹⁴C]testosterone (500 pmol, 0.07 mCi), NADPH (300 mM) in a final volume of 0.25 mL of 50 mM Tris-HCl, pH 7.4. Samples were incubated for 1 h at 30 °C. prior to addition of 250 μ L of 100% acetonitrile. The samples were then centrifuged for 10 minutes. The supernatant was transferred into glass HPLC vials and 500 μ L was injected onto a reverse-phase HPLC column. HPLC analysis was performed on a Perkin-Elmer Binary LC pump 250 system equipped with a 250x4 mm LiChrospher 100 RP-18 (5 mm) reverse-phase column (Merck, Darmstadt Germany) protected by a guard column LiChrospher 100 RP-18 (5 mm). Separation of [¹⁴C]testosterone and its metabolites was performed at 1 mL/min with a mobile phase composed of (A) 75% water and 25% acetonitrile and (B) 45% water and 55% acetonitrile. The run consisted of a 30 min linear gradient from 100% A to 100% B, 30–35 min 100%B and 35–50 min linear gradient to 100% A. Chromatographic peaks were

monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, BadWildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as a scintillation cocktail. Metabolites were tentatively identified by co-chromatography with authentic standard compounds. According to the retention time observed for the standards peaks, it was possible to identify five different peaks and calculate respective testosterone hydroxylation activity with the obtained peak areas:

- 1 - 6 β -hydroxytestosterone (6 β -OHT), peak with a retention time of 8 to 11 minutes, corresponding mostly to CYP3A activity
- 2 - 16 α -hydroxytestosterone (16 α -OHT), peak with retention time of 11-12 min corresponding to CYP2A activity
- 3 - 16 β -hydroxytestosterone (16 β -OHT), peak with retention time of 16-19 min, corresponding mostly to CYP3A activity
- 4 - Androstenedione (AD), peak with retention time of 28-30 min, corresponding to 17 β -hydroxysteroid dehydrogenase activity
- 5 - Dihydroxytestosterone (DHT), peak with retention time of 31-33 min, , corresponding to 5 α -reductase activity

One additional detected peak, with a retention time of 3-5 min was not identified.

Steroids hormones

Steroid hormones (Estradiol and Testosterone) were measured in plasma samples using a microparticle enzyme immunoassay (AxSYM Estradiol and Testosterone assay, Abbott Laboratories, Abbott Park, IL, USA) and used according to the manufacturer's instructions.

Histological procedures

Male fish gonads were fixed in ALFAC fixative solution (ethanol 70%, formaldehyde 4% and glacial acetic acid 5%) for 16 h, dehydrated in graded series of ethanol and xylene and embedded in Paraplast Plus resin (Sigma[®]) (Micron[®] EC350-2). Sections of 5 μ m thick were obtained through a semi-automatic microtome (Leica[®]) and stained with hematoxylin and eosin. The slides were observed under light photomicroscope Leica[®] and analyzed according to Leino et al., 2005 and Blazer, 2002. Male gonads were classified in 5 different stages of maturation: I – Pre-Spermatogenic; II – Early Spermatogenic; III – Mid-Spermatogenic; IV – Late-Spermatogenic; V – Late-Spawning.

Additionally, alterations such as degeneration of germ cells, atresia (degeneration of follicles), presence of parasites, centers of melanomacrophages (CMMs) and eosinophils were also analysed.

Statistical Analysis

All data were checked for outliers (Grubb's test), normality (Shapiro's test) and homoscedasticity (Bartlett's test) before performing the analysis. Either a *two-way* ANOVA test followed by the *Tukey-Kramer* post-test (parametric data) or *Kruskal-Wallis* test followed by the *Dunn's* test (nonparametric data) were utilized, with $p < 0.05$. Correlation analysis were performed with all variables and analyzed with Spearman test according to Mukaka, 2012, where correlation coefficient from: $\pm 0 - 0,3$ indicates negligible correlation; $\pm 0,3 - 0,5$ low correlation; $\pm 0,5 - 0,7$ moderate correlation; $\pm 0,7 - 1,0$ strong correlation.

RESULTS

After 2 months of exposure in Iguaçu River, about 30 tilapias per cage survived in the 4 reservoirs (SE, SS, SO and SC). There were no significant differences observed in gonadosomatic index in fish from the sites.

Chemicals

Concentrations of total PCBs and Aldrin and its metabolites in the water of SS and SO reservoirs, respectively, were above than the permitted according to Brazilian legislation thresholds (CONAMA, 2012). Moreover, Aldrin and its metabolites were detected in the water of all reservoirs (Table 2). Heptachlor and endosulfan were below the limit of detection for SS and SE reservoirs respectively, whereas PCBs and DDTs were only detected in SS reservoir. Total pesticides in the water were highest in the SS, SO and SC reservoirs.

In some sites, the pattern of chemicals detected in the water was different than what was detected in the tissues (Table). PBDEs were not detected in the water, however were present in fish from SS, SO and SC. PCBs, PBDEs, and total pesticides were predominant in individuals from SO followed by SS. When comparing the three different tissues, bioaccumulation was not the same for the four sites. In the muscle higher concentrations of PCBs and Heptachlor were observed in tilapia from SE. Fish from SS and SO, showed more bioaccumulation of PCBs and PBDEs in the liver. Chlordane and Mirex (data not shown) were detected only in the gill of SO individuals (1,27) and liver of SC (0,61), respectively, whereas HCH were detected only in the water of SS reservoir.

In general, non-coplanar PCBs, particularly congener 203, were the dominant POPs in tilapias' tissues, followed by Heptachlor > PBDEs > Endosulfan> DDTs> Chlordane> Aldrins. On the other hand, total aldrins was the predominant POP dissolved in the column of water, followed by PCBs> Endosulfan > Heptachlor >HCH >DDTs. HCB and Metoxichlor were below the limit of detection both in water and tissues.

TABLE 2. Chemical analysis of total PCBs, PBDEs and pesticides concentrations in water (ng.L⁻¹) and tissue (ng.g⁻¹) of tilapias from the four reservoirs of Iguaçu River.

		PCBs	PBDEs	Drins	DDTs	Heptachlor	Endosulfan	Σ pesticides
	<i>Conama</i>	1	-	5	2	10	56	
SE	<i>Water</i>	<LD	<LD	1,63	<LD	1,22	<LD	2,85
	<i>Liver</i>	<LD	<LD	<LD	<LD	1,95	<LD	1,95
	<i>Gill</i>	2,76	<LD	<LD	<LD	<LD	1,70	1,70
	<i>Muscle</i>	5	<LD	<LD	<LD	8,40	<LD	8,40
SS	<i>Water</i>	14,7	<LD	4,91	0,69	<LD	3,81	10,92
	<i>Liver</i>	7,72	2,22	<LD	0,54	<LD	<LD	1,94
	<i>Gill</i>	1,48	1,51	<LD	<LD	<LD	1,96	1,96
	<i>Muscle</i>	<LD	<LD	<LD	<LD	2,69	<LD	2,69
SO	<i>Water</i>	<LD	<LD	6,05	<LD	1,44	4,15	11,64
	<i>Liver</i>	8,43	3,54	<LD	2,07	<LD	<LD	4,72
	<i>Gill</i>	7,37	0,60	1,27	1,15	1,15	1,36	4,93
	<i>Muscle</i>	<LD	0,61	<LD	<LD	<LD	0,87	0,87
SC	<i>Water</i>	<LD	<LD	1,87	<LD	3,53	6,13	11,53
	<i>Liver</i>	6,06	<LD	<LD	<LD	<LD	<LD	0,61
	<i>Gill</i>	6,66	0,65	<LD	<LD	1,58	<LD	1,58
	<i>Muscle</i>	3,53	<LD	<LD	<LD	1,51	<LD	1,51

<LD = Lower than Limit of Detection. SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir.

Relative gene expression

Relative gene expression of liver *vitellogenin*, as well as *gnrh1* of male tilapia were not different between the groups of the four reservoirs (Figure 2). Despite no significant differences between groups, the two forms of aromatase were differentially expressed in ovary and neural tissue. The transcript levels of *cyp1a* gene in the liver were higher in SC and SS than SE. For the liver *cyp3a*, relative gene expression was higher for individuals from SS than SE and SC, and was also higher in SO comparing to SE (Figure 2). Gonad *cyp3a* transcript levels were significantly higher in SO than SC individuals.

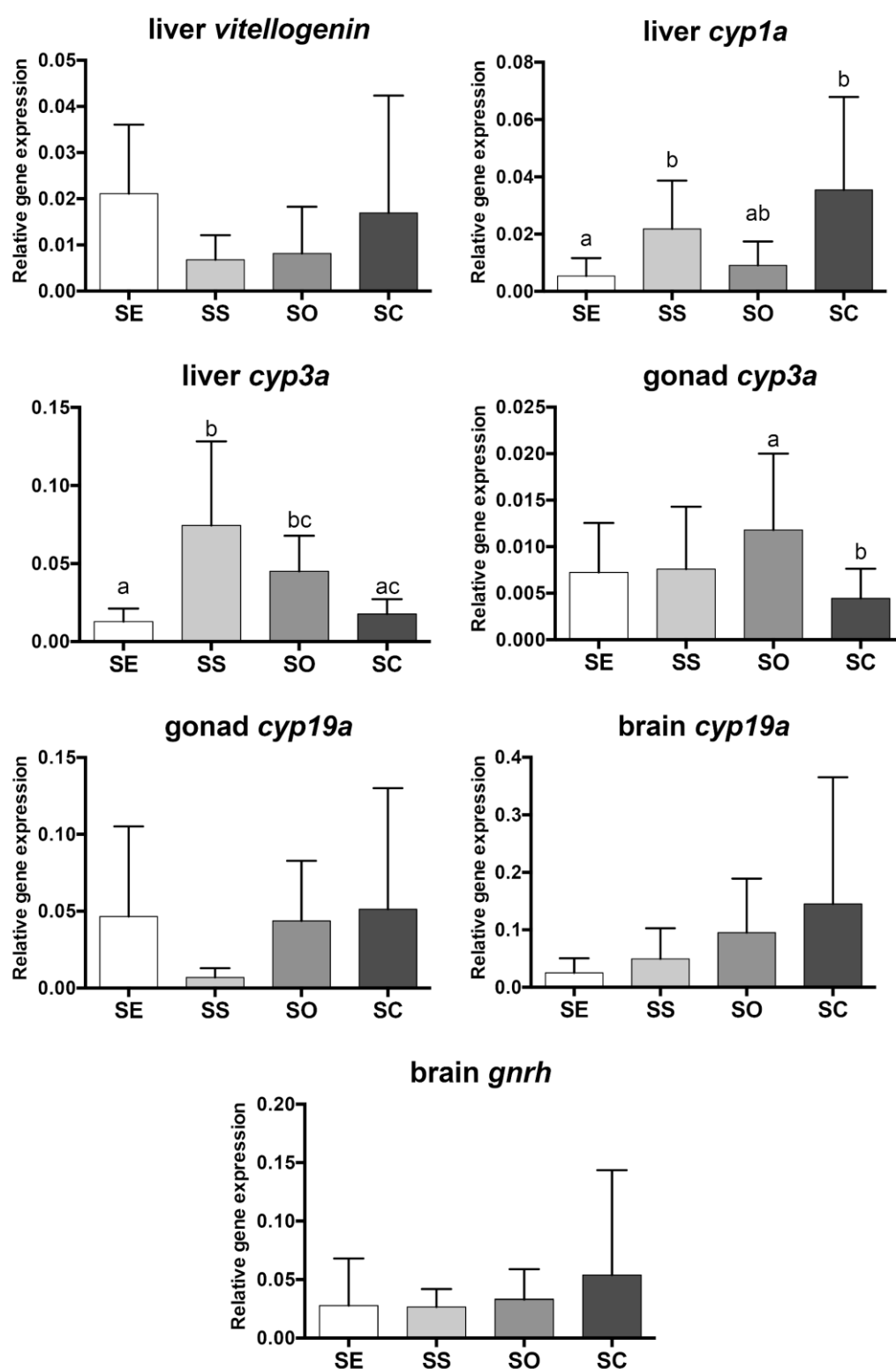


FIGURE 2. Transcriptional levels of genes of interest from *O. niloticus* from the four reservoirs of Iguaçu River. Letters indicate significant differences between the sites (a, b).

Aromatase activity and steroid hormones levels

No statistical differences were observed for aromatase activity both in gonads and brain of male fish from the four locations (Figure 3). Additionally, the steroid levels (E2 and T) in the plasma of male tilapia from the four reservoirs were not statistically different.

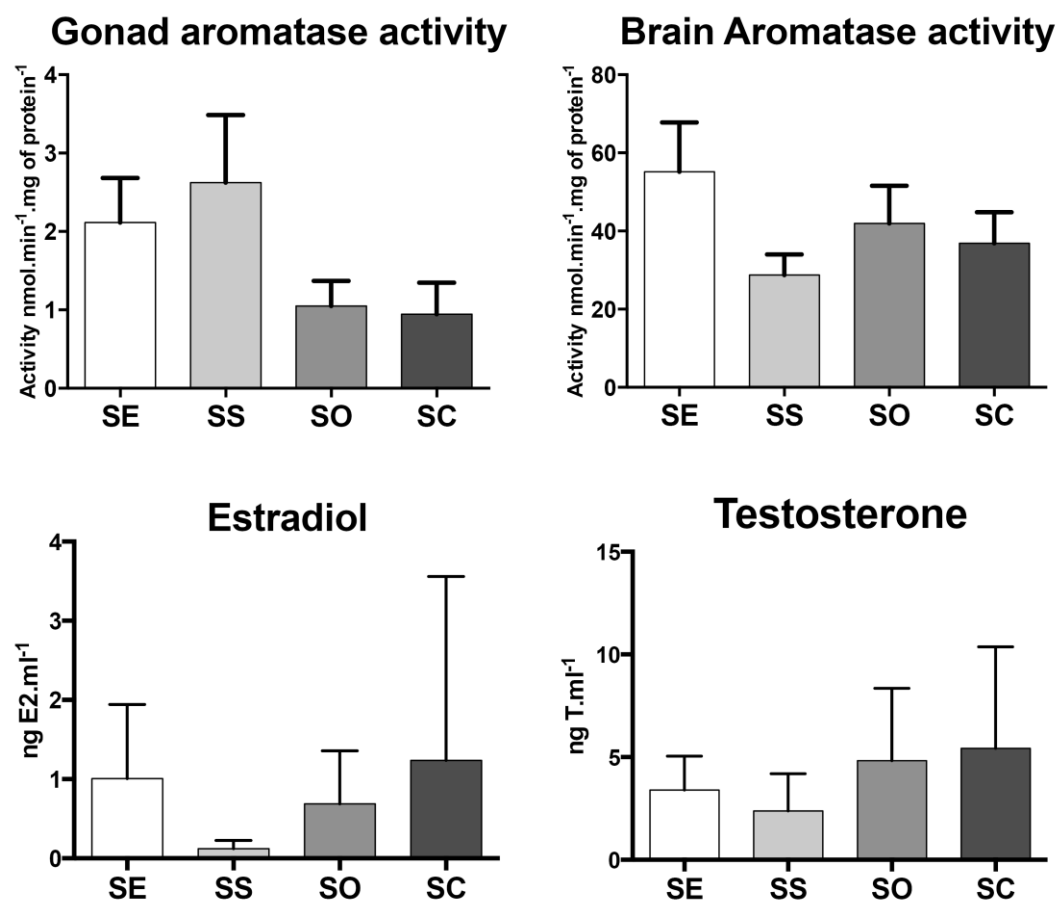


FIGURE 3. Aromatase activity in gonad and brain of *O. niloticus* from the four reservoirs of Iguaçu River. Letters indicate significant differences between the sites (a, b).

Testosterone hydroxylation (CYP3A activity)

The 6 β -OHT was the major metabolite observed in all samples. Male tilapia samples from the SS reservoir showed higher 6 β -hydroxylation than all other reservoirs (Figure 4), indicating higher activity of CYP3A for this group. Similarly, the 16 β -OHT generated was higher in tilapia from SS than SO and SC. This pattern confirmed a higher activity of CYP3A in the liver of fish from SS reservoir. For the 16 α -OHT metabolite, differences were not observed between the groups. In general, the percentage of total testosterone metabolized was higher in SS than SE group, in accordance with the higher generation of metabolites hydroxylated (6 β -OHT and 16 β -OHT).

Unlike the others metabolites, androstenedione generation was higher in SO group than SE and SS, demonstrating higher activity of 17 β -HSD in liver of males individuals from this group. Generation of dihydroxytestosterone was not different between groups, suggesting no difference in 5 α reductase activity.

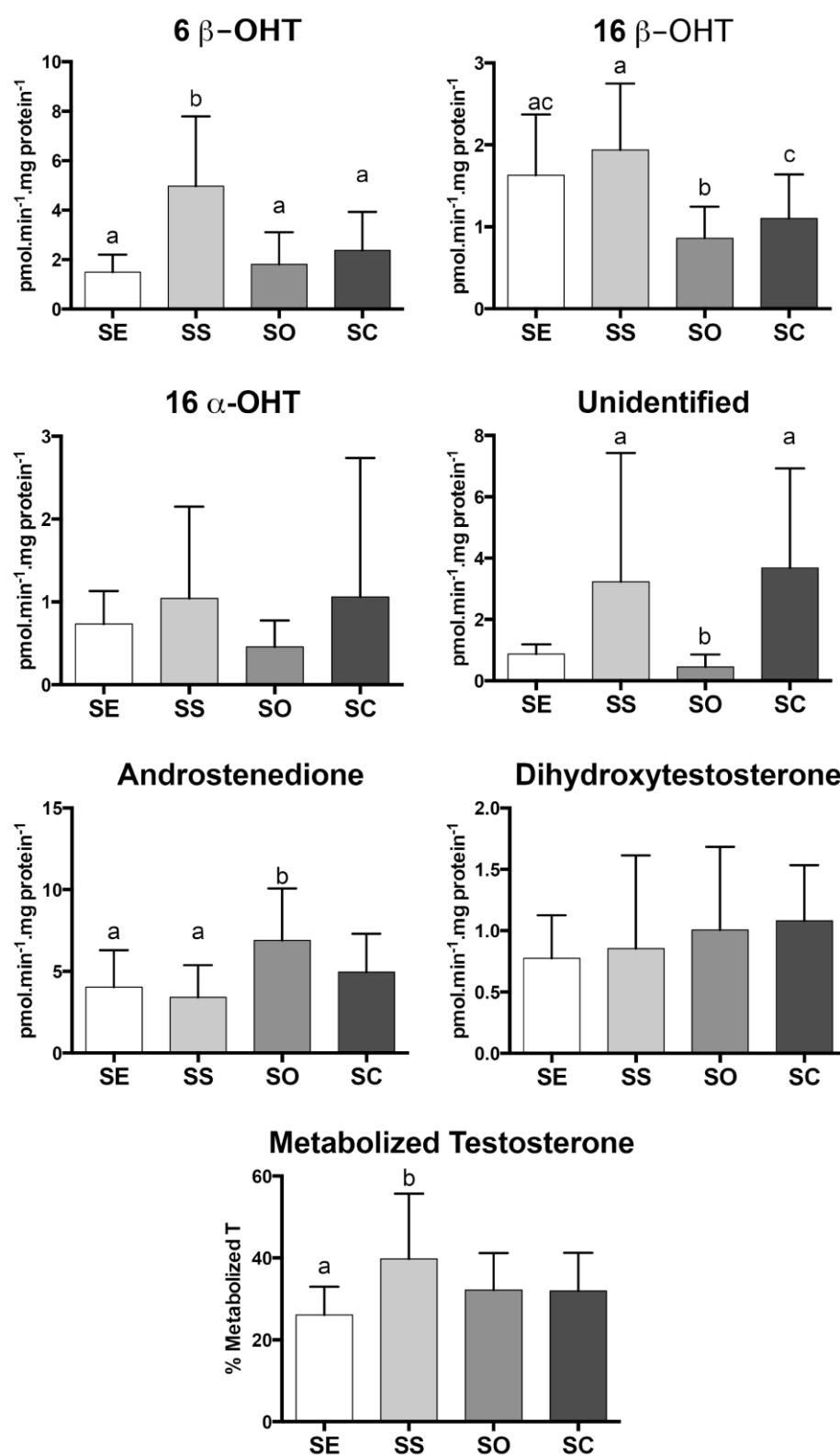


Figure 4. Testosterone hydroxylation in liver of *O. niloticus* from the four reservoirs of Iguaçu River. Testosterone hydroxylated at different positions: 6β (6β-OHT), 16α (16α-OHT), 16β (16β-OHT) and biotransformed to Androstenedione (AD) and Dihydroxytestosterone (DHT). Letters indicate significant differences between the sites (a, b).

Gonad histology

The testicular staging analysis showed that majority of male tilapia from the four reservoirs presented testes in the mid-spermatogenesis stage (Table 3), corresponding to similar proportions of germ cells from all stages of development (Figure 5). Only one individual from SE presented pre-spermatogenic testes. Eosinophilic cells were observed in 54% of male tilapia testes from SS reservoir. No significant pathological changes, such as necrosis were observed.

TABLE 3. Testicular stage of Tilapias caged in the four reservoirs of Iguaçu River.

	N	Pre-Spermatog.	Early Spermatog.	Mid-Spermatg.	Late-Spermatog.	Post-spawning	Eosinophil
SE	12	1 (8%)	0	10 (83%)	0	1 (8%)	2 (16%)
SS	11	0	2 (18%)	7 (63%)	0	2 (18%)	6 (54%)
SO	11	0	1 (9%)	10 (90%)	0	0	1 (9%)
SC	12	0	0	10 (83%)	0	2 (16%)	1 (8%)

SE = Segredo Reservoir; SS = Salto Santiago Reservoir; SO = Salto Osório Reservoir; SC = Salto Caxias Reservoir.

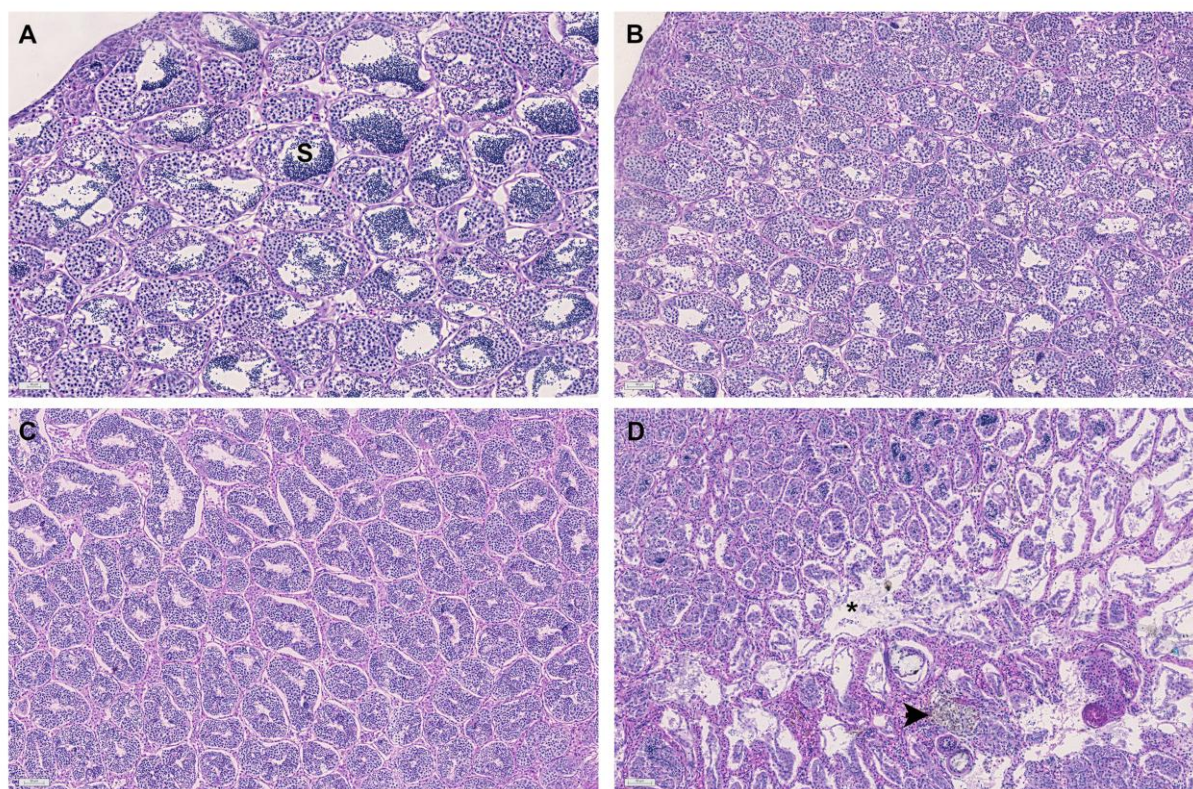


FIGURE 5. Testes section of *O. niloticus* from the five reservoirs of Iguaçu River. A- Mid-spermatogenic testes with spermatoocytes and spermatozoa (S) in the lumen of seminiferous tubules. B and C – Few spermatozoa and spermatoocytes in the seminiferous tubules. D - Empty lumen and a melanomacrophage center.

Correlation analysis

Strongest correlations of variables were verified for: Testosterone x GSI ($r=0,7$); Estradiol x Testosterone ($r=0,82$); 16α -OHT x 6β -OHT ($r=0,73$); 16β -OHT x Estradiol ($r=0,78$)(Table 4). Moderate correlations were verified for: Estradiol x GSI ($r=0,59$); Estradiol x liver cyp3a mRNA ($r=0,5$); vtg x Estradiol ($r=0,51$); gonad aromatase x 6β -OHT ($r=-0,57$); liver cyp3a mRNA x 6β -OHT ($r=0,58$); liver cyp3a mRNA x 16β -OHT ($r=0,62$); Androstenedione x liver cyp3a mRNA ($r=0,6$) and 6β -OHT x 16β -OHT ($r=0,64$).

Table 4. Correlations between different endpoints analysed in tilapias from the four reservoirs of Iguaçu River

r/p value	Testosterone	Estradiol	liver cyp3a	Brain CYP19	vtg	gnrh
GSI	0,7/ ****	0,59/0,003		0,43/0,001	0,3/0,12	
Testosterone		0,82/****	0,45/0,15	0,31/0,18	0,47/0,16	
Estradiol			0,5/0,14	0,35/0,13	0,51/0,13	0,4/0,07

r/p value	6β -OHT	16α -OHT	16β -OHT	AD
Testosterone			0,4/0,064	-0,4/0,067
Estradiol	0,45/0,03	0,38/0,07	0,78/****	-0,4/0,07
gonad cyp19	-0,57/0,002	-0,47/0,001	-0,45/0,01	0,3/0,14
liver cyp3a	0,58/0,003	0,33/0,11	0,62/****	-0,6/0,002
6β-OHT		0,73/****	0,64/****	-0,58/****
cyp1a	0,3/0,12		-0,38/0,07	

**** indicate very significant ($p < 0,001$)

4 DISCUSSION

The present study showed relevant data for risk assessment due to the occurrence of banned pesticides and organic halogenated compounds both in water and tilapia, which is one of the most cultivated species in the four reservoirs of Iguaçu River. Additionally to this data, the endocrine endpoints measured such as CYP activity, revealed the impact of the agricultural and other human activities close to the reservoirs of Iguaçu River. Overall, POP concentrations in the water and tilapia caged in the Iguaçu River were relatively lower than wild fish from other impacted aquatic environments (de Vlaming, 2008; Hao et al., 2014), but were also higher than some consumed marine fish species (Costopoulou et al., 2016; Shang et al., 2016). Nevertheless, the detected levels indicated the bioavailability of the pollutants and the bioaccumulation even with the relatively short period and form of exposure through caging.

The caged fish experimental design is suitable for detecting endocrine disrupting effects as demonstrated in this study and other previous research from different aquatic environments (Burki et al., 2006; Giesy et al., 2003; Harries et al., 2009). In comparison to field studies this strategy provides greater control because of: i) less variance of responses of fish obtained from the same lot (similar ages); ii) determination of the sex ratio; iii) controlled period of exposure and iv) the season chosen. Additionally, it is useful to understand the potential for human risk from EDCs exposure through in the consumption of farmed fish. Considering that fish is one of the major dietary sources of lipophilic chemicals such as PCBs (Costopoulou et al., 2016; Shang et al., 2016), its content may be even higher among farmed-raised than wild animals because of aquaculture feeding practices (Carubelli et al., 2007). Even lower concentrations of PCBs in fish may lead to exposure trophic levels close or exceeded to the health-based guidance values (Paiano et al., 2013; Carubelli et al., 2007).

Molecular alterations can be used as predictors for some reproductive endpoints (San-Segundo et al., 2016; Scholz and Mayer, 2008). However, changes in gene expression are not always directly related to a final effect so other levels of responses, such as proteins activities (Tyler et al., 2008) and gonad morphology (Blazer, 2002) should be used to evaluate fish health. Particularly, some reproductive effects depend on a long-term exposure or even higher doses of chemicals to elicit a

significant response. Testicular staging e.g. will likely reveal only EDC effects that profoundly influence testicular maturation (Leino et al., 2005). Although the relatively short period was probably not enough to cause relevant pathological and developmental changes in the gonad of tilapia, it was adequate to inform alterations at molecular and physiological levels, especially for CYP modulations and steroid hormone changes.

CYPs are abundantly expressed in the liver, but also in intestine, lung, and skin (Ortiz de Montellano, 2005) due to their role for metabolism of xenobiotics that enter the body from oral exposure (Guengerich, 2008). CYPs are also expressed in other organs, including kidney, brain, sertoli cells of the testis and ovary because they are responsible for other key roles including synthesis and metabolism of endogenous substances (P.B. Danielson, 2002; Wilkinson, 1996). CYP3a was also expressed in the gonads of tilapias, with higher mRNA levels in individuals from SO than SC, suggesting a metabolic role of this enzyme also found in this tissue. We confirmed the *cyp3a* expression in this organ by qPCR product sequencing that showed 92% of corresponded identity by blast software.

Both CYP 1A and 3A enzymes were overexpressed in the liver of individuals from SS reservoir and the activity of CYP3A was also higher than other reservoirs, indicating an increase in the metabolism of many drugs and endogenous steroids in those individuals. On the other hand it is possible that CYP3A was inhibited in tilapias from other reservoirs, especially for SE reservoir that showed lower expression and activity in general. Thus, normal balance of steroid levels may be compromised in these individuals. For example, estradiol concentrations in the plasma showed strong correlations with liver CYP3A mRNA and activity.

The modulation of CYP3A transcription is a common and sensitive biomarker for studies of xenobiotics metabolism and drug–drug interactions, making it important to identify or predict the agents responsible for this up/down regulation (Fenneteau et al., 2010; Guo et al., 2013; Shen et al., 2014; Shibasaki et al., 2014; Wagner et al., 2015). However, these agents are difficult to predict because CYP expression can be enhanced by a wide variety of structurally different compounds through the PXR (Konno et al., 2004; Li et al., 2008; Zhou et al., 2013). Overall, most of the studied and detected chemicals in Iguaçu River, predominant in SS and SO reservoirs, are reported as CYP3A inducers.

Non-coplanar PCBs, which were 14 fold times above the permitted concentrations in the water of SS reservoir, induce CYP3A expression in liver through binding and activation of the nuclear pregnane-X-receptor (PXR) in fish (Gräns et al., 2015), human hepatocytes (Al-Salman and Plant, 2012) and rat liver (Kopeck et al., 2010). According to Gähres et al. (2013) constitutive androstane receptor (CAR) may contribute to induction more than PXR in non-coplanar-PCB induction of CYPs, majority of PCBs found in the reservoirs, and the degree of chlorination is positively correlated with inducing potency of the congeners.

Co-planar PCBs and other dioxin-like compounds induce CYP1A expression and activity and prolonged exposure in heavily PCB-contaminated sites may impair the inducibility of CYP1A (Celander et al., 1996), while levels of CYP2 and CYP3A remained unchanged (Gräns et al., 2015). Both CYP1A and CYP3A subfamilies are involved in the metabolism of PCBs congeners in fish, whereas in crustacean this role is more related to CYP2A-like enzymes (Koenig et al., 2012). Additionally, Machala et al. (1998) associated hepatic testosterone 6 β -hydroxylation and androstenedione formation to a decrease in testosterone levels after PCB intake in bulls.

Although not detected in the water, PBDE 99 was the most abundant congener in tilapia from SS and SO. PBDE 99 is also known to increase CYP 3A and 1A gene expression (Dong et al., 2010; Li et al., 2016).

Organochlorine pesticides detected in all reservoirs of Iguaçu River, including Endosulfan, Drins, DDT, Chlordane and mirex are described as inducers of CYP (1A, 2 and 3A subfamilies) levels and activity and can consequently alter normal metabolism of steroids (Dai et al., 2001). Endosulfan, a selective inducer of CYP3A expression via PXR activation (Casabar et al., 2010; Lemaire et al., 2004), is associated with genotoxic effects through DNA-adduct formation in both fetal rat and Hep G2 cells (Dubois et al., 1996). In zebrafish, low doses of endosulfan exposure stimulates CYP and GST activities, which gradually decrease with increasing concentration and time of exposure (Dong et al., 2013).

A large number of drugs also appear to be able to reduce CYP3A activity. Therefore, the likelihood of competitive inhibition may occur when co-substrates are simultaneously administered (Danielson, 2002; Ding et al., 2016). In this case, the degree of inhibition is determined by the relative affinities of the substrates for the enzyme and their concentrations at the active site (Hodgson et al., 2012). One of the

main class of compounds known to inhibit CYP3A are azole containing fungicides (Sergent et al., 2009) and organophosphorus insecticides (Abass et al., 2009), which are also known to inhibit Acetylcholinesterase activity (Sams et al., 2000). For example, Chlorpyrifos (CPF) has been reported to have antagonistic effects on CYP enzymes in common carp gills (Fu et al., 2013).

ER transactivation and vitellogenin expression in male tilapia has been observed following exposure to (Leanos-Castaneda et al., 2002). In addition, induction and inhibition of aromatase activities (Kjeldsen et al., 2013) and altered steroids levels have also been observed following exposure (Da Cuña et al., 2013). Although significant differences were not observed for aromatase in tilapia at the caged sites, correlation analysis showed potential associations between brain aromatase and reproductive function. Brain aromatase activity was strongly positively correlated to Gonadosomatic index of male tilapias, which was also dependent to steroids levels. This finding is in accordance to Mills et al. (2014) that reported modulation of brain aromatase activity in male fish by EDCs was associated with decreased reproduction and gonadosomatic index, but not plasma vitellogenin. On the other hand, the absence of correlation between gonad aromatase and steroid levels may indicate that other factors, such as clearance rates of steroid hormones, may be influencing the balance of androgens-estrogens synthesized in the gonads.

Although most studies evaluating steroidal biosynthesis focus on gonadal aromatase (Guiguen et al., 2010), the form of brain is also involved in sexual differentiation, sexual behavior, and regulation of the reproductive cycle (Cheshenko et al., 2008; Fester et al., 2015; Tian et al., 2015). Thus, it remains to be determined whether the brain isoform is likewise affected, since its activity can be measured regardless of the maturity of fish. Interestingly, brain aromatase activity in male tilapias was much higher than in gonads, confirming the importance of this enzyme that shows a distinct pattern between mammals and fish (Hinfray et al., 2006) as sex and seasonal dependence (Chaube et al., 2015).

Conclusions

CYPs, especially the 1A and 3A subfamilies, are altered in caged fish within some of the reservoirs of the Iguaçu River. Relationships were observed between and steroids levels which may influence gonad development for individuals from the SS reservoir. Although the analyte list was limited, it is possible that pesticides and/or non- coplanar PCBs may be interfering with the normal function of CYP3A and brain aromatase which may alter steroid hormone concentrations which may impact the reproductive endocrine system in animals undergoing long-term exposure to the water of the Iguaçu River. The contribution of pesticides compared to other contaminants measured in upstream areas is unclear and deserves further study.

REFERENCES

- Abass, K., Turpeinen, M., Pelkonen, O., 2009. An evaluation of the cytochrome P450 inhibition potential of selected pesticides in human hepatic microsomes An evaluation of the cytochrome P450 inhibition potential of selected pesticides in human hepatic microsomes. *J. Environ. Sci. Heal. Part B* 44, 553–563. doi:10.1080/03601230902997766
- Al-Salman, F., Plant, N., 2012. Non-coplanar polychlorinated biphenyls (PCBs) are direct agonists for the human pregnane-X receptor and constitutive androstane receptor, and activate target gene expression in a tissue-specific manner. *Toxicol. Appl. Pharmacol.* doi:10.1016/j.taap.2012.05.016
- Baroiller, J., Cotta, H.D., Shved, N., Berishvili, G., Toguyeni, A., Fostier, A., Eppler, E., Reinecke, M., 2014. Oestrogen and insulin-like growth factors during the reproduction and growth of the tilapia *Oreochromis niloticus* and their interactions 205, 142–150. doi:10.1016/j.ygcen.2014.07.011
- Baumgartner, G., Pavanelli, S.C., Baumgartner, D., Bifi, A.G., Debona, T., Frana, V.A., 2012. Peixes do baixo rio Iguaçu.
- Belenguer, V., Martinez-Capel, F., Masiá, A., Picó, Y., 2014. Patterns of presence and concentration of pesticides in fish and waters of the júcar river (eastern spain). *J. Hazard. Mater.* 265, 271–279. doi:10.1016/j.jhazmat.2013.11.016
- Biegón, A., 2015. Frontiers in Neuroendocrinology In vivo visualization of aromatase in animals and humans. *Front. Neuroendocrinol.* 1–10. doi:10.1016/j.yfrne.2015.10.001
- Blazer, V.S., 2002. Histopathological assessment of gonadal tissue in wild fishes. *Fish Physiol. Biochem.* 26, 85–101. doi:10.1023/A:1023332216713
- Boelsterli, U.A., 2003. Mechanistic Toxicology: the molecular basis of how chemicals disrupt biological targets. London.
- Breton, T.S., DiMaggio, M.A., Sower, S.A., Berlinsky, D.L., 2015. Brain aromatase (cyp19a1b) and gonadotropin releasing hormone (gnrh2 and gnrh3) expression during reproductive development and sex change in black sea bass (*Centropristis striata*). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 181, 45–53. doi:10.1016/j.cbpa.2014.11.020
- Brodie, A., 2002. Aromatase inhibitors in breast cancer. *Trends Endocrinol. Metab.* 13, 61–65.
- Brodie, A., Lu, Q., Long, B., 1999. Aromatase and its inhibitors. *J. Steroid Biochem. Mol. Biol.* 69, 205–210.
- Burki, R., Vermeirssen, E.L.M., Körner, O., Joris, C., Burkhardt-Holm, P., Segner, H., 2006. Assessment of estrogenic exposure in brown trout (*Salmo trutta*) in a Swiss midland river: integrated analysis of passive samplers, wild and caged fish, and vitellogenin mRNA and protein. *Environ. Toxicol. Chem.* 25, 2077–2086.

doi:10.1897/05-545r.1

Burkina, V., Zlabek, V., Zamaratskaia, G., 2013. Clotrimazole, but not dexamethasone, is a potent in vitro inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout. *Chemosphere* 92, 1099–1104. doi:10.1016/j.chemosphere.2013.01.050

Carubelli, G., Fanelli, R., Mariani, G., Nichetti, S., Crosa, G., Calamari, D., Fattore, E., 2007. PCB contamination in farmed and wild sea bass (*Dicentrarchus labrax* L.) from a coastal wetland area in central Italy. *Chemosphere* 68, 1630–5. doi:10.1016/j.chemosphere.2007.04.004

Casabar, R.C.T., Das, P.C., Dekrey, G.K., Gardiner, C.S., Cao, Y., Rose, R.L., Wallace, A.D., 2010. Endosulfan induces CYP2B6 and CYP3A4 by activating the pregnane X receptor. *Toxicol. Appl. Pharmacol.* 245, 335–43. doi:10.1016/j.taap.2010.03.017

Celander, M., Stegeman, J.J., Förlin, L., 1996. CYP1A1-, CYP2B- and CYP3A-like proteins in rainbow trout (*Oncorhynchus mykiss*) liver: CYP1A1-specific down-regulation after prolonged exposure to PCB. *Mar. Environ. Res.* 42, 283–286. doi:10.1016/0141-1136(95)00045-3

Chaube, R., Rawat, A., Joy, K.P., 2015. Molecular cloning and characterization of brain and ovarian cytochrome P450 aromatase genes in the catfish *Heteropneustes fossilis*: Sex, tissue and seasonal variation in, and effects of gonadotropin on gene expression. *Gen. Comp. Endocrinol.* 221, 120–33. doi:10.1016/j.ygcen.2015.06.004

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol.* 155, 31–62. doi:10.1016/j.ygcen.2007.03.005

CONAMA, 2012. Resoluções do Conama: Resoluções vigentes publicadas entre setembro de 1984 e janeiro de 2012, Ministerio do Meio Ambiente. Brasília.

Correia, T.G., Narcizo, A.M., Bianchini, A., Moreira, R.G., 2010. Aluminum as an endocrine disruptor in female Nile tilapia (*Oreochromis niloticus*). *Comp. Biochem. Physiol. Part C* 151, 461–466. doi:10.1016/j.cbpc.2010.02.002

Costa-pierce, B.A., 2003. Rapid evolution of an established feral tilapia (*Oreochromis* spp.): the need to incorporate invasion science into regulatory structures. *Biol. Invasions* 5, 71–84.

Costopoulou, D., Vassiliadou, I., Leondiadis, L., 2016. PCDDs, PCDFs and PCBs in farmed fish produced in Greece: Levels and human population exposure assessment. *Chemosphere* 146, 511–8. doi:10.1016/j.chemosphere.2015.12.019

Da Cuña, R.H., Pandolfi, M., Genovese, G., Piazza, Y., Ansaldo, M., Lo Nostro, F.L., 2013. Endocrine disruptive potential of endosulfan on the reproductive axis of *Cichlasoma dimerus* (Perciformes, Cichlidae). *Aquat. Toxicol.* 126, 299–305. doi:10.1016/j.aquatox.2012.09.015

- Dai, D., Cao, Y., Falls, G., Levi, P.E., Hodgson, E., Rose, R.L., 2001. Modulation of Mouse P450 Isoforms CYP1A2, CYP2B10, CYP2E1, and CYP3A by the Environmental Chemicals Mirex, 2,2-Bis(p-chlorophenyl)-1,1-dichloroethylene, Vinclozolin, and Flutamide. *Pestic. Biochem. Physiol.* 70, 127–141. doi:10.1006/pest.2001.2551
- de Vlaming, V., 2008. Organochlorine Pesticides and Polychlorinated Biphenyls (PCB) Concentrations in Muscle Tissue of Fish Collected from the San Joaquin River and Sacramento River Watersheds and Delta During 2005. Davis.
- Ding, J., Lu, G., Li, Y., 2016. Interactive effects of selected pharmaceutical mixtures on bioaccumulation and biochemical status in crucian carp (*Carassius auratus*). *Chemosphere* 148, 21–31. doi:10.1016/j.chemosphere.2016.01.017
- Dong, H., Li, Z., Man, X., Zhou, J., Lu, H., Wang, S., 2010. Identification of the metabolites of polybrominated diphenyl ether 99 and its related cytochrome P450s. *J. Biomed. Res.* 24, 223–32. doi:10.1016/S1674-8301(10)60032-2
- Dong, M., Zhu, L., Shao, B., Zhu, S., Wang, J., Xie, H., Wang, J., Wang, F., 2013. The effects of endosulfan on cytochrome P450 enzymes and glutathione S-transferases in zebrafish (*Danio rerio*) livers. *Ecotoxicol. Environ. Saf.* 92, 1–9. doi:10.1016/j.ecoenv.2012.10.019
- Dubois, M., Pfohl-Leszkowicz, A., De Waziers, I., Kremers, P., 1996. Selective induction of the CYP3A family by endosulfan and DNA-adduct formation in different hepatic and hepatoma cells. *Environ. Toxicol. Pharmacol.* 1, 249–56. doi:10.1016/1382-6689(96)00018-X
- FAO, F. and A.O. of the U.S., 1983. Species Fact Sheets *Oreochromis niloticus* (Linnaeus, 1758). Fish. Aquac. Dep.
- Fenneteau, F., Poulin, P., Nekka, F., 2010. Physiologically based predictions of the impact of inhibition of intestinal and hepatic metabolism on human pharmacokinetics of CYP3A substrates. *J. Pharm. Sci.* 99, 486–514. doi:10.1002/jps.21802
- Fester, L., Brandt, N., Windhorst, S., Pröls, F., Bläute, C., Rune, G.M., 2015. Control of aromatase in hippocampal neurons. *J. Steroid Biochem. Mol. Biol.* 5–10. doi:10.1016/j.jsbmb.2015.10.009
- Fu, Y., Li, M., Liu, C., Qu, J.-P., Zhu, W.-J., Xing, H.-J., Xu, S.-W., Li, S., 2013. Effect of atrazine and chlorpyrifos exposure on cytochrome P450 contents and enzyme activities in common carp gills. *Ecotoxicol. Environ. Saf.* 94, 28–36. doi:10.1016/j.ecoenv.2013.04.018
- Gähns, M., Roos, R., Andersson, P.L., Schrenk, D., 2013. Role of the nuclear xenobiotic receptors CAR and PXR in induction of cytochromes P450 by non-dioxinlike polychlorinated biphenyls in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* 272, 77–85. doi:10.1016/j.taap.2013.05.034
- Giesy, J.P., Snyder, E.M., Nichols, K.M., Snyder, S. a, Villalobos, S. a, Jones, P.D., Fitzgerald, S.D., 2003. Examination of reproductive endpoints in goldfish (*Carassius*

auratus) exposed in situ to municipal sewage treatment plant effluent discharges in Michigan, USA. Environ. Toxicol. Chem. 22, 2416–2431. doi:10.1897/02-329

Gilannejad, N., Dorafshan, S., Paykan, F., Mahboobi, N., Asadollah, S., Martos-sitcha, J.A., Prat, F., Yúfera, M., Martínez-rodríguez, G., 2016. Vitellogenin expression in wild cyprinid *Petroleuciscus esfahani* as a biomarker of endocrine disruption along the Zayandeh Roud River , Iran 144, 1342–1350. doi:10.1016/j.chemosphere.2015.09.106

Gräns, J., Wassmur, B., Fernández-Santoscoy, M., Zanette, J., Woodin, B.R., Karchner, S.I., Nacci, D.E., Champlin, D., Jayaraman, S., Hahn, M.E., Stegeman, J.J., Celander, M.C., 2015. Regulation of pregnane-X-receptor, CYP3A and P-glycoprotein genes in the PCB-resistant killifish (*Fundulus heteroclitus*) population from New Bedford Harbor. Aquat. Toxicol. 159, 198–207. doi:10.1016/j.aquatox.2014.12.010

Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chem. Res. Toxicol. 21, 70–83. doi:10.1021/tx700079z

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.-F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165, 352–66. doi:10.1016/j.ygcen.2009.03.002

Guo, H., Liu, C., Li, J., Zhang, M., Hu, M., Xu, P., Liu, L., Liu, X., 2013. A mechanistic physiologically based pharmacokinetic-enzyme turnover model involving both intestine and liver to predict CYP3A induction-mediated drug-drug interactions. J. Pharm. Sci. 102, 2819–36. doi:10.1002/jps.23613

Hampl, R., Kubátová, J., Stárka, L., 2014. Steroids and endocrine disruptors-History, recent state of art and open questions. J. Steroid Biochem. Mol. Biol. doi:10.1016/j.jsbmb.2014.04.013

Hao, Q., Sun, Y.-X., Xu, X.-R., Yao, Z.-W., Wang, Y.-S., Zhang, Z.-W., Luo, X.-J., Mai, B.-X., 2014. Occurrence of persistent organic pollutants in marine fish from the Natuna Island, South China Sea. Mar. Pollut. Bull. 85, 274–9. doi:10.1016/j.marpolbul.2014.05.058

Hardell, S., Tilander, H., Welfinger-Smith, G., Burger, J., Carpenter, D.O., 2010. Levels of polychlorinated biphenyls (PCBs) and three organochlorine pesticides in fish from the Aleutian Islands of Alaska. PLoS One 5. doi:10.1371/journal.pone.0012396

Harries, J.E., Sheahan, D. a, Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T., Zaman, N., 2009. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout 16, 534–542. doi:10.1897/1551-5028(1997)016<0534:EAIFUK>2.3.CO;2

Hinfray, N., Porcher, J., Brion, F., 2006. Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp. Biochem. Physiol. Part C 144, 252–262.

doi:10.1016/j.cbpc.2006.09.002

Hiramatsu, N., Cheek, A.O., Sullivan, C., Matsubara, T., Hara, a, 2005. Vitellogenesis and endocrine disruption. *Biochem. Mol. Biol. Fishes* 6.

Hodgson, E., Wallace, A.D., Carolina, N., Carolina, N., 2012. Human Metabolic Interactions of Pesticides : Inhibition , Induction , and Activation. *Am. Chem. Soc.* 116–132. doi:10.1021/bk-2012-1099.ch008

Hu, W.-Y., Shi, G.-B., Hu, D.-P., Nelles, J.L., Prins, G.S., 2012. Actions of estrogens and endocrine disrupting chemicals on human prostate stem/progenitor cells and prostate cancer risk. *Mol. Cell. Endocrinol.* 354, 63–73. doi:10.1016/j.mce.2011.08.032

Huang, Y., Hu, N., Gao, X., Yan, Z., Li, S., Jing, W., Yan, R., 2015. Alterations of testosterone metabolism in microsomes from rats with experimental colitis induced by dextran sulfate sodium. *Chem. Biol. Interact.* 232, 38–48. doi:10.1016/j.cbi.2015.02.013

Hultman, M.T., Thomas, J., Erik, K., 2015. Evaluation of the sensitivity , responsiveness and reproducibility of primary rainbow trout hepatocyte vitellogenin expression as a screening assay for estrogen mimics. *Aquat. Toxicol.* 159, 233–244. doi:10.1016/j.aquatox.2014.12.013

IBGE, I.B. de G. e E., 2010. IBGE | Censo 2010. URL <http://censo2010.ibge.gov.br/> (accessed 9.29.15).

Jasinska, E.J., Goss, G.G., Gillis, P.L., Van Der Kraak, G.J., Matsumoto, J., de Souza Machado, A.A., Giacomini, M., Moon, T.W., Massarsky, A., Gagné, F., Servos, M.R., Wilson, J., Sultana, T., Metcalfe, C.D., 2015. Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge. *Sci. Total Environ.* 530-531, 140–153. doi:10.1016/j.scitotenv.2015.05.080

Kiyama, R., Wada-kiyama, Y., 2015. Estrogenic endocrine disruptors : Molecular mechanisms of action. *Environ. Int.* 83, 11–40. doi:10.1016/j.envint.2015.05.012

Kjeldsen, L.S., Ghisari, M., Bonefeld-Jørgensen, E.C., 2013. Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. *Toxicol. Appl. Pharmacol.* 272, 453–464. doi:10.1016/j.taap.2013.06.028

Koenig, S., Fernández, P., Solé, M., 2012. Differences in cytochrome P450 enzyme activities between fish and crustacea: relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). *Aquat. Toxicol.* 108, 11–7. doi:10.1016/j.aquatox.2011.10.016

Konno, Y., Sekimoto, M., Nemoto, K., Degawa, M., 2004. Sex difference in induction of hepatic CYP2B and CYP3A subfamily enzymes by nicardipine and nifedipine in rats. *Toxicol. Appl. Pharmacol.* 196, 20–8. doi:10.1016/j.taap.2003.12.009

- Kopec, A.K., Burgoon, L.D., Ibrahim-Aibo, D., Mets, B.D., Tashiro, C., Potter, D., Sharratt, B., Harkema, J.R., Zacharewski, T.R., 2010. PCB153-elicited hepatic responses in the immature, ovariectomized C57BL/6 mice: comparative toxicogenomic effects of dioxin and non-dioxin-like ligands. *Toxicol. Appl. Pharmacol.* 243, 359–71. doi:10.1016/j.taap.2009.12.003
- Leanos-Castaneda, O., Kraakb, G. Van Der, Listerb, A., Simá-Alvarez, R., Gold-Bouchot, G., 2002. o,p'-DDT induction of vitellogenesis and its inhibition by tamoxifen in Nile tilapia (*Oreochromis niloticus*). *Mar. Environ. Res.* 54, 703–707.
- Leino, R.L., Jensen, K.M., Ankley, G.T., 2005. Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Pharmacol.* 19, 85–98. doi:10.1016/j.etap.2004.05.010
- Leite, N.F., Peralta-Zamora, P., Grassi, M.T., 2011. Distribution and origin of polycyclic aromatic hydrocarbons in surface sediments from an urban river basin at the Metropolitan region of Curitiba, Brazil. *J. Environ. Sci. (China)* 23, 904–11.
- Lemaire, G., de Sousa, G., Rahmani, R., 2004. A PXR reporter gene assay in a stable cell culture system: CYP3A4 and CYP2B6 induction by pesticides. *Biochem. Pharmacol.* 68, 2347–58. doi:10.1016/j.bcp.2004.07.041
- Lephart, E.D., 1996. A review of brain aromatase cytochrome P450 22, 1–26.
- Li, D., Yang, X.-L., Zhang, S.-J., Lin, M., Yu, W.-J., Hu, K., 2008. Effects of mammalian CYP3A inducers on CYP3A-related enzyme activities in grass carp (*Ctenopharyngodon idellus*): Possible implications for the establishment of a fish CYP3A induction model. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 147, 17–29. doi:10.1016/j.cbpc.2007.07.003
- Li, H., Letcher, R.J., 2002. A high-performance-liquid-chromatography-based method for the determination of hydroxylated testosterone metabolites formed in vitro in liver microsomes from gray seal (*Halichoerus grypus*). *J. Chromatogr. Sci.* 40, 397–402.
- Li, J., Zhang, Y., Du, Z., Peng, J., Mao, L., Gao, S., 2016. Biotransformation of OH-PBDEs by pig liver microsomes: Investigating kinetics, identifying metabolites, and examining the role of different CYP isoforms. *Chemosphere* 148, 354–60. doi:10.1016/j.chemosphere.2016.01.056
- Machala, M., Neča, J., Drábek, P., Ulrich, R., Šabatová, V., Nezveda, K., Raszyk, J., Gajdušková, V., 1998. Effects of chronic exposure to PCBs on cytochrome P450 systems and steroidogenesis in liver and testis of bulls (*Bos taurus*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 120, 65–70. doi:10.1016/S1095-6433(98)10011-9
- Maenpaa, J., Perlkonen, O., Cresteil, T., Rane, A., 1993. The role of cytochrome P450 3A (CYP3A) isoform(s) in oxidative metabolism of testosterone and benzphetamine in human adult and fetal liver. *J. Steroid Biochem. Molec. Biol.* 44, 61–67.

- Mills, L.J., Gutjahr-Gobell, R.E., Zaroogian, G.E., Horowitz, D.B., Laws, S.C., 2014. Modulation of aromatase activity as a mode of action for endocrine disrupting chemicals in a marine fish. *Aquat. Toxicol.* 147, 140–50. doi:10.1016/j.aquatox.2013.12.023
- Ortiz de Montellano, P.R., 2005. *Cytochrome P450 Structure, Mechanism, and Biochemistry* Third edition, third. ed. Kluwer Academic/Plenum Publishers, New York.
- P.B. Danielson, B.S.P., 2002. The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. *Curr. Drug Metab.* 3, 561–597. doi:10.2174/1389200023337054
- Paiano, V., Generoso, C., Mandich, A., Traversi, I., Palmiotto, M., Bagnati, R., Colombo, A., Davoli, E., Fanelli, R., Fattore, E., 2013. Persistent organic pollutants in sea bass (*Dicentrarchus labrax* L.) in two fish farms in the Mediterranean Sea. *Chemosphere* 93, 338–43. doi:10.1016/j.chemosphere.2013.04.086
- Peck, L.E., Gilchrist, H.G., Mallory, C.D., Braune, B.M., Mallory, M.L., 2016. Persistent organic pollutant and mercury concentrations in eggs of ground-nesting marine birds in the Canadian high Arctic. *Sci. Total Environ.* 556, 80–88. doi:10.1016/j.scitotenv.2016.02.205
- Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 143, 303–315. doi:10.1016/j.cbpc.2006.03.004
- Rigét, F., Vorkamp, K., Bossi, R., Sonne, C., Letcher, R.J., Dietz, R., 2015. Twenty years of monitoring of persistent organic pollutants in Greenland biota. A review. *Environ. Pollut.* doi:10.1016/j.envpol.2015.11.006
- Rissato, S.R., Libânio, M., Giafferis, G.P., Gerenutti, M., 2004. Artigo. *Quim. Nova* 27, 739–743.
- Sams, C., Mason, H.J., Rawbone, R., 2000. Evidence for the activation of organophosphate pesticides by cytochromes P450 3A4 and 2D6 in human liver microsomes. *Toxicol. Lett.* 116, 217–221.
- San-Segundo, L., Guimarães, L., Fernández Torija, C., Beltrán, E.M., Guilhermino, L., Pablos, M.V., 2016. Alterations in gene expression levels provide early indicators of chemical stress during *Xenopus laevis* embryo development: A case study with perfluorooctane sulfonate (PFOS). *Ecotoxicol. Environ. Saf.* 127, 51–60. doi:10.1016/j.ecoenv.2016.01.005
- Scholz, S., Mayer, I., 2008. Molecular biomarkers of endocrine disruption in small model fish. *Mol. Cell. Endocrinol.* 293, 57–70. doi:10.1016/j.mce.2008.06.008
- Schug, T.T., Janesick, A., Blumberg, B., Heindel, J.J., 2011. Endocrine disrupting chemicals and disease susceptibility. *J. Steroid Biochem. Mol. Biol.* 127, 204–215. doi:10.1016/j.jsbmb.2011.08.007

- Sergent, T., Dupont, I., Jassogne, C., Ribonnet, L., Heiden, E. Van Der, Scippo, M., Muller, M., Mcalister, D., Pussemier, L., Larondelle, Y., Schneider, Y., 2009. CYP1A1 induction and CYP3A4 inhibition by the fungicide imazalil in the human intestinal Caco-2 cells — Comparison with other conazole pesticides. *Toxicol. Lett.* 184, 159–168. doi:10.1016/j.toxlet.2008.11.009
- Shang, X., Dong, G., Zhang, H., Zhang, L., Yu, X., Li, J., Wang, X., Yue, B., Zhao, Y., Wu, Y., 2016. Polybrominated diphenyl ethers (PBDEs) and indicator polychlorinated biphenyls (PCBs) in various marine fish from Zhoushan fishery, China. *Food Control.* doi:10.1016/j.foodcont.2016.03.008
- Shen, G., Zhuang, X., Xiao, W., Kong, L., Tan, Y., Li, H., 2014. Role of CYP3A in regulating hepatic clearance and hepatotoxicity of triptolide in rat liver microsomes and sandwich-cultured hepatocytes. *Food Chem. Toxicol.* 71, 90–6. doi:10.1016/j.fct.2014.05.020
- Shibasaki, H., Kuroiwa, M., Uchikura, S., Tsuboyama, S., Yokokawa, A., Kume, M., Furuta, T., 2014. Use of endogenous cortisol 6 β -hydroxylation clearance for phenotyping in vivo CYP3A activity in women after sequential administration of an oral contraceptive (OC) containing ethinylestradiol and levonorgestrel as weak CYP3A inhibitors. *Steroids* 87, 137–144. doi:10.1016/j.steroids.2014.05.023
- Suib, S.L., Arslan-Alaton, I., Olmez-Hanci, T., 2013. New and Future Developments in Catalysis, New and Future Developments in Catalysis. Elsevier. doi:10.1016/B978-0-444-53870-3.00012-5
- Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* 103, 173–178. doi:10.1289/ehp.95103s7173
- Tian, H., Wu, P., Wang, W., Ru, S., 2015. Disruptions in aromatase expression in the brain , reproductive behavior , and secondary sexual characteristics in male guppies (*Poecilia reticulata*) induced by tributyltin. *Aquat. Toxicol.* 162, 117–125. doi:10.1016/j.aquatox.2015.03.015
- Tournier, M., Pouech, C., Quignot, N., Lafay, F., Wiest, L., Lemazurier, E., Cren-olivé, C., Vulliet, E., 2015. Determination of endocrine disruptors and endogenic androgens and estrogens in rat serum by high-performance liquid chromatography – tandem mass spectrometry. *Steroids*. doi:10.1016/j.steroids.2015.10.011
- Trocino, A., Xiccato, G., Majolini, D., Tazzoli, M., Tulli, F., Tibaldi, E., Messina, C.M., Santulli, A., 2012. Levels of dioxin-like polychlorinated biphenyls (DL-PCBs) and metals in European sea bass from fish farms in Italy. *Food Chem.* 134, 333–338. doi:10.1016/j.foodchem.2012.02.153
- Tyler, C.R., Filby, A.L., Aerle, R. Van, Lange, A., Ball, J., Santos, E.M., 2008. Fish toxicogenomics. *Adv. Exp. Biol.* 02. doi:10.1016/S1872-2423(08)00003-3
- van Waterschoot, R. a B., Schinkel, A.H., 2011. A critical analysis of the interplay between cytochrome P450 3A and P-glycoprotein: recent insights from knockout and

transgenic mice. *Pharmacol. Rev.* 63, 390–410. doi:10.1124/pr.110.002584.

Wagner, C., Pan, Y., Hsu, V., Sinha, V., Zhao, P., 2015. Predicting the Effect of CYP3A Inducers on the Pharmacokinetics of Substrate Drugs Using Physiologically Based Pharmacokinetic (PBPK) Modeling: An Analysis of PBPK Submissions to the US FDA. *Clin. Pharmacokinet.* doi:10.1007/s40262-015-0330-y

Wang, J., Shan, R., Zhang, X., Tian, H., Wang, W., Ru, S., 2015. Development of a lipovitellin-based sandwich ELISA for quantification of vitellogenin in surface mucus and plasma of goldfish (*Carassius auratus*). *Ecotoxicol. Environ. Saf.* 120, 80–87. doi:10.1016/j.ecoenv.2015.05.010

Wilkinson, G.R., 1996. Cytochrome P4503A (CYP3A) metabolism: prediction of in vivo activity in humans. *J. Pharmacokinet. Biopharm.* 24, 475–90.

Yamamoto, F.Y., Pereira, M.V.M., Lottermann, E., Santos, G.S., Stremel, T.R.O., Doria, H.B., Gusso-Choueri, P., Campos, S.X., Ortolani-Machado, C.F., Cestari, M.M., Neto, F.F., Azevedo, J.C.R., Ribeiro, C.A.O., 2016. Bioavailability of pollutants sets risk of exposure to biota and human population in reservoirs from Iguaçu River (Southern Brazil). *Environ. Sci. Pollut. Res.* 1–18. doi:10.1007/s11356-016-6924-6

Zhou, T., Cong, S., Sun, S., Sun, H., Zou, R., Wang, S., Wang, C., Jiao, J., Goto, K., Nawata, H., Yanase, T., Zhao, Y., 2013. Identification of endocrine disrupting chemicals activating SXR-mediated transactivation of CYP3A and CYP7A1. *Mol. Cell. Endocrinol.* 365, 36–43. doi:10.1016/j.mce.2012.09.001

DISCUSSÃO FINAL

Os resultados obtidos demonstraram o impacto da atividade humana nos cinco reservatórios do Rio Iguaçu através da detecção de químicos capazes de interferir na saúde dos peixes expostos. A hipótese inicial do gradiente de poluição ao longo do percurso do rio foi refutada, já que diferentes químicos foram encontrados em todos os reservatórios em concentrações independentes da distância da Região Metropolitana de Curitiba. Além disso, as análises das respostas biológicas nos peixes confirmaram esta condição, pois também não apresentaram um padrão de acordo com os diferentes reservatórios. É necessário enfatizar que este gradiente não foi observado quando considerado o intervalo da região do baixo Iguaçu (entre os cinco reservatórios), porém o mesmo não pode ser confirmado se avaliado todo o percurso do rio, desde sua origem. Neste caso, o impacto antrópico na qualidade da água na origem do rio é certamente superior em relação aos reservatórios.

1- Químicos e as fontes de poluição ao longo dos cinco reservatórios:

A Região Metropolitana de Curitiba (RMC) foi confirmada como a principal fonte de poluentes para o Rio Iguaçu, influenciando na qualidade da água dos reservatórios mais próximos da capital, principalmente o Foz do Areia. Como já relatado anteriormente, a elevada ocorrência de cianobactérias relacionadas a concentração de matéria orgânica e outros parâmetros avaliados, demonstram a interferência de efluentes domésticos oriundos da RMC. Mesmo em outros reservatórios mais distantes (SS) ainda foi possível detectar a presença da Microcistina intracelular na água, indicando a influência destas descargas urbanas e possivelmente da atividade agrícola, devido ao uso de fertilizantes. Próximo ao reservatório de Foz do Areia também são encontradas outras cidades, como União da Vitória, e atividades antrópicas como prováveis fontes de poluentes, incluindo madeiras, vinícolas e pequenas plantações de cana de açúcar. Vários compostos podem estar sendo introduzidos nos reservatórios devido a estas atividades (TABELA 2 e 3).

Considerando o tipo de relevo mais plano da região do terceiro planalto Paranaense, a partir do reservatório de Segredo as condições para o

desenvolvimento da atividade agropecuária é mais favorável. Desta forma, as atividades agrícola e pecuária na região foram confirmadas como fontes difusas de poluentes que estão interferindo na qualidade da água, principalmente dos reservatórios SS, SO e SC. Com a maioria dos Poluentes Orgânicos Persistentes banidos há mais de 10 anos, na Convenção de Estocolmo, a detecção destes poluentes nos últimos quatro reservatórios (SE, SS, SO e SC) demonstra a persistência assim como as condições favoráveis de acumulação destes compostos nos reservatórios. Mesmo em concentrações relativamente baixas, deve-se considerar o grande volume de água dos cinco reservatórios, sendo ambientalmente relevante a detecção destes compostos tanto na água como nos peixes que permaneceram por um curto período de exposição. Por outro lado, apesar deste estudo ter sido o primeiro a quantificar estes compostos nestes reservatórios, é possível inferir que as concentrações destes poluentes há algumas décadas era muito superior e com a ação das autoridades públicas em proibir o seu uso, houve uma contribuição significativa para a redução destes químicos nos ambientes aquáticos (CLARKE et al., 2010; MAHUGIJA et al., 2015).

De fato, os principais compostos encontrados em concentrações mais relevantes foram os metais. Os metais são elementos ubíquos que podem ocorrer naturalmente nos ecossistemas, ou seja, sua ocorrência pode ser explicada por processos como a erosão natural (TABELA 2). Porém, a atividade humana pode aumentar potencialmente a concentração destes elementos no ambiente. No caso do Rio Iguaçu, prováveis fontes incluem o uso na composição de fertilizantes e pesticidas, preservação de madeiras e outras pequenas indústrias localizadas próximas ao curso do rio, principalmente relacionadas à agricultura, ou mesmo provenientes da RMC e outras cidades. Diversos estudos em campo quantificam a ocorrência de metais nos ambientes aquáticos (SANTOS et al., 2016; VOIGT et al., 2014) devido ao potencial tóxico que podem apresentar, especialmente os metais não essenciais (Cd e Cr). Entre os diversos mecanismos desencadeados, um dos mais bem conhecidos e relatados é o estresse oxidativo celular, onde diferentes órgãos são afetados (ERCAL et al., 2001; LUSHCHAK, 2011).

Os hidrocarbonetos policíclicos aromáticos, da mesma forma, são ubíquos, ocorrendo normalmente nos ambientes e também apresentam elevado potencial tóxico, com a toxicidade geralmente relacionada ao número de anéis aromáticos (Haritash & Kaushik, 2009; WHO, 2000). Entre os mais tóxicos pode-se citar o

Benzo-a-pireno, com 5 anéis aromáticos, considerado carcinogênico e mutagênico mediado pela atividade das CYPs, onde seu metabólito liga-se ao DNA (JARVIS et al., 2014). Prováveis fontes destes compostos podem estar relacionadas à grande indústria de refinaria de óleo (REPAR) localizada na RMC, assim como à atividade madeireira mais próxima ao reservatório Foz do Areia. Outras fontes não identificadas, além da atividade agropecuária, podem estar influenciando os outros reservatórios mais distantes.

As análises químicas realizadas tanto nos componentes bióticos e abióticos foram essenciais para demonstrar a presença e disponibilidade química nos reservatórios do Rio Iguaçu. De maneira geral, a concentração dos químicos foi maior nos tecidos em relação à água, com exceção dos pesticidas organoclorados (aldrins e Endosulfan), provavelmente devido à metabolização destes ou menor tendência à bioacumulação. Apesar de alguns compostos não terem sido detectados na água, a presença destes nos tecidos confirma a biodisponibilidade e a elevada diluição devido ao grande volume de água dos reservatórios. Desta forma, outros químicos, considerados como poluentes emergentes, incluindo hormônios estrogênicos, produtos farmacêuticos e industriais, detectados no Rio Iguaçu próximo à RMC, poderiam estar presentes nos reservatórios mesmo não tendo sido encontrados na água dos reservatórios (IDE et al., 2016).

2- Detecção de desreguladores endócrinos

Grande parte dos compostos detectados nos reservatórios são potencialmente capazes de exercer atividade endócrina nos organismos expostos, de acordo com a literatura (TABELA 2 e 3). Os efeitos endócrinos dos metais, pouco investigados, geralmente não estão associados à interação direta aos receptores estrogênicos, mas sim a uma alteração nos níveis hormonais. Por outro lado, os compostos aromáticos, principalmente os de maior hidrofobicidade, como os PCBs, podem apresentar maior semelhança estrutural aos hormônios esteroides e, portanto, maior capacidade de interação com seus receptores.

Porém, é importante ressaltar que estes compostos não agem isoladamente e sim em misturas complexas, o que dificulta a previsão e compreensão dos efeitos potenciais. Além do mais, a questão das concentrações em que estes químicos exercem suas atividades são controversas entre os estudos. Alguns estudos relatam

efeitos dose-dependente, outros não-monotônicos, também não há um consenso em relação aos efeitos adversos desencadeados após exposição a baixas doses (Wade et al., 2002), pois mesmo havendo um efeito este não necessariamente será deletério.

Como já esperado, as alterações reprodutivas mais significativas foram encontradas nos peixes nativos expostos cronicamente em relação às tilápias expostas por 60 dias em situação de biomonitoramento ativo. Os peixes nativos demonstraram maior potencial efeito adverso à exposição aos EDCs, como o atraso na maturação das gônadas dos peixes adultos.

3- Biomarcadores:

Dentre as diversas respostas biológicas avaliadas nos peixes foi possível concluir que os melhores biomarcadores para este estudo foram:

a) Para os peixes nativos do Rio Iguaçu expostos cronicamente:

- Atividade da Acetilcolinesterase, principalmente no músculo;
- Análise de danos genotóxicos, principalmente o ensaio Cometa nas células sanguíneas;
- Expressão da vtg associada à morfologia das gônadas em fêmeas; e
- Índice gonadossomático nos machos.

b) Tilápias expostas subcronicamente:

- Expressão gênica *cyp1a* e *3a*, e atividade da CYP3A correlacionadas aos níveis de hormônios esteroides reprodutivos; e
- Atividade da aromatase (CYP19A) no cérebro associada ao índice gonadossomático e níveis de hormônios esteroides reprodutivos.

De certa forma estes biomarcadores estão de acordo com os propostos por Van der Oost et al. (2003), que sugere como os mais significativos para uma avaliação de risco de exposição: enzimas de fase I (como a CYP1A), produtos de

biotransformação (metabólitos de PAHs na bile), parâmetros reprodutivos (vitelogenina) e genotóxicos (adutos de DNA hepáticos).

Os melhores biomarcadores utilizados para avaliação de exposição crônica dos peixes nativos demonstraram um padrão de resposta até mesmo entre as diferentes espécies de peixes para alguns casos. Estes biomarcadores mostraram diferenças significativas entre os grupos, apontando o maior comprometimento na saúde dos peixes principalmente dos reservatórios Foz do Areia e Salto Caxias. São, então, biomarcadores apropriados para demonstrar efeitos decorrentes da exposição crônica, representando efeitos intermediários entre respostas sensíveis e efeitos adversos tardios. Os biomarcadores bioquímicos, por sua vez, não foram ideais para avaliação dos efeitos crônicos na saúde dos peixes pois demonstraram menor sensibilidade e inespecificidade nas respostas entre os grupos.

Por outro lado, a avaliação subcrônica das tilápias apontou a CYP3A como um biomarcador sensível e adequado para a detecção de respostas precoces frente a exposição de químicos hidrofóbicos. Apesar de esta análise ter indicado um resultado (comprometimento dos peixes do reservatório SS) distinto do observado para os peixes nativos, é importante considerar: i) Diferentes formas de exposição entre as duas situações; ii) Diferentes espécies de peixes analisadas; iii) Épocas de coletas diferentes, havendo um ano de diferença entre as coletas, uma vez que os fatores ambientais podem influenciar na biodisponibilidade dos químicos.

A correlação entre a CYP3A com os níveis hormonais destes, por sua vez, com o índice gonadosômico, demonstra que a modulação na atividade desta enzima pode levar a alterações nos níveis hormonais e, conseqüentemente, no desenvolvimento gonadal, o que faz da CYP3A um bom biomarcador precoce de desregulação endócrina em peixes.

Em relação à atividade da aromatase no cérebro, ela demonstrou um papel importante no desenvolvimento das gônadas, devido à sua correlação ao índice gonadosômico. Possivelmente a ausência de efeitos significativos para as análises morfológicas e desenvolvimento das gônadas, assim como níveis hormonais, se deve ao curto tempo de exposição das tilápias nos reservatórios do Rio Iguaçu.

4- Espécies de peixes diferentes:

A escolha de distintas espécies de peixes foi crucial para verificar diferentes sensibilidades e formas de respostas de acordo com os seus comportamentos e hábitos alimentares. A espécie *Astyanax bifasciatus* foi a mais abundante no Rio Iguaçu, provavelmente devido à maior plasticidade do seu hábito alimentar, estratégias reprodutivas (reprodução durante o ano todo), e de certa forma à maior resistência à exposição dos químicos presentes. Por outro lado, a espécie *Crenicichla iguassuensis* parece apresentar maior sensibilidade, sendo mais sensível em relação ao lambari, possivelmente também devido ao seu hábito alimentar, por ser uma espécie topo de cadeia alimentar, onde pode biomagnificar os químicos lipofílicos. Por fim, o *Geophagus brasiliensis* que demonstrou certa sensibilidade, não apresentando uma distribuição uniforme ao longo do Rio Iguaçu, mesmo sendo uma espécie abundante e resistente em outros ecossistemas aquáticos brasileiros (Osório et al., 2014; Voigt et al., 2014). Esta distribuição pode estar relacionada tanto devido à construção das barragens, dividindo as populações de peixes, assim como às distintas formas de adaptabilidade aos ambientes. O mesmo pode ser dito para a espécie *Crenicichla iguassuensis*.

As tilápias por sua vez, apresentaram respostas distintas dos peixes nativos, mesmo sendo filogeneticamente próxima ao cará e a joaninha, pertencendo à mesma família dos teleósteos. A resistência das tilápias frente às diferentes condições ambientais favorece seu uso em experimentos em tanques redes, sendo útil para avaliações de risco de exposição no consumo desta espécie amplamente cultivada na aquicultura. Entretanto, suas respostas não devem ser extrapoladas para os efeitos em outras espécies de peixes endêmicas, que apresentam maior sensibilidade.

5 – Fatores relevantes a serem considerados

Além dos fatores já mencionados, que dificultam a interpretação dos dados obtidos em campo, como a complexidade das misturas químicas, a influência dos fatores físico-químicos e diferenças nas respostas biológicas entre as espécies, pode-se mencionar outras questões como:

- A variabilidade nas respostas entre os indivíduos de um mesmo grupo. Embora os peixes nativos possam apresentar maior variabilidade devido às diferenças de idade entre eles, as tilápias obtidas de um mesmo lote, com idades próximas, também demonstraram esta variação. Entre os biomarcadores que apresentaram maior desvio padrão em um mesmo grupo, inclui-se a expressão da vitelogenina e os níveis hormonais no plasma, o que pode explicar a falta ou pouca diferença significativa nos níveis destas respostas entre os grupos.
- No período de Junho de 2014, quatro meses antes da exposição dos peixes, houve uma das maiores cheias já registradas nos reservatórios do Rio Iguaçu, onde um dos tanques-rede já montado no reservatório SO, foi perdido devido ao elevado fluxo de água, sendo posteriormente recolocado novamente. Esta cheia, que chegou a invadir cidades, como União da Vitória, provavelmente influenciou na disponibilidade dos poluentes presentes nos reservatórios e consequentemente nas respostas dos peixes.

Considerações finais

- As análises químicas e diferentes respostas nos peixes demonstraram que os cinco reservatórios encontram-se diferentemente impactados pela ação das atividades humanas. Os reservatórios mais próximos da Região metropolitana de Curitiba são mais influenciados pela liberação de compostos provenientes de efluentes domésticos e atividades industriais, enquanto os mais distantes são mais influenciados pela atividade agrícola.
- Os biomarcadores ideais para avaliação dos riscos de exposição dependem da situação de exposição, diferindo entre as exposições crônicas e subcrônicas neste estudo.
- Como as condições dos reservatórios variaram de acordo com a época das coletas realizadas, são necessários estudos de biomonitoramento adicionais que acompanhem as mudanças e a influência das ações antrópicas nestes ambientes ao longo do tempo.
- Um maior foco ainda deve ser direcionado à redução dos riscos de exposição através do uso da água. Ações das autoridades governamentais já provaram serem efetivas com a proibição do uso de compostos tóxicos, como os PCBs, TBT e outros POPs, onde houve uma redução nas concentrações ambientais destes químicos.

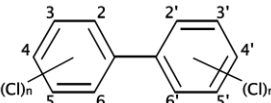
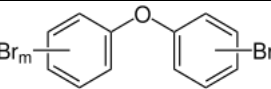
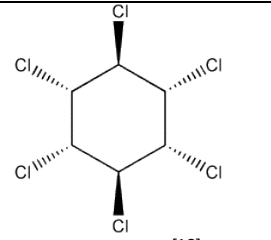
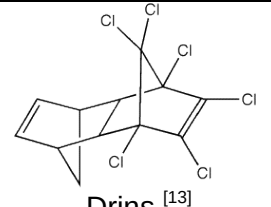
- Mesmo com avanços significativos na compreensão dos efeitos desreguladores endócrinos para os organismos, várias questões ainda precisam ser esclarecidas para proteção das populações humanas e dos animais.

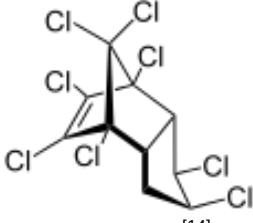
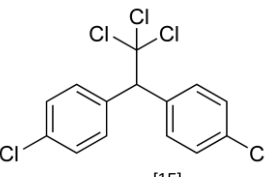
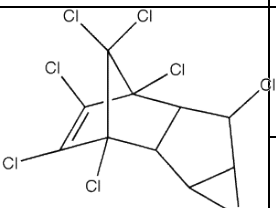
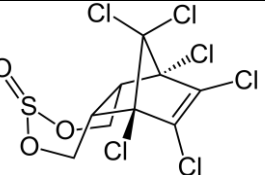
TABELA 2. Relação dos metais detectados nos cinco reservatórios do Rio Iguaçu, com o valor máximo detectado e as suas características (uso, fontes, efeitos em diferentes organismos, desregulador endócrino e mecanismos de ação) de acordo com a literatura.

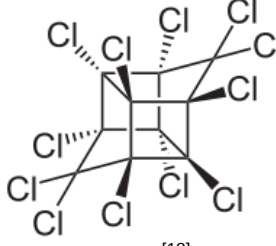
		Reservatórios	Uso, fontes	Efeitos	Desregulador Endócrino	Mecanismos de ação
METAIS	Prata (Ag) [1]	Água: <LQ	Agentes antissépticos, jóias, equipamentos elétricos	Argiria	-	Possível Mutagênico
		Sedim.: Todos (SE = 0,24)				
		Músculo: -				
	Cádmio (Cd) [2]	Água: Todos (SC 4,03)	Baterias, tintas, plásticos, refinarias de metais, fertilizantes	-Carcinógeno (pulmão, ATSDR) -Danos nos rins -Teratogênico -Efeitos nas próstatas	Possível Atividade estrogênica -Hipófise anterior; ciclo circadiano - ↓Cortisol	-↑EROs, SOD e CAT, LPO -Indução de metationeina; apoptose -Interação mecanismo de reparo de DNA - Interação com Zn e Ca ²⁺ -Inibição da síntese de cortiscosteróides via ACTH (StAR)
		Sedim.: Todos (SO 0,048)				
		Músculo: Todos (SC 12,7)				
	Cobalto (Co) [3] Essencial	Água: <LQ	Erosões naturais; Mineiração, queima de combustíveis fósseis, fertilizantes, indústrias	-Efeitos respiratórios, cardíacos, sistema nervoso central	Poucos estudos, -Atrofia testicular, -↓motilidade espermática	-Sinergismo com Zn - ↑TSH, deiodinase e EROD (sapos)
		Sedim.: Todos (SE =6,78)				
		Músculo: FA, SS, SO, SE (1,85)				
	Crômio (Cr) [4] Essencial	Água: FA (75,7), SS, SO	Preservação de madeiras, tintas, metalúrgicas, tratamento do couro	Sistema imunológico, renal, respiratório, hepático Dermatite Cr (IV) Carcinógeno (ATSDR)	Possível Atividade estrogênica -Dano no sistema reprodutivo em machos	-Agente oxidante, - Atravessa membranas biológicas (CrVI) - Liga a biomoléculas e causa LPO, PCO e dano no DNA (mutagênico, adutos e quebra do DNA) - Inibição de enzimas glutationas-dependentes
		Sedim.: FA, SS, SO, SC (1,6)				
		Músculo: Todos (SE =13,1)				
	Cobre (Cu) [5] Essencial	Água: <LQ	Erosão natural; Fungicidas, fertilizantes, Ligas metálicas, Sistemas de encanamento, preservação madeiras	Efeitos respiratórios, anormalidades hepáticas (esteatose), neurológicas, hematológicas, Disfunção nas brânquias	Possível Atividade estrogênica	- Indução da atividade da SOD e GST - Indução ROS, LPO e PCO -Danos oxidativos -Ativação da apoptose -Desregulação da osmolaridade
		Sedim.: Todos SO (76,3)				
		Músculo: Todos (SE= 3,71)				

		Reservatórios	Uso, fontes	Efeitos	Desregulador Endócrino	Mecanismos de ação
METAIS	Manganês Mn ^[6] Essencial	Água: Todos (FA=111,6)	Ligas de aço, Fertilizantes, Tintas, Cosméticos, Baterias, Gasolina	Neurotóxico (comportamento), Efeitos respiratórios e reprodutivos (danos esperma)	Poucos estudos - ↑Prolactina e cortisol	- Degradação de neurônios dopaminérgicos - Oxidação de catecolaminas (Indução de EROS) - ↑ MnSOD - ↓ AChE
		Sedim.: Todos SE (330,7)				
		Músculo: Todos (SO = 21,6)				
	Níquel (Ni) ^[7]	Água: SE, SC (6,5)	Aço inoxidável, baterias, moedas, catalizadores, galvanoplastia	- Indução de tumor - Reação alérgica (dermatite de contato) - Risco carcinogênico (pulmão)	Possível Atividade estrogênica - ↓esperma - T ₃ , T ₄	- Genotoxicidade (Cross-link proteínas-DNA, deleções, inibição do sistema de reparo do DNA) - Afeta células-T e células natural killer
		Sedim.: Todos SC (1,37)				
		Músculo: <LQ				
	Chumbo (Pb) ^[8]	Água: <LQ	Erosão natural; Sistemas de encanamento, Mineiração, solda, baterias, tintas,	Provável carcinogênico Neurotóxico Danos renais, hepáticos, cardio-vascular	Possível Atividade estrogênica - ↓esperma - T ₄ , TSH,	- - Depleção de óxido nítrico e GSH - ↑GST - Atividade ↓ALAS, ↑ALAD, ↓CYPs
		Sedim.: SO (51,0)				
		Músculo: Todos (SO=87,9)				
	Zinco (Zn) ^[9] Essencial	Água: Todos (SE=106,6)	Fertilizantes, herbicidas, pigmentos, baterias, preservação de madeiras, Catalizadores,	Sintomas gastrointestinais	Possível Atividade estrogênica	- Expressão de vtg em sapos machos - ↓atividade antioxidante - ↓colesterol LDL, metaloenzimas de Cu GPx -
		Sedim.: SO (304,4)				
		Músculo: Todos (SE = 84,77)				
		Sedim.: Todos (SC = 2586,1)				
		Músculo: -				

TABELA 3. Relação dos Poluentes Orgânicos Persistentes detectados nos quatro reservatórios do Rio Iguaçu

	Químicos	Deteção nos Reservatórios	Uso, fontes	Efeitos	Desregulador Endócrino	Mecanismos
POLUENTES ORGÂNICOS PERSISTENTES BANIDOS	 PCBs [10]	Água SS (14,7)	Sintético Aplicações industriais (plastificantes, isolantes), extensor de pesticida, banidos em 1977	Provável carcinógeno (Próstata) Hepatotóxico Toxicidade reprodutiva, Neurotoxicidade no desenvolvimento embrionário	-Provável Estrogênico -Fracos agonistas REs	-Induz expressão de vitelogenina -Agonistas receptor AhR (PCBs coplanares), PXR e CAR -Induz expressão CYPs (1A, 2, 3A)
		Tecido Todos 4 (SO: 8,43)				
	 PBDEs [11]	Água < LD	Retardantes de chamas em produtos têxteis, eletrônicos e domésticos	Neurotoxicidade e toxicidade reprodutiva no desenvolvimento embrionário	-Provável Estrogênico -Ativação receptor estrogênico e alteração dos níveis de estradiol	-Indução da expressão de CYP 1A, 2B, 3A -Inibe sinalização via AhR -Alteração dos níveis de Tiroxina
		Tecido SS, SC, SO (3,54)				
	 HCHs [12]	Água SS (1,51)	Pesticida banido em 2009* Tratamento de piolhos e sarna (γ-HCH)	Possível carcinogênico Efeitos hepáticos (tumor), renais, neurológicos e reprodutivos (degeneração de túbulos seminíferos e desregulação espermatogênese)	-Possível (anit)estrogênico - Associação com níveis de T ₄ e TSH (HCH-β) -↓Testosterona - Modulação aromatase	-Interage com receptores Gaba, interferindo na ação do neurotransmissor GABA - ↑CYPs - Ligação ao DNA (não-mutagênico) - Interação com membranas celulares -↓GSH, SOD e CAT, ↑LPO e O ₂ ⁻
		Tecido < LD				
	 Drins [13]	Água Todos 4 (SO: 6,05)	Inseticida banido 1974, usado para milho e coton	Danos no fígado (tumor) Efeitos adversos no sistema nervoso (convulsões e tremores), rins, reprodutivos, imunológicos	Possível fraco estrogênico (Dieldrin) - Indução aromatase (Aldrin)	- Antagonista de receptor GABA - ↑síntese de DNA (fase S), proliferação celular -↑dano oxidativo (MDA, OH ⁸ dG), -↓α-tocoferol no fígado
		Tecido SO (1,27)				

	Químicos	Reservatórios	Uso, fontes	Efeitos	Desregulador Endócrino	Mecanismos
POLUENTES ORGANICO PERSISTENTES BANIDOS	 Clordanas ^[14]	Água < LD Tecido SS, SO (2,65)	Pesticida sintético (termitecida) banido em 1988 Controle de formigas em transformadores	Possível carcinogênico Hepatotoxicidade, neurotoxicidade, toxicidade no desenvolvimento Efeito gastrointestinal e reprodutivos (↓fertilidade)	- Indução aromatase - Alteração no metabolismo de esteróides	- Ligação irreversível a macromoléculas - ↑LPO e O ₂ ^{•-} - ↑AChE - Inibição comunicações GAP - Alteração epigenética - Inibição ATPase, estimula PkC
	 DDTs ^[15]	Água SS (0,69) Tecido	Sintético Inseticida banido 1972 Controle da Malária (África)	Provável carcinógeno (EPA) Efeitos no sistema nervoso, reprodutivo, imune e endócrino Tumores no fígado, pulmão, adrenais	Possível - Estrogênico, interação RES(α,β) - Desregulação T ₃ , T ₄	- Ativação do receptor CAR - ↑níveis CYP2B, 3A2 - Inibição comunicações GAP - Danos oxidativos no DNA (8-OHdG), e LPO, ↑EROs - Focos eosinófilos hepáticos - Inibição do transporte de Ca ²⁺
	 Heptacloro ^[16]	Água SE, SO, SC (3,53) Tecido Todos 4 (SE 8,4)	Pesticida sintético, inseticida banido em 1974 usado para milho, pequenos grãos Controle de formigas	Provável carcinogênico Danos no fígado Efeitos reprodutivos, hepáticos desenvolvimento e sistema nervoso	Possível - ↓Testosterona, - Modulação estradiol Progesterona, - ↑LH, Cortisol, - Sem interação RES - Desregulação T ₄ - Inibição aromatase	- Inibição comunicações GAP - Não-genotóxico, carcinogênico □p53 - Mitogênico (Estimula PkC MAPKs) - apoptose, afeta Bcl-2 e citocromo c - Induz entrada de Ca ²⁺ intracelular
	 Endosulfan ^[17]	Água SS, SO, SC (6,13) Tecido SE, SO, SS (1,96)	Pesticida (cafe), inseticida para vegetais e frutas, uso cancelado em 2010 (uso restrito)	Neurotoxicidade no desenvolvimento embrionário Espermatozóides anormais, diminuição de motilidade e quantidade de esperma	-Estrogênico, Interação REα -Desregulação T ₃ - Indução aromatase - ↓Testosterona	-Induz níveis e atividade de CYPs 2B e 3A4 dose e PXR-dependente -Indução de CYP 3A associada a formação de adutos de DNA em hepatócitos -↑metabolismo e excreção de testosterona -Induz estresse oxidativo, alterando atividade de enzimas antioxidantes -Inibição canais GABA

 Mirex ^[18]	Água < LD	Inseticida sintético banido em 1978, usado no controle de formigas e como retardante de chama	-Efeitos hepáticos (aumento do fígado) -Ovulação e espermatogênese reduzida em ratos	-Provável antiandrogênico - ↑corticosterona, hipertrofia adrenal	-Induz CYPs (1A, 2, 3A) e metabolismo da Testosterona - ↓GSH, glicogênio, -↑lipídeos, vacuolização - Desregulação secreção biliar
	Tecido SC (0,61)				

Referências tabelas:

- 1 - (WHO, 1996)
- 2 - (Satarug et al., 2009; Who, 2011)(Jiménez-Ortega et al., 2012) (Sandhu and Vijayan, 2011)(Rani et al., 2014)(Kiyama and Wada-kiyama, 2015) (Kumar and Singh, 2010)
- 3 - (Bresson et al., 2013; Falfushynska et al., 2016; US EPA, OAR, 2000)
- 4 - (ATSDR, 2013; Mattagajasingh et al., 2008; Nickens et al., 2010)
- 5 - (ATSDR, 2015; Denier et al., 2009; Lushchak, 2011; Luzio et al., 2013; Wei and Yang, 2015)
- 6 - (ATSDR, 2012)
- 7 - (EPA, 2000; WHO, 2005)
- 8 - (ATSDR, 2007; WHO, 2010)
- 9 - (Denier et al., 2009) (EPA, 2010)
- 10 - (Gräns et al., 2015; World Health Organization, 2000; Yaglova and Yaglov, 2016)
- 11 - (Dong et al., 2010; Wahl et al., 2010)
- 12-(ATSDR, 2005; EPA, 1998)
- 13- (EPA, 2003)
- 14-(ATSDR, 1994; Koshlukova and Reed, 2014)
- 15-(ATSDR, 2002; Harada et al., 2016)
- 16- (Hansen et al., 2006; Okoumassoun et al., 2003; WHO, 2006)
- 17-(Casabar et al., 2010; Dong et al., 2013; Stanley et al., 2009)
- 18- (ATSDR, 1995; Dai et al., 2001)

Referencias para mais de uma citação:

Chakraborty et al., 2011; Freire et al., 2013; Laville et al., 2006

REFERÊNCIAS

Amiard-Triquet, C., Amiard, J.-C., Mouneyrac, C., 2015. *Aquatic Ecotoxicology: Advancing Tools for Dealing with Emerging Risks*. Elsevier Science, London.

ANA, A.N. de águas, 2004. Indicadores de Qualidade- Índice de qualidade das águas (IQA). URL <http://portalpnqa.ana.gov.br/indicadores-indice-aguas.aspx> (accessed 3.22.16).

Ankley, G.T., Bencic, D.C., Breen, M.S., Collette, T.W., Conolly, R.B., Denslow, N.D., Edwards, S.W., Ekman, D.R., Garcia-Reyero, N., Jensen, K.M., Lazorchak, J.M., Martinović, D., Miller, D.H., Perkins, E.J., Orlando, E.F., Villeneuve, D.L., Wang, R.L., Watanabe, K.H., 2009. Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action. *Aquat. Toxicol.* 92, 168–178. doi:10.1016/j.aquatox.2009.01.013

Araldi, R.P., de Melo, T.C., Mendes, T.B., de Sá Júnior, P.L., Nozima, B.H.N., Ito, E.T., de Carvalho, R.F., de Souza, E.B., de Cassia Stocco, R., 2015. Using the comet and micronucleus assays for genotoxicity studies: A review. *Biomed. Pharmacother.* 72, 74–82. doi:10.1016/j.biopha.2015.04.004

Arukwe, A., Goksøyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp. Hepatol.* 2, 4. doi:10.1186/1476-5926-2-4

ATSDR, 2013. Chromium (Cr) Toxicity: What Are the Physiologic Effects of Chromium Exposure? | ATSDR - Environmental Medicine & Environmental Health Education - CSEM. URL <http://www.atsdr.cdc.gov/csem/csem.asp?csem=10&po=10> (accessed 3.24.16).

ATSDR, 2005. Toxicological profile for alpha-, beta-, gamma-, and Delta-Hexachlorocyclohexane 377.

ATSDR, 1994. Toxicological Profile for Chlordane.

ATSDR, A. for T.S. and D.R., 2015. ATSDR - Toxicological Profile: Copper. URL <http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=206&tid=37> (accessed 3.24.16).

ATSDR, A. for T.S. and D.R., 2012. Toxicological Profile for Manganese, Public Health. Georgia.

ATSDR, A. for T.S. and D.R., 2008. ATSDR - Chemical Classes Home Page. URL <http://www.atsdr.cdc.gov/substances/ToxChemicalClasses.asp> (accessed 3.21.16).

ATSDR, A. for T.S. and D.R., 2007. ATSDR - Toxicological Profile. URL <http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=96&tid=22> (accessed 3.25.16).

ATSDR, A. for T.S. and D.R., 2002. ATSDR - Public Health Statement: DDT, DDE, DDD. URL <http://www.atsdr.cdc.gov/phs/phs.asp?id=79&tid=20> (accessed 3.25.16).

ATSDR, A. for T.S. and D.R., 1995. Toxicological profile for Mirex and Chlordane,

Toxicol. Ind. Health. doi:10.1177/074823379501100601

Backhaus, T., Blanck, H., 2010. Hazard and Risk Assessment of Chemical Mixtures under REACH State of the Art, Gaps and Options for Improvement. Malin Carlgren, Gothenburg.

Bars, R., Fegert, I., Gross, M., Lewis, D., Weltje, L., Weyers, A., Wheeler, J.R., Galay-Burgos, M., 2012. Risk assessment of endocrine active chemicals: Identifying chemicals of regulatory concern. *Regul. Toxicol. Pharmacol.* 64, 143–154. doi:10.1016/j.yrtph.2012.06.013

Barucca, M., Canapa, A., Olmo, E., Regoli, F., 2006. Analysis of vitellogenin gene induction as a valuable biomarker of estrogenic exposure in various Mediterranean fish species. *Environ. Res.* 101, 68–73. doi:10.1016/j.envres.2005.07.002

Batiha, M.A., Kadhum, A.A.H., Batiha, M.M., Takriff, M.S., Mohamad, A.B., 2010. MAFRAM- a new fate and risk assessment methodology for non-volatile organic chemicals. *J. Hazard. Mater.* 181, 1080–7. doi:10.1016/j.jhazmat.2010.05.125

Baumgartner, G., Pavanelli, S.C., Baumgartner, D., Bifi, A.G., Debona, T., Frana, V.A., 2012. Peixes do baixo rio Iguaçu.

Beketov, M.A., Liess, M., 2012. Ecotoxicology and macroecology - Time for integration. *Environ. Pollut.* 162, 247–254. doi:10.1016/j.envpol.2011.11.011

Beyer, A., Biziuk, M., 2009. Environmental fate and global distribution of polychlorinated biphenyls. *Rev. Environ. Contam. Toxicol.* 201, 137–58. doi:10.1007/978-1-4419-0032-6_5

Boelsterli, U.A., 2003. Mechanistic Toxicology: the molecular basis of how chemicals disrupt biological targets. London.

Borgert, C.J., Mihaich, E.M., Quill, T.F., Marty, M.S., Levine, S.L., Becker, R. a., 2011. Evaluation of EPA's Tier 1 Endocrine Screening Battery and recommendations for improving the interpretation of screening results. *Regul. Toxicol. Pharmacol.* 59, 397–411. doi:10.1016/j.yrtph.2011.01.003

Bresson, C., Darolles, C., Carmona, A., Gautier, C., Sage, N., Roudeau, S., Ortega, R., Ansoborlo, E., Malard, V., 2013. Cobalt chloride speciation, mechanisms of cytotoxicity on human pulmonary cells, and synergistic toxicity with zinc. *Metallomics* 5, 133–43.

Brito, I., Arruda Freire, C., Yamamoto, F.Y., Silva de Assis, H.C., Rodrigues Souza-Bastos, L., Cestari, M.M., de Castilhos Ghisi, N., Prodocimo, V., Filipak Neto, F., de Oliveira Ribeiro, C.A., 2012. Monitoring water quality in reservoirs for human supply through multi- biomarker evaluation in tropical fish. *J. Environ. Monit.* 14, 615. doi:10.1039/c2em10461j

Brown, J., Ph, D., Walker, S., Steinman, K., Royal, F., 2003. ENDOCRINE MANUAL FOR REPRODUCTIVE NON-DOMESTIC SPECIES. Smithson. Natl. Zool. Park. Conserv. Res. Cent. (internal Publ.

Browning, J., Thomas, N., 2016. An assessment of the tsunami risk in Muscat and Salalah, Oman, based on estimations of probable maximum loss. *Int. J. Disaster Risk Reduct.* 16, 75–87. doi:10.1016/j.ijdrr.2016.02.002

Bui, T.T., Giovanoulis, G., Cousins, A.P., Magnér, J., Cousins, I.T., de Wit, C.A., 2016. Human exposure, hazard and risk of alternative plasticizers to phthalate esters. *Sci. Total Environ.* 541, 451–67. doi:10.1016/j.scitotenv.2015.09.036

Cachada, A., Ferreira da Silva, E., Duarte, A.C., Pereira, R., 2016. Risk assessment of urban soils contamination: The particular case of polycyclic aromatic hydrocarbons. *Sci. Total Environ.* 551-552, 271–284. doi:10.1016/j.scitotenv.2016.02.012

Calò, M., Casella, S., Panzera, M., Lo Cascio, P., Alberghina, D., Piccione, G., 2012. Activation of the Ahr signalling pathway by polychlorobiphenyls causes a marked induction of cytochrome P450 only after depletion of vitellogenin in *Sparus aurata*. *Environ. Toxicol. Pharmacol.* 34, 735–42. doi:10.1016/j.etap.2012.09.014

Campion, S., Aubrecht, J., Boekelheide, K., Brewster, D.W., Vaidya, V.S., Anderson, L., Burt, D., Dere, E., Hwang, K., Pacheco, S., Saikumar, J., Schomaker, S., Sigman, M., Goodsaid, F., 2013. The current status of biomarkers for predicting toxicity. *Expert Opin. Drug Metab. Toxicol.* 9, 1391–408. doi:10.1517/17425255.2013.827170

Carballo, M., Aguayo, S., De La Torre, A., Muñoz, M.J., 2005. Plasma vitellogenin levels and gonadal morphology of wild carp (*Cyprinus carpio*) in a receiving rivers downstream of Sewage Treatment Plants. *Sci. Total Environ.* 341, 71–79. doi:10.1016/j.scitotenv.2004.08.021

Carneiro, C., Andreoli, C.V., Cunha, C. de L. da N., Gobbi, E.F., 2014. Reservoir Eutrophication: Preventive Management: An applied example of Integrated Basin Management Interdisciplinary Research. IWA Publishing.

Casabar, R.C.T., Das, P.C., Dekrey, G.K., Gardiner, C.S., Cao, Y., Rose, R.L., Wallace, A.D., 2010. Endosulfan induces CYP2B6 and CYP3A4 by activating the pregnane X receptor. *Toxicol. Appl. Pharmacol.* 245, 335–43. doi:10.1016/j.taap.2010.03.017

Chakraborty, T., Katsu, Y., Zhou, L.Y., Miyagawa, S., Nagahama, Y., Iguchi, T., 2011. Estrogen receptors in medaka (*Oryzias latipes*) and estrogenic environmental contaminants: an in vitro-in vivo correlation. *J. Steroid Biochem. Mol. Biol.* 123, 115–21. doi:10.1016/j.jsbmb.2010.11.015

Charles A Janeway, J., Travers, P., Walport, M., Shlomchik, M.J., 2001. The structure of a typical antibody molecule.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008a. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol.* 155, 31–62. doi:10.1016/j.ygcen.2007.03.005

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008b. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and

consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol.* 155, 31–62. doi:10.1016/j.ygcen.2007.03.005

Clarke, B.O., Porter, N.A., Marriott, P.J., Blackbeard, J.R., 2010. Investigating the levels and trends of organochlorine pesticides and polychlorinated biphenyl in sewage sludge. *Environ. Int.* 36, 323–9. doi:10.1016/j.envint.2010.01.004

Cobbina, S.J., Chen, Y., Zhou, Z., Wu, X., Feng, W., Wang, W., Mao, G., Xu, H., Zhang, Z., Wu, X., Yang, L., 2015. Low concentration toxic metal mixture interactions: Effects on essential and non-essential metals in brain, liver, and kidneys of mice on sub-chronic exposure. *Chemosphere* 132, 79–86. doi:10.1016/j.chemosphere.2015.03.013

Collins, A., Koppen, G., Valdiglesias, V., Dusinska, M., Kruszewski, M., Møller, P., Rojas, E.,

Colović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., Vasić, V.M., 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. *Curr. Neuropharmacol.* 11, 315–35. doi:10.2174/1570159X11311030006

Committee, I.A., Testing, R., 2012. Information on OECD Work Related to Endocrine Disrupters. Appraisal 1–8.

CONAMA, 2012. Resoluções do Conama: Resoluções vigentes publicadas entre setembro de 1984 e janeiro de 2012, Ministerio do Meio Ambiente. Brasília.

Cordova, M.M., Finardi, E.C., Ribas, F.A.C., de Matos, V.L., Scuzziato, M.R., 2014. Performance evaluation and energy production optimization in the real-time operation of hydropower plants. *Electr. Power Syst. Res.* 116, 201–207. doi:10.1016/j.epsr.2014.06.012

Craig, Z.R., Wang, W., Flaws, J.A., 2011. Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction* 142, 633–46. doi:10.1530/REP-11-0136

Daga, V.S., Gubiani, É. a., 2012. Variations in the endemic fish assemblage of a global freshwater ecoregion: Associations with introduced species in cascading reservoirs. *Acta Oecologica* 41, 95–105. doi:10.1016/j.actao.2012.04.005

Dai, D., Cao, Y., Falls, G., Levi, P.E., Hodgson, E., Rose, R.L., 2001. Modulation of Mouse P450 Isoforms CYP1A2, CYP2B10, CYP2E1, and CYP3A by the Environmental Chemicals Mirex, 2,2-Bis(p-chlorophenyl)-1,1-dichloroethylene, Vinclozolin, and Flutamide. *Pestic. Biochem. Physiol.* 70, 127–141. doi:10.1006/pest.2001.2551

Darbre, P.D., 2015. Endocrine Disruption and Human Health. Elsevier Science.

Dekant, W., Colnot, T., 2013. Endocrine effects of chemicals : Aspects of hazard identification and human health risk assessment. *Toxicol. Lett.* 223, 280–286. doi:10.1016/j.toxlet.2013.03.022

Denier, X., Hill, E.M., Rotchell, J., Minier, C., 2009. Estrogenic activity of cadmium,

copper and zinc in the yeast estrogen screen. *Toxicol. In Vitro* 23, 569–73.
doi:10.1016/j.tiv.2009.01.006

Desforbes, J.P.W., Peachey, B.D.L., Sanderson, P.M., White, P. a., Blais, J.M., 2010. Plasma vitellogenin in male teleost fish from 43 rivers worldwide is correlated with upstream human population size. *Environ. Pollut.* 158, 3279–3284.
doi:10.1016/j.envpol.2010.07.017

Diamanti-Kandarakis, E., 2009. Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement. *Endocr. Reviews* 30, 293–342.

Ding, J., Lu, G., Li, Y., 2016. Interactive effects of selected pharmaceutical mixtures on bioaccumulation and biochemical status in crucian carp (*Carassius auratus*). *Chemosphere* 148, 21–31. doi:10.1016/j.chemosphere.2016.01.017

Dong, H., Li, Z., Man, X., Zhou, J., Lu, H., Wang, S., 2010. Identification of the metabolites of polybrominated diphenyl ether 99 and its related cytochrome P450s. *J. Biomed. Res.* 24, 223–32. doi:10.1016/S1674-8301(10)60032-2

Dong, M., Zhu, L., Shao, B., Zhu, S., Wang, J., Xie, H., Wang, J., Wang, F., 2013. The effects of endosulfan on cytochrome P450 enzymes and glutathione S-transferases in zebrafish (*Danio rerio*) livers. *Ecotoxicol. Environ. Saf.* 92, 1–9.
doi:10.1016/j.ecoenv.2012.10.019

Dorevitch, S., DeFlorio-Barker, S., Jones, R.M., Liu, L., 2015. Water quality as a predictor of gastrointestinal illness following incidental contact water recreation. *Water Res.* 83, 94–103. doi:10.1016/j.watres.2015.06.028

Dorevitch, S., Panthi, S., Huang, Y., Li, H., Michalek, A.M., Pratap, P., Wroblewski, M., Liu, L., Scheff, P.A., Li, A., 2011. Water ingestion during water recreation. *Water Res.* 45, 2020–8. doi:10.1016/j.watres.2010.12.006

Dhawan, A., Benzie, I., Coskun, E., Moretti, M., Speit, G., Bonassi, S., 2014. The comet assay as a tool for human biomonitoring studies: the ComNet project. *Mutat. Res. Rev. Mutat. Res.* 759, 27–39. doi:10.1016/j.mrrev.2013.10.001

Dwyer, F.J., Doane, T.R., Hinman, M.L., 1997. *Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment*, Sixth Volume, Issue 1317, sixth. ed. ASTM International, Philadelphia.

EDSTAC, 1998. Endocrine Disruptor Screening and Testing Advisory Committee.

Edwards, R., 1999. *Immunodiagnosics: A Practical Approach*. OUP Oxford.

Eichelbaum, M., Burk, O., 2001. CYP3A genetics in drug metabolism. *Nat. Med.* 7, 285–287. doi:10.1038/85417

EPA, 2002. National primary drinking water regulations: Long Term 1 Enhanced Surface Water Treatment Rule. Final rule. *Fed. Regist.* 67, 1811–44. doi:EPA 816-F-09-004

EPA, E.P.A., 2010. Toxicological Review of Zinc and Compounds. *Rev. Lit. Arts Am.*

39, 759– 786. doi:<http://www.epa.gov/iris/toxreviews/0070tr.pdf>

EPA, E.P.A., 2003. Health Effects Support Document for Aldrin/Dieldrin.

EPA, E.P.A., 2000. Nickle Compounds | Technology Transfer Network Air Toxics Web site | US EPA. Off. Air Qual. Plan. Stand. URL <https://www3.epa.gov/airtoxics/hlthef/nickel.html> (accessed 3.25.16).

EPA, E.P.A., 1998. Ambient Water Quality Valur for Protection of Sources of Potable Water - beta-Hexachlorocyclohexane, Journal of Chemical Information and Modeling. doi:10.1017/CBO9781107415324.004

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic Metals inducing oxidative stress.pdf. Curr. Top. Med. Chem. 1, 529–539.

Falfushynska, H., Gnatyshyna, L., Fedoruk, O., Sokolova, I.M., Stoliar, O., 2016. Endocrine activities and cellular stress responses in the marsh frog *Pelophylax ridibundus* exposed to cobalt, zinc and their organic nanocomplexes. Aquat. Toxicol. 170, 62–71. doi:10.1016/j.aquatox.2015.11.006

Ferreira, J.H.I., Camacho, J.R., Malagoli, J.A., Júnior, S.C.G., 2016. Assessment of the potential of small hydropower development in Brazil. Renew. Sustain. Energy Rev. 56, 380–387. doi:10.1016/j.rser.2015.11.035

Fowler, B.A., 2012. Biomarkers in toxicology and risk assessment. EXS 101, 459–70. doi:10.1007/978-3-7643-8340-4_16

Freeman, S., Kaufman-Shriqui, V., Berman, T., Varsano, R., Shahar, D.R., Manor, O., 2015. Children's diets, pesticide uptake, and implications for risk assessment: an israeli case study. Food Chem. Toxicol. 87, 88–96. doi:10.1016/j.fct.2015.11.009

Freire, C., Koifman, R.J., Sarcinelli, P.N., Simões Rosa, A.C., Clapauch, R., Koifman, S., 2013. Long-term exposure to organochlorine pesticides and thyroid status in adults in a heavily contaminated area in Brazil. Environ. Res. 127, 7–15. doi:10.1016/j.envres.2013.09.001

Freire, C.A., Souza-bastos, L.R., Chiesse, J., Tincani, F.H., Piancini, L.D.S., Randi, M.A.F., Prodocimo, V., Cestari, M.M., Silva-de-assis, H.C., Abilhoa, V., Vitule, J.R.S., Bastos, L.P., Oliveira-ribeiro, C.A. De, 2015. A multibiomarker evaluation of urban , industrial , and agricultural exposure of small characins in a large freshwater basin in southern Brazil 13263–13277. doi:10.1007/s11356-015-4585-5

Frenzilli, G., Nigro, M., Lyons, B.P., 2009. The Comet assay for the evaluation of genotoxic impact in aquatic environments. Mutat. Res. - Rev. Mutat. Res. 681, 80–92. doi:10.1016/j.mrrev.2008.03.001

Frische, T., Bachmann, J., Frein, D., Jufferholz, T., Kehrer, A., Klein, A., Maack, G., Stock, F., Stolzenberg, H.C., Thierbach, C., Walter-Rohde, S., 2013. Identification, assessment and management of “endocrine disruptors” in wildlife in the EU substance legislation-Discussion paper from the German Federal Environment Agency (UBA). Toxicol. Lett. 223, 306–309. doi:10.1016/j.toxlet.2013.03.004

- Gauthier, P.T., Norwood, W.P., Prepas, E.E., Pyle, G.G., 2014. Metal-PAH mixtures in the aquatic environment: A review of co-toxic mechanisms leading to more-than-additive outcomes. *Aquat. Toxicol.* 154, 253–269. doi:10.1016/j.aquatox.2014.05.026
- Gelbke, H.P., Kayser, M., Poole, A., 2004. OECD test strategies and methods for endocrine disruptors. *Toxicology* 205, 17–25. doi:10.1016/j.tox.2004.06.034
- Gilannejad, N., Dorafshan, S., Paykan, F., Mahboobi, N., Asadollah, S., Martositcha, J.A., Prat, F., Yúfera, M., Martínez-rodríguez, G., 2016. Vitellogenin expression in wild cyprinid *Petroleuciscus esfahani* as a biomarker of endocrine disruption along the Zayandeh Roud River, Iran 144, 1342–1350. doi:10.1016/j.chemosphere.2015.09.106
- Goding, J.W., 1996. Monoclonal Antibodies, Monoclonal Antibodies. Elsevier. doi:10.1016/B978-012287023-1/50049-3
- Gore, A.C., 2010. Neuroendocrine targets of endocrine disruptors. *Hormones (Athens)*. 9, 16–27.
- Gräns, J., Wassmur, B., Fernández-Santoscoy, M., Zanette, J., Woodin, B.R., Karchner, S.I., Nacci, D.E., Champlin, D., Jayaraman, S., Hahn, M.E., Stegeman, J.J., Celander, M.C., 2015. Regulation of pregnane-X-receptor, CYP3A and P-glycoprotein genes in the PCB-resistant killifish (*Fundulus heteroclitus*) population from New Bedford Harbor. *Aquat. Toxicol.* 159, 198–207. doi:10.1016/j.aquatox.2014.12.010
- Gray, J.S., 2002. Biomagnification in marine systems: The perspective of an ecologist. *Mar. Pollut. Bull.* 45, 46–52. doi:10.1016/S0025-326X(01)00323-X
- Grill, G., Khan, U., Lehner, B., Nicell, J., Ariwi, J., 2016. Risk assessment of down-the-drain chemicals at large spatial scales: Model development and application to contaminants originating from urban areas in the Saint Lawrence River Basin. *Sci. Total Environ.* 541, 825–38. doi:10.1016/j.scitotenv.2015.09.100
- Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. *Chem. Res. Toxicol.* 21, 70–83. doi:10.1021/tx700079z
- Gunasekera, R., Ishizawa, O., Aubrecht, C., Blankespoor, B., Murray, S., Pomonis, A., Daniell, J., 2015. Developing an adaptive global exposure model to support the generation of country disaster risk profiles. *Earth-Science Rev.* 150, 594–608. doi:10.1016/j.earscirev.2015.08.012
- Gupta, R.C., 2014. Biomarkers in Toxicology. Academic Press, Kentucky.
- Hampl, R., Kubátová, J., Stárka, L., 2014a. Steroids and endocrine disruptors-History, recent state of art and open questions. *J. Steroid Biochem. Mol. Biol.* doi:10.1016/j.jsbmb.2014.04.013
- Hampl, R., Kubátová, J., Stárka, L., 2014b. open questions. *J. Steroid Biochem. Mol. Biol.* doi:10.1016/j.jsbmb.2014.04.013
- Han, R., Zhao, M., 2012. Evaluation on the Coordination of Economy and

Environment with scarce Water Resources in Shandong Peninsula, China. *Procedia Environ. Sci.* 13, 2236– 2245. doi:10.1016/j.proenv.2012.01.212

Hansen, M.E., Pessah, I.N., Matsumura, F., 2006. Heptachlor epoxide induces a non-capacitative type of Ca^{2+} entry and immediate early gene expression in mouse hepatoma cells. *Toxicology* 220, 218–31. doi:10.1016/j.tox.2006.01.006

Harada, T., Takeda, M., Kojima, S., Tomiyama, N., 2016. Toxicity and Carcinogenicity of Dichlorodiphenyltrichloroethane (DDT). *Toxicol. Res.* 32, 21–33. doi:10.5487/TR.2016.32.1.021

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *J. Hazard. Mater.* 169, 1–15. doi:10.1016/j.jhazmat.2009.03.137

Harlow, E., Lane, D., 1988. *Antibodies a Laboratory Manual*.

Hiramatsu, N., Cheek, A.O., Sullivan, C., Matsubara, T., Hara, a, 2005. Vitellogenesis and endocrine disruption. *Biochem. Mol. Biol. Fishes* 6.

Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C. V., Hara, A., 2006. Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. *Mar. Biol.* 149, 35–47. doi:10.1007/s00227-005-0214-z

Hoffman, D.J., Rattner, B.A., Burton, G.A., 2003. *Handbook of ecotoxicology*, 2nd edition.

Holmstrup, M., Bindesb I, A.M., Oostingh, G.J., Duschl, A., Scheil, V., K??hler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. *Sci. Total Environ.* 408, 3746– 3762. doi:10.1016/j.scitotenv.2009.10.067

Hultman, M.T., Thomas, J., Erik, K., 2015. Evaluation of the sensitivity , responsiveness and reproducibility of primary rainbow trout hepatocyte vitellogenin expression as a screening assay for estrogen mimics. *Aquat. Toxicol.* 159, 233–244. doi:10.1016/j.aquatox.2014.12.013

IAP, I.A. do P., 2008. *Qualidade das aguas dos Reservatórios do Estado do Paraná 2005 a 2008*.

IBGE, I.B. de G. e E., 2010. IBGE | Censo 2010. URL <http://censo2010.ibge.gov.br/> (accessed 9.29.15).

Jain, K.K., 2010. *The Handbook of Biomarkers*. Springer Science & Business Media.

Jamin, E.L., Riu, A., Douki, T., Debrauwer, L., Cravedi, J.-P., Zalko, D., Audebert, M., 2013. Combined genotoxic effects of a polycyclic aromatic hydrocarbon (B(a)P) and an heterocyclic amine (PhIP) in relation to colorectal carcinogenesis. *PLoS One* 8, e58591. doi:10.1371/journal.pone.0058591

Jarvis, I.W.H., Dreij, K., Mattsson, Å., Jernström, B., Stenius, U., 2014. Interactions

between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. *Toxicology* 321, 27–39. doi:10.1016/j.tox.2014.03.012

Jeng, H.A., 2014. Exposure to endocrine disrupting chemicals and male reproductive health. *Front. public Heal.* 2, 55. doi:10.3389/fpubh.2014.00055

Jiménez-Ortega, V., Cano Barquilla, P., Fernández-Mateos, P., Cardinali, D.P., Esquifino, A.I., 2012. Cadmium as an endocrine disruptor: correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin. *Free Radic. Biol. Med.* 53, 2287– 97. doi:10.1016/j.freeradbiomed.2012.10.533

Jorgensen, E., 2010. *Ecotoxicology*. Academic Press.

Kapoor, B.G., Khanna, B., 2004. *Ichthyology Handbook*. Springer Science & Business Media.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D.M., Sinks, T., Tilson, H. a, 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* 104 Suppl , 715–740. doi:10.1289/ehp.96104s4715

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for the several activities of the glutathione S transferases. *J. Biol. Chem.* 251, 6183–6188.

Kim, B.-M., Lee, M.C., Kang, H.-M., Rhee, J.-S., Lee, J.-S., 2015. Genomic organization and transcriptional modulation in response to endocrine disrupting chemicals of three vitellogenin genes in the self-fertilizing fish *Kryptolebias marmoratus*. *J. Environ. Sci.* doi:10.1016/j.jes.2015.08.006

Kirsch-Volders, M., Gonzalez, L., Carmichael, P., Kirkland, D., 2009. Risk assessment of genotoxic mutagens with thresholds: a brief introduction. *Mutat. Res.* 678, 72–5. doi:10.1016/j.mrgentox.2009.05.001

Kiyama, R., Wada-kiyama, Y., 2015. Estrogenic endocrine disruptors : Molecular mechanisms of action. *Environ. Int.* 83, 11–40. doi:10.1016/j.envint.2015.05.012

Knez, J., 2013. Endocrine-disrupting chemicals and male reproductive health. *Reprod. Biomed. Online* 26, 440–8. doi:10.1016/j.rbmo.2013.02.005

Knobil, E., Neill, J.D., 2006. *Knobil and Neill's Physiology of Reproduction, Volume 2*. Gulf Professional Publishing.

Kolle, S.N., Ramirez, T., Kamp, H.G., Buesen, R., Flick, B., Strauss, V., van Ravenzwaay, B., 2012. A testing strategy for the identification of mammalian, systemic endocrine disruptors with particular focus on steroids. *Regul. Toxicol. Pharmacol.* 63, 259–278. doi:10.1016/j.yrtph.2012.04.004

Kong, L., Tang, M., Zhang, T., Wang, D., Hu, K., Lu, W., Wei, C., Liang, G., Pu, Y., 2014. Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats. *Int. J. Mol. Sci.* 15, 21253–69. doi:10.3390/ijms151121253

Koshlukova, S.E., Reed, N.R., 2014. Encyclopedia of Toxicology, Encyclopedia of Toxicology. Elsevier. doi:10.1016/B978-0-12-386454-3.00111-1

Kühnel, D., Nickel, C., 2014. The OECD expert meeting on ecotoxicology and environmental fate-towards the development of improved OECD guidelines for the testing of nanomaterials. *Sci. Total Environ.* 472, 347–53. doi:10.1016/j.scitotenv.2013.11.055

Kumar, P., Singh, A., 2010. Cadmium toxicity in fish: An overview | GEF Bulletin of Biosciences - Academia.edu. GEF Bull. Biosci. URL http://www.academia.edu/2346003/Cadmium_toxicity_in_fish_An_overview (accessed 8.5.15).

Laane, R.W.P.M., Vethaak, A.D., Gandrass, J., Vorkamp, K., Köhler, A., Larsen, M.M., Strand, J., 2013. Chemical contaminants in the Wadden Sea: Sources, transport, fate and effects. *J. Sea Res.* 82, 10–53. doi:10.1016/j.seares.2013.03.004

Laville, N., Balaguer, P., Brion, F., Hinfrey, N., Casellas, C., Porcher, J.-M., Aït-Aïssa, S., 2006. Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line. *Toxicology* 228, 98–108. doi:10.1016/j.tox.2006.08.021

Leenaars, M., Hendriksen, C.F.M., 2005. Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *ILAR J.* 46, 269–279. doi:10.1093/ilar.46.3.269

Lephart, E.D., 1996. A review of brain aromatase cytochrome P450 22, 1–26.

Lewis, M., Pryor, R., Wilking, L., 2011. Fate and effects of anthropogenic chemicals in mangrove ecosystems: a review. *Environ. Pollut.* 159, 2328–46. doi:10.1016/j.envpol.2011.04.027

Lindim, C., van Gils, J., Cousins, I.T., 2016. A large-scale model for simulating the fate & transport of organic contaminants in river basins. *Chemosphere* 144, 803–10. doi:10.1016/j.chemosphere.2015.09.051

Liu, Y., Ma, J., Yan, H., Ren, Y., Wang, B., Lin, C., Liu, X., 2016. Bioaccessibility and health risk assessment of arsenic in soil and indoor dust in rural and urban areas of Hubei province, China. *Ecotoxicol. Environ. Saf.* 126, 14–22. doi:10.1016/j.ecoenv.2015.11.037

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 101, 13–30. doi:10.1016/j.aquatox.2010.10.006

Luzio, A., Monteiro, S.M., Fontainhas-Fernandes, A.A., Pinto-Carnide, O., Matos, M., Coimbra, A.M., 2013. Copper induced upregulation of apoptosis related genes in zebrafish (*Danio rerio*) gill. *Aquat. Toxicol.* 128–129, 183–9. doi:10.1016/j.aquatox.2012.12.018

MacKay, D., Fraser, A., 2000. Bioaccumulation of persistent organic chemicals: Mechanisms and models. *Environ. Pollut.* 110, 375–391. doi:10.1016/S0269-7491(00)00162-7

- Mahugija, J.A.M., Henkelmann, B., Schramm, K.-W., 2015. Levels and patterns of organochlorine pesticides and their degradation products in rainwater in Kibaha Coast Region, Tanzania. *Chemosphere* 118, 12–9. doi:10.1016/j.chemosphere.2014.05.051
- Manders, T.N., Höffken, J.I., van der Vleuten, E.B.A., 2016. Small-scale hydropower in the Netherlands: Problems and strategies of system builders. *Renew. Sustain. Energy Rev.* 59, 1493–1503. doi:10.1016/j.rser.2015.12.100
- Marin, M.G., Matozzo, V., 2004. Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments. *Mar. Pollut. Bull.* 48, 835–9. doi:10.1016/j.marpolbul.2004.02.037
- Marx, a, Sherry, J., Hansen, P.D., Hock, B., 2001. A new monoclonal antibody against vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Chemosphere* 44, 393–399.
- Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environ. Int.* 34, 531–545. doi:10.1016/j.envint.2007.09.008
- Mattagajasingh, S.N., Misra, B.R., Misra, H.P., 2008. Carcinogenic chromium(VI)-induced protein oxidation and lipid peroxidation: Implications in DNA-protein crosslinking. *J. Appl. Toxicol.* doi:10.1002/jat.1364
- Mena, S., Ortega, A., Estrela, J.M., 2009. Oxidative stress in environmental-induced carcinogenesis. *Mutat. Res. - Genet. Toxicol. Environ. Mutagen.* 674, 36–44. doi:10.1016/j.mrgentox.2008.09.017
- Migliore, L., Coppedè, F., 2009. Environmental-induced oxidative stress in neurodegenerative disorders and aging. *Mutat. Res. - Genet. Toxicol. Environ. Mutagen.* 674, 73–84. doi:10.1016/j.mrgentox.2008.09.013
- Mills, L.J., Gutjahr-Gobell, R.E., Zaroogian, G.E., Horowitz, D.B., Laws, S.C., 2014. Modulation of aromatase activity as a mode of action for endocrine disrupting chemicals in a marine fish. *Aquat. Toxicol.* 147, 140–50. doi:10.1016/j.aquatox.2013.12.023
- Mlynarcikova, A., Fickova, M., Scsukova, S., 2014. Impact of endocrine disruptors on ovarian steroidogenesis. *Endocr. Regul.* 48, 201–24.
- Morales, S., Álvarez, C., Acevedo, C., Diaz, C., Rodriguez, M., Pacheco, L., 2015. An overview of small hydropower plants in Colombia: Status, potential, barriers and perspectives. *Renew. Sustain. Energy Rev.* 50, 1650–1657. doi:10.1016/j.rser.2015.06.026
- Nagababu, E., Chrest, F.J., Rifkind, J.M., 2003. Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase. *Biochim. Biophys. Acta* 1620, 211–7.
- Nagler, J.J., Davis, T.L., Modi, N., Vijayan, M.M., Schultz, I., 2010. Intracellular, not membrane, estrogen receptors control vitellogenin synthesis in the rainbow trout.

Gen. Comp. Endocrinol. 167, 326–330. doi:10.1016/j.ygcen.2010.03.022

Newman, M.C., 2014. Fundamentals of Ecotoxicology: The Science of Pollution, Fourth Edition. CRC Press.

Nickens, K.P., Patierno, S.R., Ceryak, S., 2010. Chromium genotoxicity: A double-edged sword. Chem. Biol. Interact. 188, 276–88. doi:10.1016/j.cbi.2010.04.018

Nicolas, J.M., 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquat. Toxicol. 45, 77–90. doi:10.1016/S0166-445X(98)00095-2

Nie, Y., Qiang, Z., Zhang, H., Ben, W., 2012. Fate and seasonal variation of endocrine-disrupting chemicals in a sewage treatment plant with A/A/O process. Sep. Purif. Technol. 84, 9–15. doi:10.1016/j.seppur.2011.01.030

Norris, D.O., Carr, J.A., 2005. Endocrine Disruption: Biological Bases for Health Effects in Wildlife and Humans. Oxford University Press, New York.

NRDC, N.R.D.C., 1998. Endocrine Disruptors | NRDC [WWW Document]. URL <http://www.nrdc.org/health/effects/qendoc.asp> (accessed 3.23.16).

OECD, O.F.E.C.-O.A.D., 2012. Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption.

Okoumassoun, L.-E., Averill-Bates, D., Marion, M., Denizeau, F., 2003. Possible mechanisms underlying the mitogenic action of heptachlor in rat hepatocytes. Toxicol. Appl. Pharmacol. 193, 356–69.

Ortiz de Montellano, P.R., 2005. Cytochrome P450 Structure, Mechanism, and Biochemistry Third edition, third. ed. Kluwer Academic/Plenum Publishers, New York.

Osório, F.H.T., Silva, L.F.O., Piancini, L.D.S., Azevedo, A.C.B., Liebel, S., Yamamoto, F.Y.,

Philippi, V.P., Oliveira, M.L.S., Ortolani-Machado, C.F., Filipak Neto, F., Cestari, M.M., da Silva de Assis, H.C., de Oliveira Ribeiro, C.A., 2014. Water quality assessment of the Tubarão River through chemical analysis and biomarkers in the Neotropical fish *Geophagus brasiliensis*. Environ. Sci. Pollut. Res. 21, 9145–9160. doi:10.1007/s11356-013-1512-5

P.B. Danielson, B.S.P., 2002. The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. Curr. Drug Metab. 3, 561–597. doi:10.2174/1389200023337054

Pahs, S., Republic, F., Bap, O., Pah, T., 2000. Polycyclic aromatic hydrocarbons (PAHs). WHO Reg. Off. Eur. Copenhagen, Denmark 3, 1–24.

Pait, A.S., Nelson, J.O., 2003. Vitellogenesis in male *Fundulus heteroclitus* (killifish) induced by selected estrogenic compounds. Aquat. Toxicol. 64, 331–342. doi:10.1016/S0166-445X(03)00060-2

- Palumbo, A.J., Koivunen, M., Tjeerdema, R.S., 2009. Optimization and validation of a California halibut environmental estrogen bioassay using a heterologous ELISA. *Sci. Total Environ.* 407, 953–961. doi:10.1016/j.scitotenv.2008.09.046
- Pérez-Díaz, J.I., Chazarra, M., García-González, J., Cavazzini, G., Stoppato, A., 2015. Trends and challenges in the operation of pumped-storage hydropower plants. *Renew. Sustain. Energy Rev.* 44, 767–784. doi:10.1016/j.rser.2015.01.029
- Pisoschi, A.M., Pop, A., 2015. The role of antioxidants in the chemistry of oxidative stress: A review. *Eur. J. Med. Chem.* 97, 55–74. doi:10.1016/j.ejmech.2015.04.040
- Poulos, T.L., Johnson, E.R., 2005. Structures of Cytochrome P450 Enzymes. *Cytochrome P450 Struct. Mech. Biochem.* 87–114. doi:10.1007/b139087
- Pramanik, S., Roy, K., 2013. Environmental toxicological fate prediction of diverse organic chemicals based on steady-state compartmental chemical mass ratio using quantitative structure-fate relationship (QSFR) models. *Chemosphere* 92, 600–7. doi:10.1016/j.chemosphere.2013.03.065
- Price, J.I., Janmaat, J., Sugden, F., Bharati, L., 2016. Water storage systems and preference heterogeneity in water-scarce environments: A choice experiment in Nepal's Koshi River Basin. *Water Resour. Econ.* 13, 6–18. doi:10.1016/j.wre.2015.09.003
- Pundir, C.S., Chauhan, N., 2012. Acetylcholinesterase inhibition-based biosensors for pesticide determination: a review. *Anal. Biochem.* 429, 19–31. doi:10.1016/j.ab.2012.06.025
- Ran, Y., Lannerstad, M., Herrero, M., Van Middelaar, C.E., De Boer, I.J.M., 2016. Assessing water resource use in livestock production: A review of methods. *Livest. Sci.* 187, 68–79. doi:10.1016/j.livsci.2016.02.012
- Rani, A., Kumar, A., Lal, A., Pant, M., 2014. Cellular mechanisms of cadmium-induced toxicity: a review. *Int. J. Environ. Health Res.* 24, 378–99. doi:10.1080/09603123.2013.835032
- Reinen, J., Suter, M.J.F., Vögeli, a. C., Fernandez, M.F., Kiviranta, H., Eggen, R.I.L., Vermeulen, N.P.E., 2010. Endocrine disrupting chemicals-Linking internal exposure to vitellogenin levels and ovotestis in *Abramis brama* from Dutch surface waters. *Environ. Toxicol. Pharmacol.* 30, 209–223. doi:10.1016/j.etap.2010.07.004
- Sachdev, H.S., Akella, A.K., Kumar, N., 2015. Analysis and evaluation of small hydropower plants: A bibliographical survey. *Renew. Sustain. Energy Rev.* 51, 1013–1022. doi:10.1016/j.rser.2015.06.065
- Sanderson, J.T., 2006. The steroid hormone biosynthesis pathway as a target for endocrine- disrupting chemicals. *Toxicol. Sci.* 94, 3–21. doi:10.1093/toxsci/kfl051
- Sandhu, N., Vijayan, M.M., 2011. Cadmium-mediated disruption of cortisol biosynthesis involves suppression of corticosteroidogenic genes in rainbow trout. *Aquat. Toxicol.* 103, 92–100. doi:10.1016/j.aquatox.2011.02.011

Santos, D.M., Santos, G.S., Cestari, M.M., de Oliveira Ribeiro, C.A., de Assis, H.C.S., Yamamoto, F., Guiloski, I.C., de Marchi, M.R.R., Montone, R.C., 2014. Bioaccumulation of butyltins and liver damage in the demersal fish *Cathorops spixii* (Siluriformes, Ariidae). *Environ. Sci. Pollut. Res.* 21, 3166–3174. doi:10.1007/s11356-013-2280-y

Santos, D.R., Yamamoto, F.Y., Filipak Neto, F., Randi, M.A.F., Garcia, J.E., Costa, D.D.M., Liebel, S., Campos, S.X., Voigt, C.L., de Oliveira Ribeiro, C.A., 2016. The applied indicators of water quality may underestimate the risk of chemical exposure to human population in reservoirs utilized for human supply-Southern Brazil. *Environ. Sci. Pollut. Res. Int.* doi:10.1007/s11356-015-5995-0

Satarug, S., Garrett, S.H., Sens, M.A., Sens, D.A., 2009. Cadmium, Environmental Exposure, and Health Outcomes. *Environ. Health Perspect.* 118, 182–190. doi:10.1289/ehp.0901234

Schlenk, D., 2005. Pesticide biotransformation in fish, in: Mommsen, T.P. (Ed.), *Molecular Biology*. Elsevier.

Scholz, S., Mayer, I., 2008. Molecular biomarkers of endocrine disruption in small model fish. *Mol. Cell. Endocrinol.* 293, 57–70. doi:10.1016/j.mce.2008.06.008

Schrader, T.J., 2016. *Encyclopedia of Food and Health*, Encyclopedia of Food and Health. Elsevier. doi:10.1016/B978-0-12-384947-2.00476-1

SEMA, S. de E. do M.A. e R.H., 2010. *Bacias Hidrográficas do Paraná*. Curitiba.

Serpe, C.T.A., 2014. Análise da composição e abundância da comunidade fitoplanctônica em quatro reservatórios do rio Iguaçu.

Smeets, J.M.W., Wamsteker, J., Roth, B., Everaarts, J., van den Berg, M., 2002. Cytochrome P4501A induction and testosterone hydroxylation in cultured hepatocytes of four fish species. *Chemosphere* 46, 163–72. doi:10.1016/S0045-6535(01)00054-6

Sobus, J.R., Tan, Y.-M., Pleil, J.D., Sheldon, L.S., 2011. A biomonitoring framework to support exposure and risk assessments. *Sci. Total Environ.* 409, 4875–84. doi:10.1016/j.scitotenv.2011.07.046

SPECKER, J., 1994. Vitellogenesis in fishes : status and perspectives. *Perspect. Comp. Endocrinol.* 304–315.

Stanley, K.A., Curtis, L.R., Simonich, S.L.M., Tanguay, R.L., 2009. Endosulfan I and endosulfan sulfate disrupts zebrafish embryonic development. *Aquat. Toxicol.* 95, 355–61. doi:10.1016/j.aquatox.2009.10.008

Stils, H.F., 2005. Adjuvants and Antibody Production: Dispelling the Myths Associated with Freund's Complete and Other Adjuvants. *ILAR J.* 46, 280–293. doi:10.1093/ilar.46.3.280

Sudhir-Yadav, Balwinder-Singh, Humphreys, E., Kukal, S.S., 2013. *Agricultural Sustainability*, Agricultural Sustainability. Elsevier. doi:10.1016/B978-0-12-404560-

- Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* 103, 173–178. doi:10.1289/ehp.95103s7173
- Tabrez, S., Ahmad, M., 2010. Cytochrome P450 system as a toxicity biomarker of industrial wastewater in rat tissues. *Food Chem. Toxicol.* 48, 998–1001. doi:10.1016/j.fct.2009.12.024
- Thomas, C., Mackey, M.M., Diaz, A.A., Cox, D.P., 2009. Hydroxyl radical is produced via the Fenton reaction in submitochondrial particles under oxidative stress: implications for diseases associated with iron accumulation. *Redox Rep.* 14, 102–8. doi:10.1179/135100009X392566
- Tyler, C.R., Filby, A.L., Aerle, R. Van, Lange, A., Ball, J., Santos, E.M., 2008. Fish toxicogenomics. *Adv. Exp. Biol.* 02. doi:10.1016/S1872-2423(08)00003-3
- US EPA, OAR, O. of A.Q.P. and S., 2000. Cobalt Compounds | Technology Transfer Network Air Toxics Web site | US EPA [WWW Document]. URL <https://www3.epa.gov/airtoxics/hlthef/cobalt.html> (accessed 3.24.16).
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39, 44–84. doi:10.1016/j.biocel.2006.07.001
- Van der Linde, A., Jan Hendriks, A., Sijm, D.T.H.M., 2001. Estimating biotransformation rate constants of organic chemicals from modeled and measured elimination rates. *Chemosphere* 44, 423–435. doi:10.1016/S0045-6535(00)00213-7
- Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol.* 13, 57–149. doi:10.1016/S1382-6689(02)00126-6
- Vesterinen, J., Pouta, E., Huhtala, A., Neuvonen, M., 2010. Impacts of changes in water quality on recreation behavior and benefits in Finland. *J. Environ. Manage.* 91, 984–994. doi:10.1016/j.jenvman.2009.12.005
- Voigt, C.L., da Silva, C.P., Doria, H.B., Randi, M.A.F., de Oliveira Ribeiro, C.A., de Campos, S.X., 2014. Bioconcentration and bioaccumulation of metal in freshwater Neotropical fish *Geophagus brasiliensis*. *Environ. Sci. Pollut. Res. Int.* doi:10.1007/s11356-014-3967-4
- Wade, M.G., Parent, S., Finnson, K.W., Foster, W., Younglai, E., McMahon, A., Cyr, D.G., Hughes, C., 2002. Thyroid toxicity due to subchronic exposure to a complex mixture of 16 organochlorines, lead, and cadmium. *Toxicol. Sci.* 67, 207–18.
- Wahl, M., Guenther, R., Yang, L., Bergman, A., Straehle, U., Strack, S., Weiss, C., 2010. Polybrominated diphenyl ethers and arylhydrocarbon receptor agonists: Different toxicity and target gene expression. *Toxicol. Lett.* 198, 119–26. doi:10.1016/j.toxlet.2010.06.001
- Walker, C.H., Sibly, R.M., Hopkin, S.P., Peakall, D.B., 2005. Principles of Ecotoxicology, Third Edition. CRC Press.

- Wang, J., Bing, X., Yu, K., Tian, H., Wang, W., Ru, S., 2015a. Preparation of a polyclonal antibody against goldfish (*Carassius auratus*) vitellogenin and its application to detect the estrogenic effects of monocrotophos pesticide. *Ecotoxicol. Environ. Saf.* 111, 109–116. doi:10.1016/j.ecoenv.2014.10.007
- Wang, J., Shan, R., Zhang, X., Tian, H., Wang, W., Ru, S., 2015b. Development of a lipovitellin- based sandwich ELISA for quantification of vitellogenin in surface mucus and plasma of goldfish (*Carassius auratus*). *Ecotoxicol. Environ. Saf.* 120, 80–7. doi:10.1016/j.ecoenv.2015.05.010
- Wang, W., Gao, L., Liu, P., Hailu, A., 2014. Relationships between regional economic sectors and water use in a water-scarce area in China: A quantitative analysis. *J. Hydrol.* 515, 180–190. doi:10.1016/j.jhydrol.2014.04.057
- Watts, M., Pankhurst, N.W., Pryce, a., Sun, B., 2003. Vitellogenin isolation, purification and antigenic cross-reactivity in three teleost species. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 134, 467–476. doi:10.1016/S1096-4959(02)00288-9
- Wedebye, E.B., Dybdahl, M., Nikolov, N.G., Jónsdóttir, S.Ó., Niemelä, J.R., 2015. QSAR screening of 70,983 REACH substances for genotoxic carcinogenicity, mutagenicity and developmental toxicity in the ChemScreen project. *Reprod. Toxicol.* 55, 64–72. doi:10.1016/j.reprotox.2015.03.002
- Wei, K., Yang, J., 2015. Oxidative damage induced by copper and beta-cypermethrin in gill of the freshwater crayfish *Procambarus clarkii*. *Ecotoxicol. Environ. Saf.* 113, 446–53. doi:10.1016/j.ecoenv.2014.12.032
- Weydert, C.J., Cullen, J.J., 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat. Protoc.* 5, 51–66. doi:10.1038/nprot.2009.197
- WHO, 2012. WHO | State of the science of endocrine disrupting chemicals - 2012.
- WHO, 2011. Cadmium in Drinking-water 16.
- WHO, 1996. Silver in Drinking-water. *Guidel. Drink. Qual.* 2, 1–9.
- WHO, 2010. Exposure to Lead: A major public health concern. *World Heal. Organ.* 6.
- WHO, 2006. Concise International Chemical Assessment Document 70 - HEPTACHLOR. *Elem. Mercur. Inorg. Mercur. Compd. Hum. Heal. Asp.* 61.
- WHO, 2005. Nickel in Drinking-water, Environmental Health.
- WHO, W.H.O., IPCS, I.P. on C.S., 2004. IPCS risk assessment terminology. Geneva.
- WHO, IPCS, I.P. on C.S., 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors, International Programme on Chemical Safety.
- Wilkinson, G.R., 1996. Cytochrome P4503A (CYP3A) metabolism: prediction of in vivo activity in humans. *J. Pharmacokinet. Biopharm.* 24, 475–90.

World Health Organization, 2000. General description Polychlorinated biphenyls (PCBs) 1–22.

Yaglova, N. V., Yaglov, V. V., 2016. The effect of long-term exposure to low doses of endocrine disruptor ddt on serum levels of thyroid protein autoantigenes and antithyroid autoantibodies. *Biomed. Khim.* 62, 73–78.

Yang, F., Huang, W., Xie, W., Lu, C., Liu, W., 2015. Targeted analytical toxicology: simultaneous

determination of 17 α -ethynylestradiol and the estrogen-induced vitellogenin biomarker. *Environ. Int.* 74, 119–24. doi:10.1016/j.envint.2014.10.015

Yu, B., Xu, L., 2016. Review of ecological compensation in hydropower development. *Renew. Sustain. Energy Rev.* 55, 729–738. doi:10.1016/j.rser.2015.10.038

Zhou, T., Cong, S., Sun, S., Sun, H., Zou, R., Wang, S., Wang, C., Jiao, J., Goto, K., Nawata, H., Yanase, T., Zhao, Y., 2013. Identification of endocrine disrupting chemicals activating SXR-mediated transactivation of CYP3A and CYP7A1. *Mol. Cell. Endocrinol.* 365, 36–43. doi:10.1016/j.mce.2012.09.001

Zhu, Y., Price, O.R., Tao, S., Jones, K.C., Sweetman, A.J., 2014. A new multimedia contaminant fate model for China: how important are environmental parameters in influencing chemical persistence and long-range transport potential? *Environ. Int.* 69, 18–27. doi:10.1016/j.envint.2014.03.020

Ziech, D., Franco, R., Georgakilas, A.G., Georgakila, S., Malamou-Mitsi, V., Schoneveld, O., Pappa, A., Panayiotidis, M.I., 2010. The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. *Chem. Biol. Interact.* 188, 334–339. doi:10.1016/j.cbi.2010.07.010

APÊNDICES

Algumas metodologias não detalhadas nos artigos, foram descritas nesta seção:

1. ANÁLISES BIOQUÍMICAS

As amostras de fígado foram descongeladas e fragmentos de aproximadamente 200 mg de tecido foram homogeneizados em tampão Tris HCl 20mM, EDTA 0,1M, PMSF 1mM pH 7,4 (volume final de 2 mL). Para o músculo e cérebro as amostras foram homogeneizadas em Tampão Fosfato 0,1M, pH 7,5 e 8,0 respectivamente. Em seguida foram centrifugadas numa velocidade de rotação de 12.000 g, a 4° C durante 30 minutos. O sobrenadante foi aliqotado em diferentes tubos (2 mL), com volumes conhecidos, para evitar o congelamento das amostras. Antes de realizar cada análise, as amostras foram diluídas de acordo com o método a ser utilizado, a fim de alcançar uma faixa de concentração de proteínas desejada (1mg de proteínas/ml para a atividade das enzimas).

1.1 Quantificação protéica no plasma

As concentrações de proteínas totais das alíquotas de plasma, fígado, gônadas, músculo e cérebro foram determinadas para normalizar os dados obtidos nos diferentes ensaios bioquímicos (BRADFORD, 1976). Na microplaca foram adicionados 10 µL do sobrenadante de cada amostra diluída seguidos de 250 µL do reativo de Bradford® (Coomassie brilliant blue, Sigma Aldrich). Para o branco foi adicionado PBS. Foram empregadas duas réplicas de cada amostra e a leitura foi realizada em espectrofotômetro a um comprimento de onda de 595 nm.

1.2 Peroxidação de lipídios (LPO)

Os danos causados nos lipídios foram avaliados de acordo com o Método FOX (JIANG et al., 1991, 1992). O princípio do método se baseia na rápida oxidação do Fe^{+2} mediada por peróxidos sob condições ácidas e posterior formação do complexo Fe^{+3} – laranja de xilenol na presença do estabilizador hidroxitolueno

butilado, que absorve luz a um comprimento de onda de 550-570 nm. Em um microtubo de 2 mL foram pipetados 200 μ L da amostra centrifugada concentrada acrescida de 1800 μ L do meio de reação. Este foi preparado na hora, utilizando metanol 100%, Xilenol Laranja 100 μ M, H_2SO_4 25 mM, hidroxitolueno butilado 4mM e $\text{FeSO}_4\cdot\text{NH}_4$ 25 μ M. Após 20 minutos de reação à temperatura ambiente, as amostras foram centrifugadas a 9.000g. Para a leitura, 300 μ L do sobrenadante foram pipetados em microplacas e a absorbância foi medida a 550-570 nm em um espectrofotômetro de microplaca.

1.3 *Glutathione S-transferase (GST)*

A atividade da enzima GST foi mensurada de acordo com a formação de um tioéter a partir da reação do substrato CNDB com o GSH, catalisada pela GST (KEEN et al., 1976 com modificações). Na microplaca foram adicionados 20 μ L de amostra, acrescidos de 180 μ L do meio de reação com uma pipeta multicanal. O meio de reação foi preparado a partir de tampão fosfato a 0,1M pH 6,5; solução de GSH a 3mM (Sigma Aldrich) e solução de CDNB a 3mM (Sigma Aldrich). A absorbância foi medida em um comprimento de onda de 340 nm durante 2 min em intervalos de 12 segundos. A atividade enzimática foi determinada de acordo com o delta absorbância no intervalo de 1 minuto.

1.4 *Glutathione reduzida (GSH)*

A concentração de Glutathione reduzida (GSH) foi determinada a partir da reação com o DTNB 2,5 mM (ácido 5,5-ditiobis(2-nitrobenzóico). Para precipitar proteínas das amostras foi adicionado TCA (ácido tricloroacético) para chegar a uma concentração final 10%. A mistura foi centrifugada e 50 μ L do sobrenadante foi pipetado na microplaca seguido de 230 μ L de tampão tris-base a 0,4M, pH8,9 e 20 μ L de DTNB 2,5mM. A leitura foi realizada a 415 nm.

1.5 Carbonilação de proteínas (PCO)

A concentração de proteínas oxidadas/carboniladas foi determinada de acordo com sua reação com a 2,4-dinitrofenil-hidrazina (DNPH) formando dinitrofenil hidrazonas detectadas a um comprimento de onda de 358-370 nm (LEVINE et al., 1994). A cada 200 µl de amostra foram adicionados 500 µl de reagente (DNPH a 10 mM, HCl 2M) e mantidos a 30-37° C por 90 minutos. Para a precipitação das proteínas foi adicionado TCA a 28% e centrifugados a 9.000g por 10 minutos. O pellet foi lavado três vezes por ressuspensão (em etanol/acetato de etila) e centrifugação. As proteínas foram solubilizadas com cloreto de guanidina a 6 M e posteriormente foram centrifugadas para eliminar resíduos não solúveis. As carbonilas foram determinadas espectrofotometricamente a 360 nm.

1.6 Acetilcolinesterase (AChE)

Para avaliar a atividade da AChE foi utilizado o método de ELLMAN et al. (1961) que tem como princípio a hidrólise do substrato acetiltiocolina pela AChE, em tiocolina e acetato. A tiocolina resultante reage com o DTNB gerando o ânion 5-Tio-2-nitrobenzoato responsável pelo aparecimento de coloração amarela que pode ser monitorado pelo aumento de absorbância a 405 nm. Amostras de músculo e cérebro foram descongeladas e homogeneizadas em tampão fosfato pH 7,5 e 8,0, respectivamente. Após a quantificação de proteínas totais das amostras, estas foram diluídas. Em uma microplaca foram adicionados 50 µL da amostra e com uma pipeta multicanal, 200 µL de DTNB 0,75mM. A leitura foi realizada a 405 nm durante 4 minutos em intervalos de 30 segundos.

2 ANÁLISES DE GENOTOXICIDADE

As análises de genotoxicidade foram realizadas em parceria com o laboratório de Mutagênese Ambiental (departamento de Genética – UFPR).

2.1 Ensaio Cometa

A metodologia do ensaio cometa em eritrócitos foi realizada segundo Speit e Hartmann (1999) modificada para hepatócitos por Ferraro et al. (2004). Primeiramente 10µL do sangue aliquotado em 1mL soro bovino fetal foi diluído em 120µL de agarose de baixo ponto de fusão e plotados sobre uma lâmina coberta com agarose ultrapura. As lâminas foram colocadas em uma solução de lise composta de NaCl (2.5 M), EDTA (100 mM), Tris (10 mM), NaOH (0,8%), N-lauril-sarcosinato (10%), Triton X-100 (1%) e DMSO (10%) por 72 h a 4°C. Posteriormente as lâminas foram imersas em uma solução de NaOH (10N) e EDTA (200 mM), pH>13 por 25 minutos, para desnaturar o DNA. Então foi realizada a eletroforese a 300mA e 25 V por 25 minutos. As lâminas foram neutralizadas em 0,4M Tris, pH 7,5 e fixadas em etanol por 10 minutos. Os cometas foram corados com 0,02 g/ml de brometo de etídeo e contabilizados usando um microscópio de epifluorescência Leica DMLS2. Foram analisados visualmente 100 cometas por indivíduo e contabilizados em cinco classes de danos segundo Collins (2004).

2.2 Teste de Micronúcleo Písceo

A frequência de micronúcleos em eritrócitos foi determinada segundo a técnica descrita por Heddle (1973) para a montagem das lâminas. Com o sangue retirado da veia caudal foi realizado um esfregaço numa lâmina de vidro limpa posteriormente fixada com etanol por 30 minutos, e corado com laranja de acridina. Foram analisados 2000 eritrócitos por peixe num aumento de 1000x em um microscópio de epifluorescência. Além dos micronúcleos foram contabilizadas as alterações morfológicas nucleares segundo Carrasco et al. (1990) e a frequência de eritrócitos policromáticos segundo Ueda et al. (1992).

3. ANÁLISES MORFOLÓGICAS

3.1 Fígado

Índice de bernet

As alterações histopatológicas do fígado foram analisadas de acordo com uma metodologia adaptada da proposta por Bernet (1999). As principais alterações avaliadas no fígado foram: necroses; infiltração leucocitária; vacuolização; neoplasia; diferenciação tecidual e parasitas. Para cada alteração foi atribuído um fator de importância (w) que varia de 1 a 3 (TABELA 1). A relevância de uma lesão depende de sua importância patológica, ou seja, como ela afeta a função do órgão:

- 1- Importância patológica mínima, a lesão é facilmente reversível quando a exposição ao irritante é cessada;
- 2- Importância patológica moderada, a lesão é reversível na maioria dos casos se o estressor é neutralizado;
- 3- Importância patológica marcada, a lesão geralmente é irreversível, levando a perda parcial ou total da função do órgão.

Cada lâmina analisada recebeu um índice de lesão que representa o somatório do valor obtido em cada alteração encontrada. Para atribuir um valor para cada alteração é necessário multiplicar o fator de importância da alteração (w) pelo valor de pontuação que varia de 0 a 6 de acordo com o grau de extensão da alteração.

TABELA 1 - Fator de importância e valor de pontuação atribuídos para cada lesão em fígado.

	Necrose	Resposta inflamatória	Vacuolização	CMM	Neoplasia	Diferenciação tecidual	Parasitas
Fator de importância	3	1	2	2	3	3	2
	Sem alteração	Leve ocorrência	Ocorrência moderada	Ocorrência severa			
Valor de pontuação	0	2	4	6			

3.2 Gônadas

São órgãos pareados que, quando maduros, podem ocupar grande parte da cavidade abdominal. São envoltos por um peritônio, composto por um mesotelio e uma camada de tecido conectivo, a túnica albugínea.

Tanto para análise dos ovários como para os testículos, a classificação dos estágios de desenvolvimento representa um método fundamental para avaliação dos efeitos dos DEs. Outros métodos utilizados incluem as análises histopatológicas, inclusive a porcentagem de folículos atrésicos (acima de 20% indica taxa anormal), diâmetros dos túbulos seminíferos, degeneração das células germinativas, hiperplasia ou hipertrofia das células de Sertoli e de Leydig.

A classificação dos estágios do desenvolvimento das células germinativas dos peixes, pode ser feita pela contagem da porcentagem dos estágios presentes, com o uso de uma ocular (contagem de 1 campo no aumento de 10x) :

Fêmeas:

1. Oogônias: Menores células da linhagem reprodutiva ocorrendo em grupos ou ninhos, com volume reduzido do citoplasma, núcleo grande e nucléolo único e central;
2. Crescimento primário: Ovócitos primários ainda não completamente envoltos por células foliculares, também presentes em ninhos com núcleo oval e poucos nucléolos com tamanho variável;
3. Alvéolo cortical: Surgimento do alvéolo cortical (vesículas de vitelo) e pequenas gotículas lipídicas
4. Pré-vitelogênica: Aparecimento de vesículas de vitelo, inicialmente poucas e pequenas, até variando a várias de tamanhos-variados, vesículas germinais localizadas centralmente são redondas a ovais com vários nucléolos periféricos
5. Vitelogênico: Vesícula germinal perde os nucléolos, move para a periferia e é degradado, vesículas de vitelo frequentemente preenchem todo o ovócito e uma vesícula germinativa podem não ser evidente.
6. Maduro/ ovócito em desova: Completa degradação da vesícula germinativa, vesículas de vitelo se fundem e podem ser maiores que os alvéolos corticais (difícilmente visualizado, portanto apenas os 5 estágios foram considerados)

Folículo vazio – Quando o ovócito é liberado e restam somente as células foliculares hipertrofiadas com espaço vazio interior.

Folículos atrésicos – São ovócitos que não foram liberados e sofreram degeneração, sendo uma massa acidófila amorfa envolta por células foliculares hipertrofiadas

Machos

Os testículos apresentam um tecido conjuntivo, contínuo à túnica albugínea, que separam os túbulos seminíferos. Nos testículos maduros os espermatozoides estão presentes no lúmen dos túbulos seminíferos. Além das células germinativas duas outras células apresentam papel importante: i) As células de Sertoli são pequenas e de difícil localização, apresentam estrutura em formato triangular e situada próximas à periferia dos túbulos seminíferos. Envolvem um grupo de células germinativas formando um cisto; ii) As células de Leydig estão presentes em grupos no tecido conectivo entre os túbulos seminíferos, apresentam núcleo oval e citoplasma estreito

1. Espermatogônias primárias: Células grandes nas periferias dos túbulos, tem um núcleo levemente corado com nucléolo proeminente
2. Espermatogônias secundárias: grupo de células de tamanho-médio com núcleo redondo, levemente basófilo, várias divisões mitóticas resultam nos espermatócitos primários
3. Espermatócitos primários: células pequenas com núcleo menor e mais basófilo que as espermatogônias, vão para meiose I para produzir espermatócitos
4. Espermatócitos secundários: Pequenas células com pequeno núcleo e mais basófilo que os espermatócitos primários, vão para meiose II para produzir espermátides
5. Espermátide tem núcleo pequeno intensamente basófilo, eles amadurecem a espermatozoides

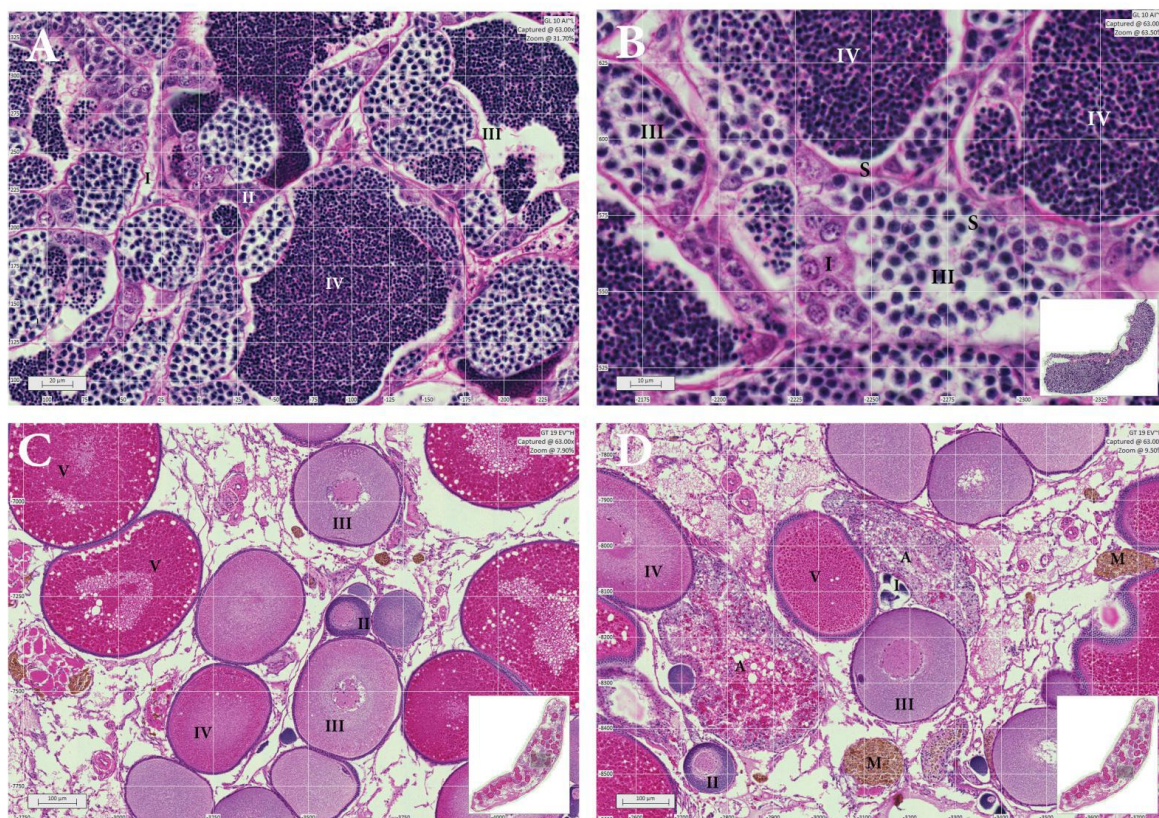


FIGURA 1. Metodologia para contagem das células germinativas em diferentes estágios de desenvolvimento e análise histopatológica das gônadas dos peixes do Rio Iguaçu. A – Contagem da porcentagem das células germinativas do testículo de *A. bifasciatus* em 2 campos distintos no aumento de 20X. B – Aumento em 40x para demonstrar detalhes das células nos diferentes estágios e as células de Sertoli. C – Contagem da porcentagem dos ovócitos do ovário de *C. iguassuensis* em 2 campos distintos no aumento de 5X. B – Ocorrência de atresia(A) e centros de melanomacrófagos(M).

RESULTADOS COMPLEMENTARES

CAPÍTULO I

TABELA 2. Índices somáticos (fator de condição e índice hepatossomático) dos peixes coletados nos cinco reservatórios do Rio Iguaçu.

Espécies		n	K		HSI	
			Verão		Inverno	
FA	<i>A. bifasciatus</i>	20	1,31 ($\pm$ 0,21)	1,31 ($\pm$ 0,07) a	0,60 ($\pm$ 0,32) *	0,97 ($\pm$ 0,48) *
	<i>G. brasiliensis</i>	20	1,39 ($\pm$ 0,14)	1,59 ($\pm$ 0,08)	1,36 ($\pm$ 0,42) a*	3,33 ($\pm$ 0,26) a*
	<i>C. iguassuensis</i>	6	0,85 ($\pm$ 0,07)	1,09	1,18 ($\pm$ 0,64)	0,97
SE	<i>A. bifasciatus</i>	20	1,34 ($\pm$ 0,14) a	1,20 ($\pm$ 0,10)	0,48 ($\pm$ 0,20)	0,63 ($\pm$ 0,21)
	<i>G. brasiliensis</i>	20	1,50 ($\pm$ 0,13)	1,48 ($\pm$ 0,09)	0,81 ($\pm$ 0,48) b	0,58 ($\pm$ 0,05) b
	<i>C. iguassuensis</i>	3	0,88 ($\pm$ 0,07)	-	0,93 ($\pm$ 0,41)	-
SS	<i>A. bifasciatus</i>	20	1,19 ($\pm$ 0,14) b	1,17 ($\pm$ 0,09) b	0,71 ($\pm$ 0,59)	0,69 ($\pm$ 0,19)
	<i>G. brasiliensis</i>	20	1,45 ($\pm$ 0,74)	1,66 ($\pm$ 0,07)	1,46 ($\pm$ 0,57) a	1,97 ($\pm$ 0,32) b
	<i>C. iguassuensis</i>	2	1,15 ($\pm$ 0,90)	0,82 ($\pm$ 0,08)	1,72 ($\pm$ 1,39)	1,05 ($\pm$ 0,17)
SO	<i>A. bifasciatus</i>	9	1,22 ($\pm$ 0,11)	1,26 ($\pm$ 0,08)	0,74 ($\pm$ 0,36)	0,62 ($\pm$ 0,23)
	<i>G. brasiliensis</i>	9	1,32 ($\pm$ 0,28)	-	1,05 ($\pm$ 0,28)	-
	<i>C. iguassuensis</i>	17	0,84 ($\pm$ 0,09)	-	0,98 ($\pm$ 0,37)	-
SC	<i>A. bifasciatus</i>	20	1,33 ($\pm$ 0,12) a	1,33 ($\pm$ 0,08) a	0,46 ($\pm$ 0,08)	0,69 ($\pm$ 0,61)
	<i>G. brasiliensis</i>	3	1,51 ($\pm$ 0,35)	-	0,83 ($\pm$ 0,16)	-
	<i>C. iguassuensis</i>	20	1,17 ($\pm$ 0,16)	1,08 ($\pm$ 0,27)	0,77 ($\pm$ 0,28)	1,01 ($\pm$ 0,60)

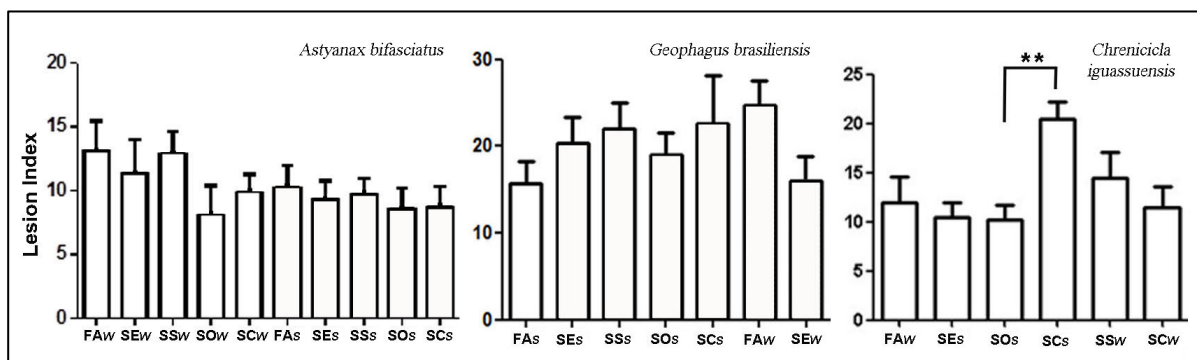


FIGURA 1. Índice de lesão do fígado dos peixes coletados nos cinco reservatórios do Rio Iguaçu.

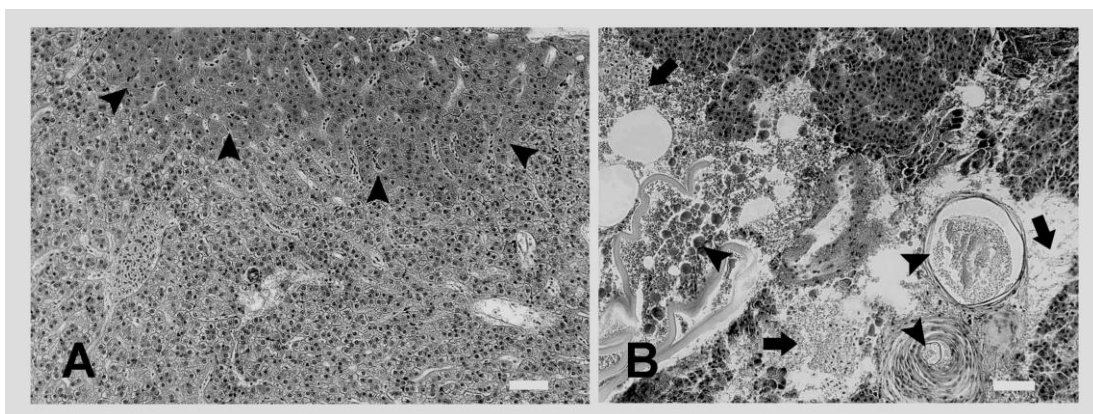


FIGURA 2. Secção do fígado de *A. Bifasciatus* corado com Hematoxilina e Eosina. A – Foco basófilo (ponta da seta) (barra da escala = 50 μ m). B – Área de necrose (setas) relacionadas à presença de parasitas (ponta das setas) (barra da escala = 50 μ m).

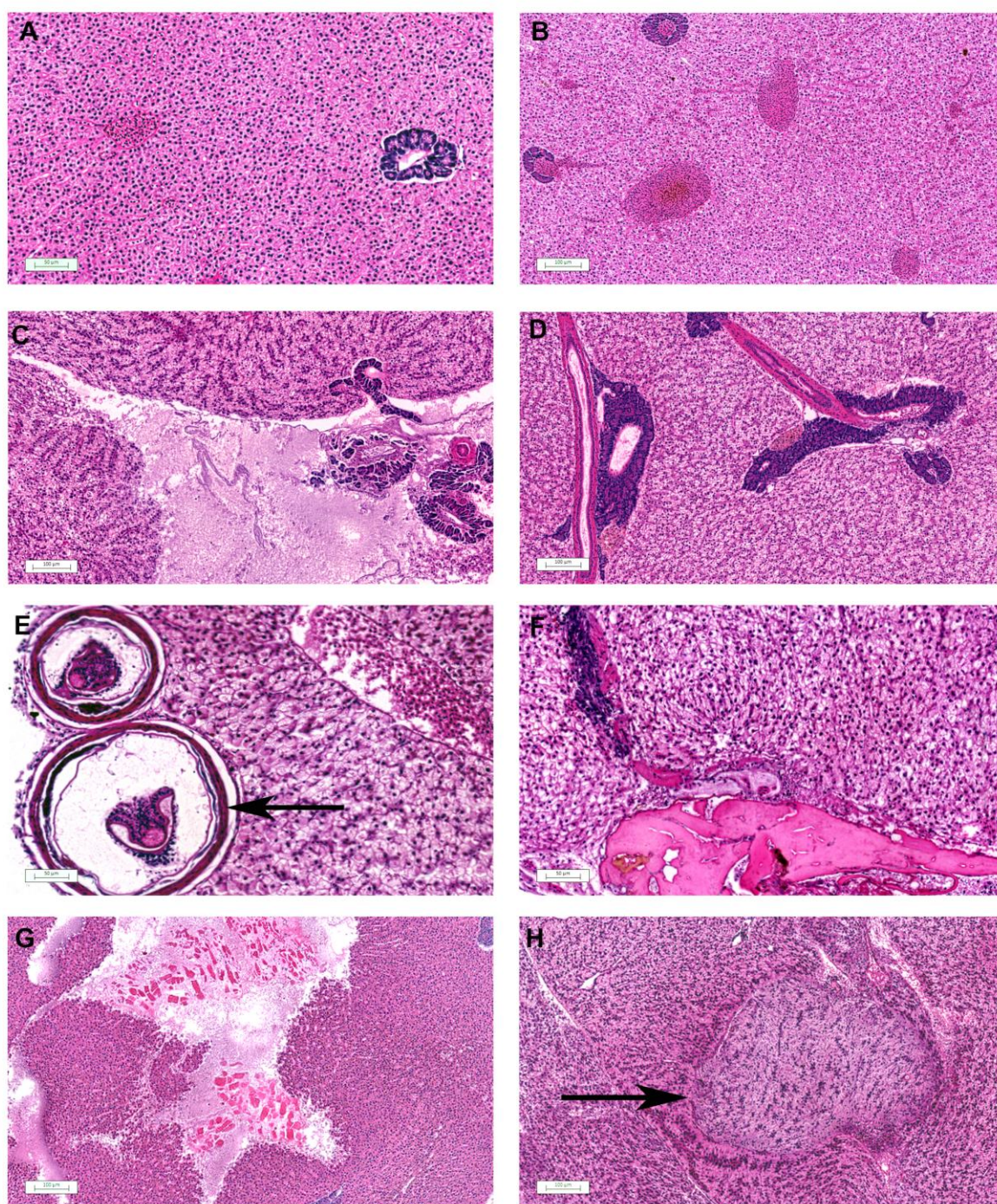


FIGURA 3. Histopatologia do fígado de *G. brasiliensis* coletados nos cinco reservatórios do Rio Iguaçu. A – Parênquimas hepático normal sem alterações. B – Distúrbio circulatório, congestão vascular. C – Extensa área de necrose do parênquima hepático. D – Granulomatose peritubular, inflamação dos ductos biliares. E – Parasitas pluricelulares, hepatócitos vacualizados. F – Fibrose hepática. G – Necrose e fibrose hepática. H – Neoplasia, tumor benigno encapsulado.

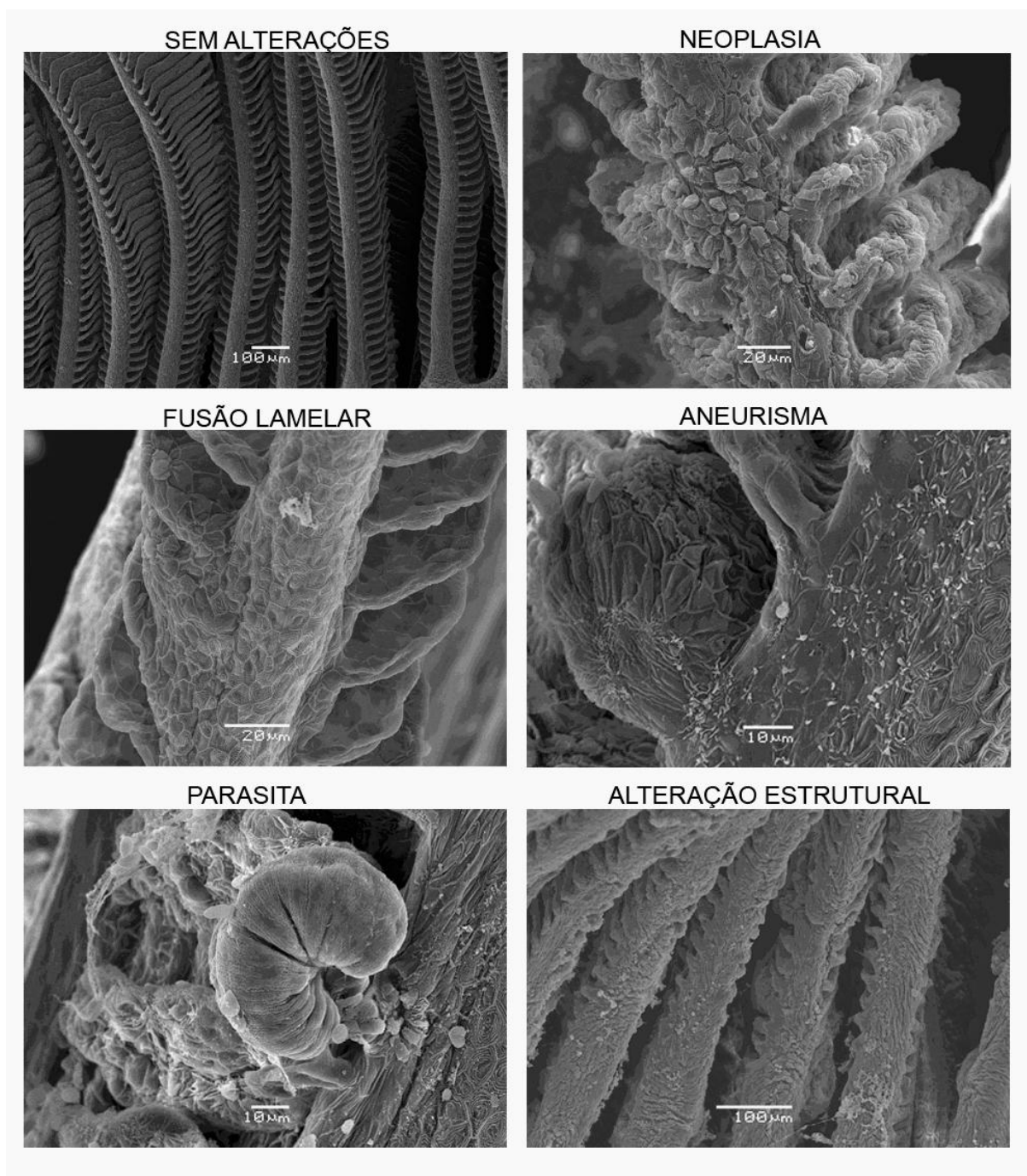


FIGURA 4. Principais alterações visualizadas nas brânquias de *G. brasiliensis* através da microscopia eletrônica de varredura.

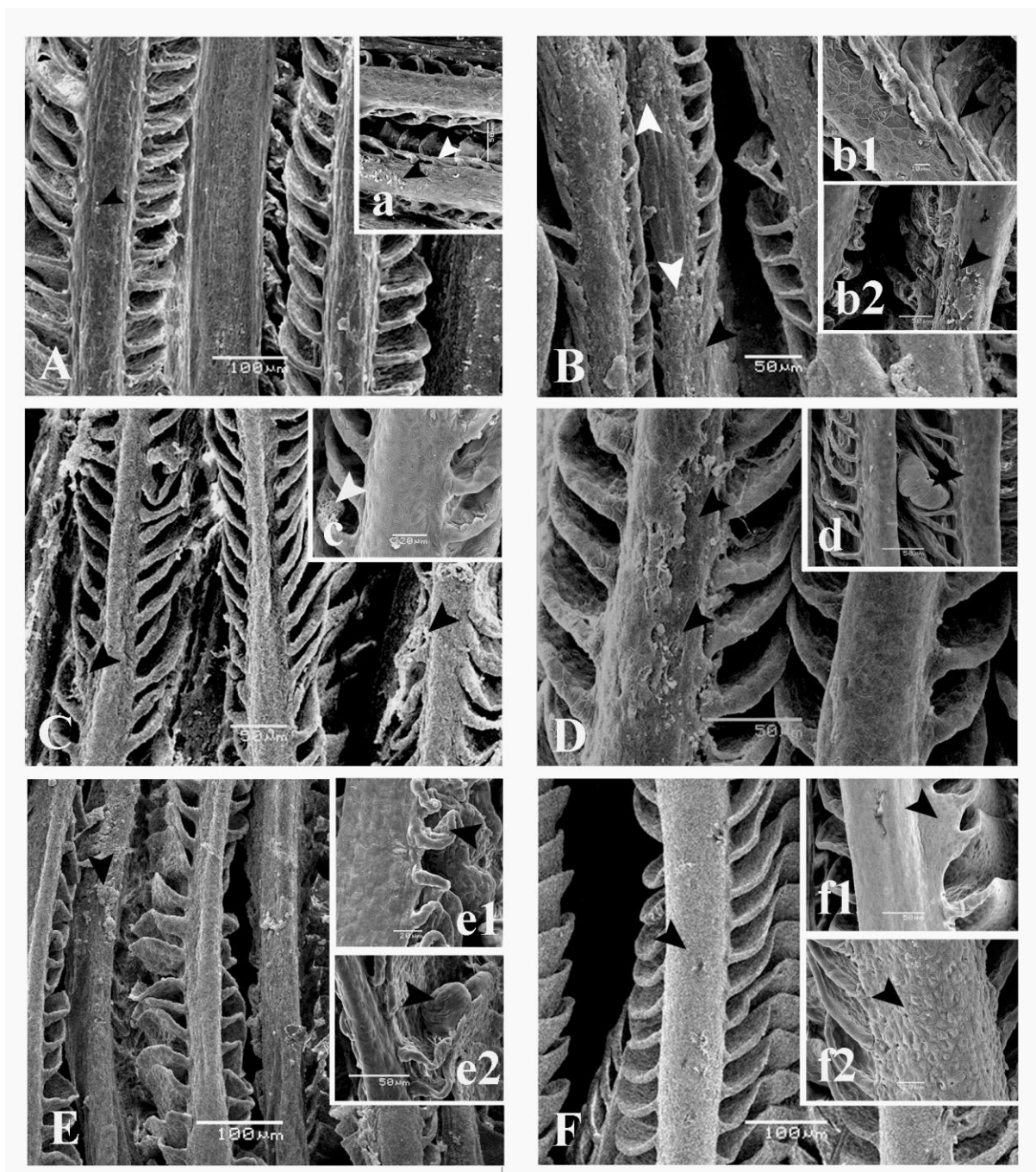


FIGURA 5. Microscopia eletrônica de varredura das brânquias de *C. Iguassuensis*. A – FA inverno: Danos foram observados ambos em lamelas primárias e secundárias (ponta das setas). B – FA verão: Necrose na lamela secundária do epitélio (ponta das setas brancas) , b2 e danos da lamela secundária (b1). C – SE verão: observe o intenso desarranjo da lamela secundária, incluindo fusão (ponta da seta). Em detalhe (c) morte celular são observadas no epitélio da lamela secundária. D – SS inverno: Área de necrose no epitélio da lamela no epitélio da lamela secundária (ponta da seta) e parasita (d). E – SO verão: área de necrose no epitélio da lamela secundária (ponta da seta) e desarranjo da lamela secundária (e2). F – SC verão: danos no epitélio da lamela primária (ponta da seta) e fusão da lamela secundária (f1).

Table 3. Summary of factor loadings (after Varimax rotation) of the FA/PCA applied on the data matrices of *A. Bifasciatus* in two steps: (A) accounted for all cases; (B) accounted for all variables. The variance of the principal factors is given in percentage of the total variance in the original matrix. Loading cut-off was set at 0.55 and 0.60, respectively.

	(A)				(B)		
	F1	F2	F3	F4	F1	F2	F3
% variance	22.049	19.126	15.691	12.173	41.90 7	38.179	19.91 5
Comet assay	-0.146	-0.165	0.400	-0.301	0.882	0.472	-0.013
Micronucleus	-0.171	-0.460	0.059	0.044	-0.915	0.356	0.188
Blebed	0.138	0.828	0.126	0.328	0.302	-0.811	0.501
Notched	-0.087	0.936	-0.220	0.202	0.059	-0.949	0.311
Mononuclear alterations	-0.066	0.914	-0.019	0.338	0.049	-0.934	0.354
Polychromatic erythrocytes	0.530	-0.318	0.297	-0.631	-0.004	0.969	0.245
LPO	0.061	0.715	-0.629	-0.092	0.109	-0.931	0.349
GST	-0.277	-0.194	0.548	0.146	-0.595	-0.746	-0.301
Catalase	0.315	0.085	0.461	0.592	-0.077	-0.906	0.416
AChE muscle	0.407	-0.695	-0.135	0.271	-0.208	0.267	-0.941
AChE Brain	0.866	-0.187	-0.050	0.035	-0.764	0.108	-0.636
Hepatosomatic index	0.055	-0.088	-0.857	0.067	-0.629	0.386	-0.675
Gonadosomatic index	-0.386	-0.219	-0.731	0.216	0.159	0.285	-0.945
Histopathological index	-0.671	-0.365	-0.277	-0.069	0.044	0.710	-0.703
Condition factor	-0.216	0.044	0.489	-0.130	-0.135	0.221	0.966
Niwater	-0.194	0.208	0.416	-0.745	0.678	0.552	0.485
Cdwater	-0.317	0.446	0.570	-0.517	0.369	0.073	0.926
Znwater	-0.044	-0.222	-0.105	-0.795	-0.353	0.843	0.407
Crwater	0.141	-0.822	-0.356	0.379	-0.677	0.354	-0.645
Mnwater	-0.326	-0.633	-0.373	0.128	-0.852	0.431	-0.297
Ag sed	0.155	-0.143	0.623	0.303	0.638	-0.343	0.689
Pb sed	0.981	0.074	-0.120	0.094	-	-	-
Cd sed	0.545	0.348	0.024	-0.016	0.830	0.277	0.484
Cr sed	0.143	-0.078	0.310	-0.059	-0.915	0.356	0.188
Cu sed	0.981	0.081	-0.051	0.126	-0.090	-0.979	-0.180
Mn sed	-0.358	0.365	0.511	0.526	0.082	-0.971	0.225
Zn sed	0.959	0.160	-0.157	0.093	0.920	-0.363	-0.145
Ni sed	0.531	0.386	0.628	0.001	0.315	-0.924	0.215
Cr bb	-0.020	-0.032	0.178	-0.911	0.362	0.920	0.151
Cu bb	0.473	-0.416	0.294	0.359	0.977	0.213	-0.014
Mn bb	0.270	0.577	-0.285	0.203	-0.834	-0.464	0.298
Cd bb	-0.240	-0.096	0.139	-0.757	0.707	0.620	0.340
Zn bb	-0.149	0.011	0.238	0.317	-0.296	-0.936	-0.189
Pb bb	0.819	-0.409	0.013	0.319	-0.851	-0.107	-0.515
Co bb	-0.112	0.125	-0.011	-0.680	-	-	-
PAHs 6 rings	-	-	-	-	0.451	-0.175	-0.875
PAHs 5 rings	-	-	-	-	0.913	0.104	-0.394
PAHs 4 rings	-	-	-	-	0.925	0.311	0.219
PAHs 2 rings	-	-	-	-	-0.976	0.154	-0.155

Table 4. Summary of factor loadings (after Varimax rotation) of the FA/PCA applied on the data matrices of *G. Brasiliensis* in two steps: (A) accounted for all cases; (B) accounted for all variables. The variance of the principal factors is given in percentage of the total variance in the original matrix. Loading cut-off was set at 0.55.

	(A)				(B)			
	F1	F2	F3	F4	F1	F2	F3	F4
% variance	29.88	24.23	19.45	10.68	35.33	25.71	18.82	15.06
	0	7	0	6	3	6	1	3
Comet assay	0.603	0.518	0.280	0.196	0.684	0.224	0.664	-0.186
Micronucleus	0.809	-0.335	-0.186	0.002	0.913	-0.279	-0.262	0.054
Blebed	0.838	-0.032	0.292	-0.103	0.793	0.375	-0.312	0.167
Notched	0.910	-0.150	0.117	0.125	0.992	0.053	-0.093	-0.049
Mononuclear alterations	0.916	-0.131	0.104	0.104	0.994	0.036	-0.082	-0.031
Polychromatic erythrocytes	-0.179	0.856	-0.008	0.149	-0.312	-0.189	0.876	-0.267
Catalase	0.534	0.308	0.542	0.347	0.378	0.803	0.173	-0.396
AChE muscle	0.853	0.302	0.174	-0.265	0.824	0.242	0.300	0.260
AChE Brain	-0.620	0.554	-0.324	-0.063	-0.891	-0.371	0.237	-0.082
LPO	-0.232	0.877	-0.176	0.363	-0.339	-0.139	0.784	-0.501
Protein Carbonization	0.232	0.332	-0.386	0.372	-0.141	-0.206	-0.023	-0.856
GST	-0.174	-0.951	0.039	-0.125	0.019	0.059	-0.944	0.317
GPx	0.520	-0.737	0.192	0.250	0.584	0.251	-0.713	-0.162
GSH	0.074	0.927	0.189	-0.171	-0.041	0.156	0.967	0.112
Hepatosomatic index	-0.627	-0.505	-0.059	-0.376	-0.557	-0.256	-0.547	0.504
Gonadosomatic index	-0.455	-0.247	0.695	-0.031	-0.362	0.878	0.006	0.153
Histopathological index	-	-	-	-	0.353	-0.274	-0.795	-0.315
Condition factor	-0.053	-0.821	-0.439	0.038	0.287	-0.657	-0.690	0.080
Niwater	0.837	-0.204	-0.386	-0.104	0.913	-0.279	-0.262	0.054
Cdwater	0.702	0.154	-0.617	0.153	0.732	-0.568	0.216	-0.280
Znwater	0.465	0.098	-0.231	-0.627	0.437	-0.114	0.244	0.846
Crwater	-0.570	0.059	0.583	-0.456	-0.516	0.486	0.249	0.607
Mnwater	-0.457	0.332	-0.084	-0.754	-0.513	-0.275	0.326	0.740
Ag sed	0.480	0.272	0.128	0.531	0.716	-0.014	0.232	-0.658
Pb sed	0.159	0.053	0.957	0.047	0.097	0.968	-0.027	0.061
Cd sed	0.375	-0.601	0.539	0.219	0.647	0.454	-0.554	-0.016
Cr sed	0.055	0.557	0.656	-0.413	0.011	0.560	0.629	0.484
Cu sed	0.148	0.100	0.953	0.094	0.079	0.973	0.001	0.007
Mn sed	-0.301	0.133	-0.233	0.841	-0.123	-0.255	0.262	-0.900
Zn sed	0.159	0.049	0.959	0.056	0.102	0.969	-0.027	0.054
Ni sed	0.395	0.455	0.402	0.598	0.251	0.614	0.315	-0.677
Crbb	-	-	-	-	-0.574	0.503	0.407	-0.439
Cubbb	-	-	-	-	-0.258	0.611	0.096	-0.718
Mnbb	-	-	-	-	-0.201	0.743	0.462	0.307
Cdbb	-	-	-	-	0.882	-0.295	-0.345	0.087
Znbb	-	-	-	-	-0.007	0.730	-0.515	-0.359
Cobb	-	-	-	-	-0.438	-0.074	-0.656	0.444
PAHs 6 rings	-	-	-	-	0.928	-0.249	-0.242	0.063
PAHs 5 rings	-	-	-	-	-0.052	0.266	0.856	0.287
PAHs 4 rings	-	-	-	-	-0.051	0.880	0.433	0.034
PAHs 2 rings	-	-	-	-	-0.403	0.135	0.778	0.009

TABLE 5. Summary of factor loadings (after Varimax rotation) of the FA/PCA applied on the data matrices of *C. iguassuensis* in two steps: (A) accounted for all cases; (B) accounted for all variables. The variance of the principal factors is given in percentage of the total variance in the original matrix. Loading cut-off was set at 0.55 and 0.60, respectively.

	(A)				(B)		
	F1	F2	F3	F4	F1	F2	F3
% variance	27.52	25.11	21.31	16.62	45.54	31.49	22.97
Comet assay	-0.393	-0.207	0.237	-0.824	-0.945	0.189	0.267
Blebed	0.211	0.530	-0.677	0.408	0.838	0.497	0.227
Notched	0.496	0.140	-0.569	0.597	0.956	0.277	-0.097
Mononuclear alterations	0.469	0.308	-0.554	0.603	0.970	0.241	0.030
Policrromatic erythrocytes	0.607	0.554	-0.313	-0.461	0.694	0.377	0.614
Catalase	-0.173	-0.436	0.552	0.674	-0.202	-0.800	-0.565
AChE muscle	0.713	0.481	-0.024	-0.103	0.942	-0.152	0.298
AChE Brain	0.923	-0.020	0.050	-0.018	0.927	-0.253	-0.278
LPO	0.297	0.906	0.027	-0.273	0.383	-0.029	0.923
Protein Carbonilation	0.224	0.956	0.039	-0.144	0.410	-0.098	0.907
GST	-0.793	-0.091	-0.211	0.020	-0.860	0.409	-0.303
Hepatosomatic index	-0.024	-0.322	0.317	0.876	0.176	-0.755	-0.632
Gonadosomatic index	0.640	-0.518	-0.332	0.164	0.597	0.168	-0.784
Condition factor	-0.801	-0.187	-0.241	-0.343	-0.850	0.507	-0.142
Niwater	-0.893	0.005	-0.244	0.188	-0.893	0.426	-0.144
Cdwater	-0.668	0.220	-0.627	0.120	-0.383	0.919	0.096
Znwater	0.151	-0.916	0.023	-0.159	-0.200	0.056	-0.978
Crwater	0.663	-0.531	0.441	0.259	0.436	-0.651	-0.621
Mnwater	0.615	-0.579	-0.290	0.295	0.573	0.089	-0.815
Agshed	0.090	0.170	0.056	-0.943	-0.515	0.476	0.713
Pbshed	0.173	0.089	0.958	0.038	-0.111	-0.991	0.072
Cdshed	-0.825	0.084	0.551	0.085	-0.869	-0.448	0.209
Crshed	-0.195	0.132	0.003	0.835	0.438	-0.648	-0.624
Cused	0.152	0.174	0.936	0.091	-0.076	-0.990	0.119
Mnsed	-0.059	0.762	-0.474	0.157	0.398	0.416	0.818
Znsed	0.164	0.094	0.960	0.045	-0.115	-0.990	0.078
Nised	-0.142	0.834	0.356	0.110	0.060	-0.557	0.829
PAHs 6rings	-	-	-	-	0.977	-0.168	0.132
PAHs 5 rings	-	-	-	-	0.988	-0.086	0.126
PAHs 4 rings	-	-	-	-	0.810	-0.568	0.146
PAHs 2 rings	-	-	-	-	-0.302	0.946	-0.118

CAPÍTULO III

TABELA 6. Detecção de Poluentes Orgânicos Persistentes na água dos quatro reservatórios do Rio Iguaçu

PCBs	SE	SS	SO	SC
PCB 8	< LD	3,62	< LD	< LD
PCB 201	< LD	9,56	< LD	< LD
PCB 203	< LD	1,48	< LD	< LD
Σ 51 PCBs	< LD	14,7	< LD	< LD
Pesticidas (OCs)	SE	SS	SO	SC
α-HCH	< LD	< LD	< LD	< LD
β-HCH	< LD	1,51	< LD	< LD
γ-HCH	< LD	< LD	< LD	< LD
δ-HCH	< LD	< LD	< LD	< LD
Σ HCHs	< LD	1,51	< LD	< LD
Aldrin	0,68	0,78	1,85	1,87
Isodrin	0,95	0,85	2,99	< LD
Dieldrin	< LD	4,06	1,21	< LD
Endrin	< LD	< LD	< LD	< LD
Σ Drins	1,63	4,91	6,05	1,87
α-clordana	< LD	< LD	< LD	< LD
γ-clordana	< LD	< LD	< LD	< LD
oxi-clordana	< LD	< LD	< LD	< LD
Σ clordanas	< LD	< LD	< LD	< LD
o,p'-DDE	< LD	< LD	< LD	< LD
p,p'-DDE	< LD	< LD	< LD	< LD
o,p'-DDD	< LD	< LD	< LD	< LD
p,p'-DDD	< LD	0,69	< LD	< LD
o,p'-DDT	< LD	< LD	< LD	< LD
p,p'-DDT	< LD	< LD	< LD	< LD
Σ DDTs	< LD	0,69	< LD	< LD
HCB	< LD	< LD	< LD	< LD
Heptacloro	< LD	< LD	< LD	< LD
Heptacloro Ep.A	< LD	< LD	1,44	2,75
Heptacloro Ep.B	1,22	< LD	< LD	0,78
Endossulfan I	< LD	< LD	< LD	< LD
Endossulfan II	< LD	3,81	4,15	6,13
Metoxicloro	< LD	< LD	< LD	< LD
Mirex	< LD	< LD	< LD	< LD
Σ POCs	2,85	11,70	11,64	11,53

Congêneros de PCBs não detectados - 18, 31, 28, 33, 52, 49, 44, 74, 40, 66, 95, 56/60, 101, 99, 97, 81, 87, 77, 110, 151, 123, 149, 118, 114, 153, 132, 105, 141, 138, 158, 126, 187, 183, 128, 167, 174, 156, 157, 180, 169, 170, 189, 195, 194, 206, 209

E de PBDEs: 28, 100, 99, 154, 153 e 183

TABELA 7. Detecção de PCBs e PBDEs nos tecidos (Brânquias, músculo e fígado) das tilápias mantidas nos quatro reservatórios do Rio Iguaçu

PCBs	SE brânq.	SE musc.	SE fíga.	SS brânq.	SS musc.	SS fíga.	SO brânq.	SO musc.	SO fíga.	SC brânq.	SC musc.	SC fíga.
PCB 95 non dioxin-like	< LD	< LD	< LD	< LD	< LD	0,58	0,57	< LD	0,87	0,53	< LD	< LD
PCB 99 non coplanar	< LD	< LD	< LD	< LD	< LD	0,52	< LD	< LD	< LD	< LD	< LD	< LD
PCB 105 dioxin-like	< LD	< LD	< LD	< LD	< LD	0,65	< LD	< LD	< LD	< LD	< LD	0,63
PCB 158 non-dioxin	< LD	< LD	< LD	< LD	< LD	1,74	< LD	< LD	3,20	< LD	< LD	< LD
PCB 201	< LD	< LD	< LD	< LD	< LD	< LD	0,82	< LD	< LD	< LD	< LD	0,56
PCB 203 non dioxin	1,72	4,48	< LD	< LD	< LD	4,23	4,99	< LD	4,36	4,60	2,35	4,87
PCB 195 non dioxin	< LD	< LD	< LD	1,48	< LD	< LD	< LD	< LD	< LD	1,53	< LD	< LD
PCB 194 non coplanar	1,04	0,52	< LD	< LD	< LD	< LD	0,99	< LD	< LD	< LD	1,18	< LD
Σ 51 PCBs	2,76	5,00	< LD	1,48	< LD	7,72	7,37	< LD	8,43	6,66	3,53	6,06
Σ 7 PCBs	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD

PBDEs	SE brânq.	SE musc.	SE fíga.	SS brânq.	SS musc.	SS fíga.	SO brânq.	SO musc.	SO fíga.	SC brânq.	SC musc.	SC fíga.
BDE 28	< LD	< LD	< LD	0,59	< LD	1,33	0,60	< LD	< LD	< LD	< LD	< LD
BDE 47	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
BDE 100	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	1,10	0,65	< LD	< LD
BDE 99	< LD	< LD	< LD	0,92	< LD	0,89	< LD	0,61	2,44	< LD	< LD	< LD
BDE 154	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
BDE 153	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
BDE 183	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Σ PBDEs	< LD	< LD	< LD	1,51	< LD	2,22	0,60	0,61	3,54	0,65	< LD	< LD

Congêneros de PCBs não detectados nos tecidos dos peixes: 8, 18, 31, 28, 33, 52, 49, 44, 74, 70, 66, 56,60, 101, 97, 81, 110, 151,123, 149, 118, 114, 153, 132, 141, 138, 126, 187, 183, 128, 167, 174, 177, 156, 157, 180, 169, 170, 189, 206, 209

TABELA 8. Detecção de pesticidas organoclorados nos tecidos (Brânquias, músculo e fígado) das tilápias mantidas nos quatro reservatórios do Rio Iguaçu

Pesticidas (OCs)	SE brânq.	SE musc.	SE fíga.	SS brânq.	SS musc.	SS fíga.	SO brânq.	SO musc.	SO fíga.	SC brânq.	SC musc.	SC fíga.
Aldrin	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Isodrin	< LD	< LD	< LD	< LD	< LD	< LD	1,27	< LD	< LD	< LD	< LD	< LD
Dieldrin	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Endrin	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Σ Drins	< LD	< LD	< LD	< LD	< LD	< LD	1,27	< LD	< LD	< LD	< LD	< LD
α-clordana	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
γ-clordana	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
oxi-clordana	< LD	< LD	< LD	< LD	< LD	1,40	< LD	< LD	2,65	< LD	< LD	< LD
Σ clordanas	< LD	< LD	< LD	< LD	< LD	1,40	< LD	< LD	2,65	< LD	< LD	< LD
o,p'-DDE	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
p,p'-DDE	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
o,p'-DDD	< LD	< LD	< LD	< LD	< LD	0,54	< LD	< LD	2,07	< LD	< LD	< LD
p,p'-DDD	< LD	< LD	< LD	< LD	< LD	< LD	0,52	< LD	< LD	< LD	< LD	< LD
o,p'-DDT	< LD	< LD	< LD	< LD	< LD	< LD	0,63	< LD	< LD	< LD	< LD	< LD
p,p'-DDT	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Σ DDTs	< LD	< LD	< LD	< LD	< LD	0,54	1,15	< LD	2,07	< LD	< LD	< LD
HCB	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Heptacloro	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Heptacloro Ep.A	< LD	1,39	< LD	< LD	2,69	< LD	1,15	< LD	< LD	< LD	1,51	< LD
Heptacloro Ep.B	< LD	7,01	1,95	< LD	< LD	< LD	< LD	< LD	< LD	1,58	< LD	< LD
Endossulfan I	1,70	< LD	< LD	1,96	< LD	< LD	1,36	0,87	< LD	< LD	< LD	< LD
Endossulfan II	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Metoxicloro	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD
Mirex	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	< LD	0,61
Σ POCs	1,70	8,40	1,95	1,96	2,69	1,94	4,93	0,87	4,72	1,58	1,51	0,61

