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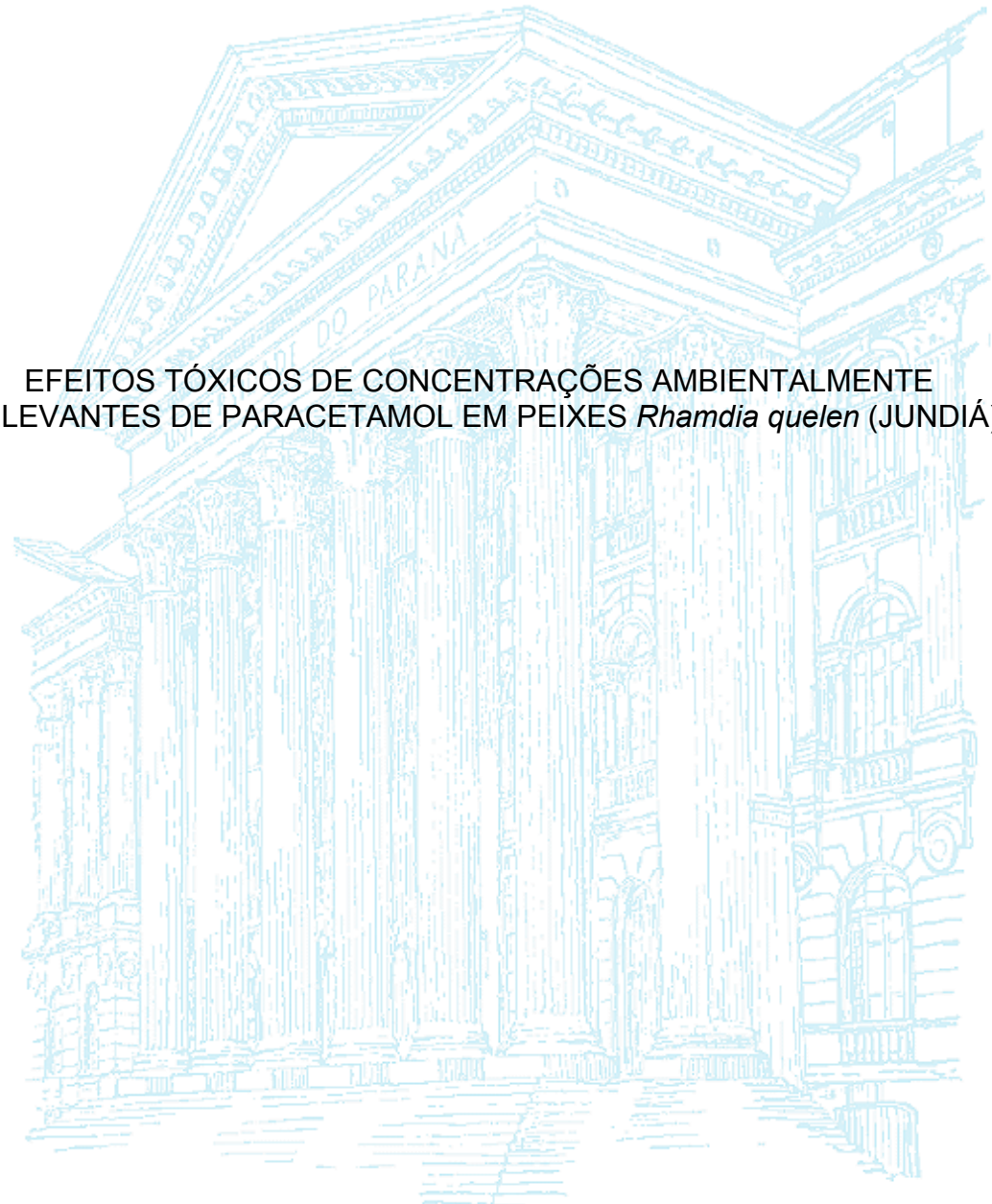
MAIARA CAROLINA PERUSSOLO



EFEITOS TÓXICOS DE CONCENTRAÇÕES AMBIENTALMENTE RELEVANTES DE PARACETAMOL EM PEIXES *Rhamdia quelen* (JUNDIÁ)

CURITBA
2018

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Orientadora: Profa. Dra. Helena Cristina Silva de Assis

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **MAIARA CAROLINA PERUSSOLO** intitulada: **Efeitos tóxicos de concentrações ambientalmente relevantes de paracetamol em peixes Rhamdia quelen (jundiá)**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 22 de Fevereiro de 2018.

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Presidente da Banca Examinadora (UFPR)

IZONETE CRISTINA GUILOSKI
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CINTIA MARA RIBAS DE OLIVEIRA
Avaliador Externo (UP)

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Esta dissertação é apresentada na forma de artigo científico para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e um artigo científico abordando os experimentos realizados, com resultados e discussão, bem como conclusão. O artigo científico foi formatado conforme as normas propostas pelo periódico ao qual foi submetido.

Dedico esta dissertação a minha mãe, que desde sempre me apoiou e estimulou a correr atrás dos meus sonhos (sejam eles quais forem!), a minha irmã, que aguentou com paciência meus períodos bons e os não tão bons e ao meu pai, que – tenho certeza- está feliz e vibrante por mais esse ciclo que encerro, onde quer que esteja.

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“Eu não me vejo na palavra
Fêmea: alvo de caça,
conformada vítima

Prefiro queimar o mapa,
traçar de novo a estrada,
ver cores nas cinzas
e a vida reinventar.”

Francisco, el Hombre em “Triste, louca ou má”.

RESUMO

A presença de fármacos em compartimentos ambientais torna-se cada vez mais comum, visto que além do descarte incorreto a atual tecnologia de tratamento não os elimina totalmente. Este trabalho avaliou efeitos tóxicos de concentrações de paracetamol encontradas em ambientes aquáticos em peixes *Rhamdia quelen* utilizando biomarcadores bioquímicos, genéticos, fisiológicos, hematológicos e morfológicos. Os animais foram divididos em quatro grupos, sendo um controle acetona (solvente) e três expostos a 0.25, 2.5 e 25 $\mu g.L^{-1}$ de paracetamol por via hídrica. Após 14 dias os peixes foram anestesiados com benzocaína 1% e realizada a coleta de sangue para contagem de leucócitos e trombócitos e ensaio cometa. Posteriormente, os peixes foram eutanasiados através de secção medular, sendo brânquias e rim posterior coletados para análises dos biomarcadores. Amostras de água foram coletadas durante o experimento (0, 24 e 48 h, 7 e 14 dias) para análise química. Os resultados apontaram para a capacidade genotóxica do paracetamol, sendo o fármaco capaz de aumentar os danos em DNA em sangue e brânquias na concentração de 25 $\mu g.L^{-1}$ e em rim posterior nas concentrações de 2.5 e 25 $\mu g.L^{-1}$. Além disso, o fármaco alterou o sistema antioxidante de brânquias e rim em todas as concentrações testadas e causou lipoperoxidação em brânquias no grupo 2.5 $\mu g.L^{-1}$. A concentração total de espécies reativas de oxigênio (EROs) em brânquias não sofreu alterações entre os grupos, enquanto no rim houve uma redução da concentração de EROs no grupo 0.25 $\mu g.L^{-1}$ quando comparado ao controle. A contagem de leucócitos e trombócitos foi reduzida no grupo de maior concentração. Houve uma redução da concentrações de íons magnésio, potássio e sódio, além da redução de atividade da enzima anidrase carbônica em tecido branquial. O paracetamol foi capaz de promover elevação de epitélio respiratório, fusão de lamelas secundárias e vasodilatação em brânquias de animais do grupo 25 $\mu g.L^{-1}$. No rim posterior, foram observados núcleos picnóticos, degeneração glomerular e infiltração de leucócitos e adipócitos no grupo 25 $\mu g.L^{-1}$. Os resultados demonstram que o paracetamol foi genotóxico para sangue, rim posterior e brânquias, além de ter seu caráter nefrotóxico evidenciado em *R. quelen*. Estes dados apontam para a necessidade de monitoramento das concentrações de paracetamol encontradas nos compartimentos aquáticos a fim de evitar riscos para a saúde animal e humana.

Palavras- chave: Paracetamol; peixe; estresse oxidativo; nefrotoxicidade,

ABSTRACT

The presence of pharmaceutical drugs in environmental compartments becomes increasingly common, since the besides incorrect disposal, the current treatment technology does not totally eliminate them. This study evaluated the toxic effects of paracetamol concentrations found in aquatic environments in *Rhamdia quelen* fish using biochemical, genetic, physiological, hematological and morphological biomarkers. The animals were divided into four groups, one acetone (solvent) and three exposed to 0.25, 2.5 and 25 $\mu g.L^{-1}$ paracetamol via hidric. After 14 days the fish were anesthetized with 1% benzocaine and blood was taken for leukocyte and thrombocytes count and comet assay. Subsequently, the fish were euthanized through a medullary section, and gills and posterior kidney were collected for analysis. Water was sampled during the experiment (0, 24 and 48 h, 7 and 14 days) for chemical analysis. The results showed the genotoxic capacity of paracetamol, since the drug was able to increase DNA damage in blood and gills at concentrations of 25 $\mu g.L^{-1}$ and in posterior kidney at concentrations of 2.5 and 25 $\mu g.L^{-1}$. In addition, the pharmaceutical altered the antioxidant system of gills and kidney at all tested concentrations and caused gills lipoperoxidation in the 2.5 $\mu g.L^{-1}$ group. The total concentration of reactive oxygen species (ROS) in the gills was not statistically significant among the groups, whereas in the kidney a reduction in the concentration of ROS was observed in the group 0.25 $\mu g.L^{-1}$ when compared to the control group. The count of defence cells (leukocytes and thrombocytes) were reduced in the highest concentration. There was a reduction of magnesium, potassium and sodium ions concentrations, as well as the reduction of the enzymatic activity of the carbonic anhydrase in gill tissue. Paracetamol was able to promote respiratory epithelial elevation, secondary lamella fusion and vasodilation in gills of 25 $\mu g.L^{-1}$ animals. In the posterior kidney, pyknotic cells, glomerular degeneration and infiltration of leukocytes and adipocytes were observed in the 25 $\mu g.L^{-1}$ group. The results demonstrate that paracetamol was genotoxic for blood, posterior kidney and gills, besides having its nephrotoxic character evidenced in *R. quelen*. The data point to the need to monitoring the concentrations of paracetamol found in aquatic compartments, in order to avoid risks to the aquatic animal and human health.

Keywords: Acetaminophen; fish; oxidative stress; nephrotoxicity.

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LISTA DE SIGLAS E ABREVIATURAS

AINE (NSAID)	-	Anti-inflamatório não esteroidal
ALFAC	-	Álcool, Formol e Ácido acético
ANOVA	-	Análise de variância
ANVISA	-	Agência Nacional de Vigilância Sanitária
EAC (CAA)	-	Ensaio de anidrase carbônica
CAT	-	Catalase
CEUA	-	Comitê de Ética no Uso de Animais
COX	-	Cicloxigenase
CYP	-	Sistema citocromo oxidase, Citocromo P450
DCF	-	Diclorofluoresceína
DCFH-DA	-	20-70- diclorofluoresceína-diacetato
EROS (ROS)	-	Espécies reativas de oxigênio
FDA	-	*Food and Drug Administration*
GPx	-	Glutationa peroxidase
GSH	-	Glutationa reduzida
GST	-	Glutationa-S-Transferase
HE	-	Hematoxilina- Eosina
CLAE (HPLC)	-	Cromatografia líquida de alta eficiência
IBRv2	-	Índice de Integração de Biomarcadores
LPO	-	Lipoperoxidação
MGGW	-	May Grünwald-Giemsa-Wright
NAPQI	-	N-acetil-p-benzo-quinonamina
SOD	-	Superóxido dismutase
SNC	-	Sistema nervoso central
UNIOESTE	-	Universidade do Oeste do Paraná
UFPR	-	Universidade Federal do Paraná
UV	-	Ultravioleta

SUMÁRIO

1 INTRODUÇÃO

Com o aumento do consumo e utilização de fármacos humanos e veterinários, a presença dessas substâncias no ambiente aquático tornou-se alvo de muitos estudos ecotoxicológicos pelo mundo (AL-KHAZRAJY & BOXALL, 2016; BIEL-MAESO *et al.*, 2018; HOYETT *et al.*, 2016; PAPAGEORGIOU *et al.*, 2016). Sua chegada aos efluentes acontece, na maioria das vezes, pelo destino incorreto de esgotos domésticos, industriais e hospitalares (EBELE *et al.*, 2017). Além desse fator, as unidades de tratamento de esgoto não possuem, em sua maior parte, tecnologias que possibilitem a total retirada desses contaminantes da água (TROMBINI *et al.*, 2016). A introdução contínua desses contaminantes aos efluentes, mesmo que em baixas concentrações, fornece potencial toxicidade aos organismos alí presentes (AL-KHAZRAJY & BOXALL, 2016; TROMBINI *et al.,*, 2016) causando danos diversos a organismos como algas, daphnias e peixes, por exemplo (SANTOS *et al.*, 2013).

Algumas das classes de fármacos de maior relevância quando se considera a contaminação de compartimentos ambientais aquáticos são os antibióticos, anti-inflamatórios não- esteroidais (AINEs), hormônios e β-bloqueadores (ÁLVAREZ-MUÑOZ *et al.*, 2015). AINEs são as drogas mais frequentemente detectadas em ambiente aquático, tendo como seus principais representantes os fármacos Ibuprofeno, Dicloflenaco, Paracetamol e Naproxeno (BÁCSI *et al.*, 2016). Esses fármacos agem, resumidamente, inibindo as isoformas da enzima cicloxigenase (COX) - COX 1 e COX 2- de forma seletiva ou não (STANCOVÁ *et al.*, 2015). Vandermeersch *et al.* (2015) citam o paracetamol e o diclofenaco como os fármacos mais encontrados em organismos aquáticos como peixes e moluscos, variando das concentrações 4,9 até 490 $ng.g^{-1}$. Oliveira *et al.* (2015) demonstraram, em estudo que expôs de forma subcrônica (14 dias) indivíduos da espécie *Daphnia magna* a três diferentes concentrações (0.01, 0.1 e 1 $mg.L^{-1}$) de paracetamol e diclofenaco, uma atividade inibitória de colinesterases.

A presença do paracetamol em corpos hídricos é relatada frequentemente em concentrações que variam de 31.97 a 0.05 $\mu g.L^{-1}$ (BIEL-MAESO *et al.*, 2018; CHEN *et al.*, 2016; HUBER *et al.*, 2016; SPONGBERG *et al.*, 2011). Seus efeitos tóxicos já relatados incluem desregulação endócrina em peixes (GUILOSKI *et al.*, 2017), estresse oxidativo em moluscos (CORREIA *et al.*, 2016), alterações bioquímicas e

hepatotoxicidade em peixes (JYOTSNA, 2016), por exemplo. Considerando os efeitos do paracetamol no modelo animal desde estudo, o peixe *Rhamdia quelen*, poucos são os relatos. O peixe *R. quelen*, conhecido como jundiá, é uma espécie nativa da América do Sul (BARCELLOS *et al.*, 2010). A espécie é considerada um bom modelo experimental em estudos ecotoxicológicos, visto que fatores como adaptação ao ambiente laboratorial, manutenção, custos e cuidados são facilitados (CARNEIRO & MIKOS, 2005).

Apesar de, em estudos ecotoxicológicos, as respostas a contaminantes serem avaliadas separadamente há necessidade de estudos que analisem de forma geral e integrada o potencial efeito tóxico do fármaco paracetamol em peixes juvenis da espécie *Rhamdia quelen* a fim de esclarecer e aprofundar conhecimentos já existentes contribuindo, também, para fortalecer a imprescindibilidade de uma legislação que promova o controle da concentração de fármacos em ambiente aquático.

2 REVISÃO DE LITERATURA

2.1 FÁRMACOS EM AMBIENTE AQUÁTICO

Com o avanço tecnológico, aumento populacional e de estimativa de vida, cada vez mais novos fármacos vêm sendo disponibilizados para uso humano e também veterinário. Entre os meses de Janeiro e Dezembro de 2017 foram aprovadas 155 novas drogas nos Estados Unidos (FDA, 2017). No Brasil, apenas no ano de 2015, foram aprovados 742 novos medicamentos (ANVISA, 2016). Após o consumo, boa parte desses fármacos são excretados ainda em sua forma original, além de diferentes metabólitos (BIEL-MAESO *et al.*, 2018; BURKINA *et al.*, 2016), sendo encaminhados para estações de tratamento de esgoto as quais, por sua vez, possuem um sistema de tratamento convencional limitado e ineficaz na retirada de moléculas bioativas de fármacos (BIEL-MAESO *et al.*, 2018).

Além do consumo, fármacos chegam ao ambiente aquático por diferentes vias (Figura 1). O descarte incorreto de fármacos, além de resquícios industriais de farmacêuticas são grandes precursores da presença desses contaminantes no meio aquático (SANJUAN-REYES *et al.*, 2015).

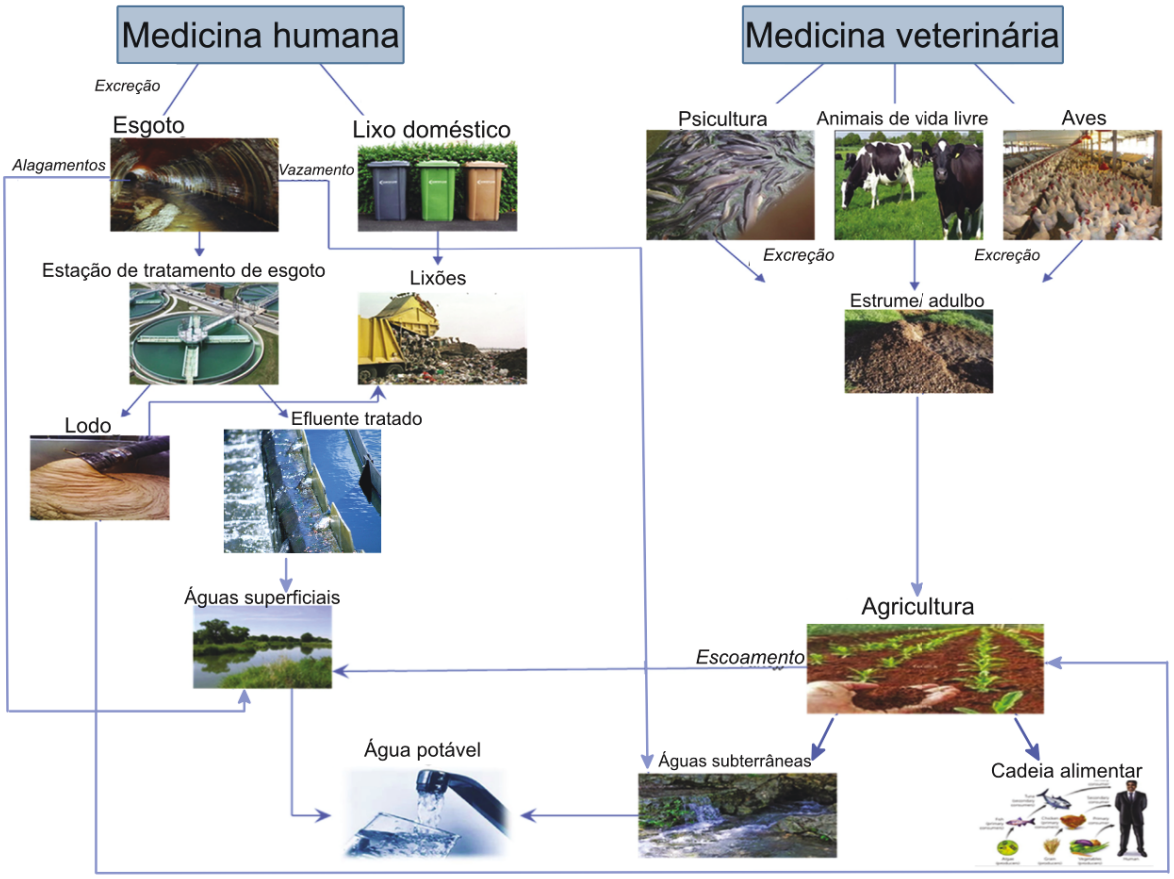


Figura 1: Principais vias de chegada de fármacos ao ambiente aquático (Fonte: adaptado de Ebele *et al.*, 2017).

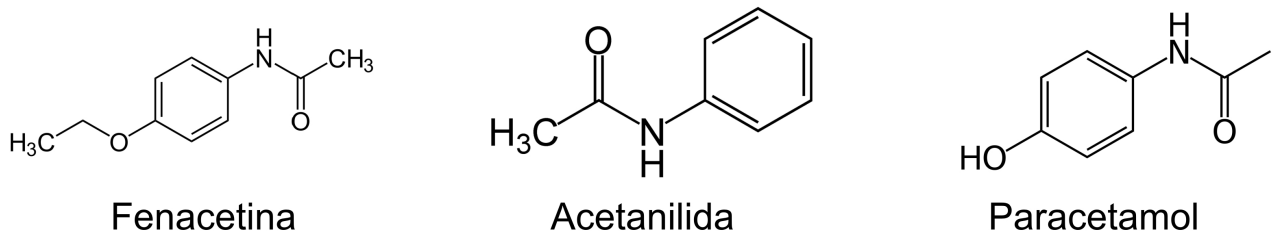
A detecção de fármacos em efluentes tornou-se bastante comum, sendo essas análises foco de estudos (BU *et al.*, 2016; OSORIO *et al.*, 2016). As baixas concentrações encontradas, aliadas à introdução constante dessas substâncias ao ambiente e à falta de conhecimento sobre os possíveis efeitos tóxicos aos organismos expostos classifica-os como contaminantes emergentes (GOGOI *et al.*, 2018).

A ação de fármacos sob organismos aquáticos têm sido foco de estudos ecotoxicológicos (GONZALEZ-REY & BEBIANNO, 2011; OVERTURF *et al.*, 2016; XIA *et al.*, 2017). Entre os fármacos estudados estão as classes mais comumente encontradas em análises de efluentes: analgésicos, anti-inflamatórios não-esteroidais (AINEs) e antibióticos (ÁLVAREZ-MUÑOZ *et al.*, 2015; BIEL-MAESO *et al.*, 2018). Dentre os AINEs, destacam-se os fármacos ibuprofeno, dipirona, paracetamol, diclofenaco e naproxeno. Xia *et al.* (2017) demonstraram, em ensaio agudo, aumento na velocidade de eclosão de ovos e alterações em comportamento motor de embriões de *Danio rerio* expostos, 6 horas após a fecundação, aos

fármacos ibuprofeno, diclofenaco e paracetamol nas concentrações de 5, 50 e 500 $\mu g.L^{-1}$. A exposição a maior concentração de ibuprofeno e diclofenaco reduziu o tempo de incubação dos embriões, além de reduzir movimento espontâneo e distância de natação dos animais. Stancová *et al.* (2015) atestaram, em experimento subcrônico (14 dias), o efeito tóxico do fármaco naproxeno. Indivíduos *Danio rerio* expostos as concentrações de 1 e 100 $\mu g.L^{-1}$ apresentaram aumento nos níveis de RNA mensageiro (*m*RNA*)* da enzima catalase em ambas concentrações de fármaco testadas. Além disso, os níveis de *m*RNA para a enzima GST apresentaram-se aumentados na concentração de 100 $\mu g.L^{-1}$. Em relação a dipirona, Pamplona *et al.* (2011) verificaram a redução no número de eritrócitos, trombócitos e no valor de hematócrito de peixes *Rhamdia quelen* expostos as concentrações de 0.5, 5 e 50 $\mu g.L^{-1}$ durante 15 dias. Outros efeitos tóxicos já demonstrados com AINEs incluem alteração da integridade do tecido renal, osmorregulação e alteração do sistema antioxidante de diferentes espécies de peixes expostos ao diclofenaco (LUBIANA *et al.*, 2016; NÄSLUND *et al.*, 2017; SARAVANAN & MANOHARAN *et al.*, 2011) e alteração de parâmetros hematológicos de peixes (SARAVANAN *et al.*, 2012), estresse oxidativo e desregulação endócrina de mariscos (GONZALEZ-REY & BEBIANNO, 2011) expostos ao ibuprofeno.

2.2 PARACETAMOL

O paracetamol (N-acetil-p-aminofenol), um fármaco utilizado como analgésico não opióide e antipirético, foi descoberto em 1887 - como metabólito ativo dos fármacos fenacetina e acetanilida- e teve sua comercialização iniciada apenas em 1950, quando dúvidas sobre sua toxicidade foram esclarecidas (CANDIDO *et al.*, 2017). As estruturas químicas dos fármacos fenacetina, acetanilida e paracetamol estão representadas na Figura 2.



Fenacetina Acetanilida Paracetamol

Figura 2: Estrutura química dos fármacos fenacetina, acetanilida e paracetamol. (Fonte: Sociedade Brasileira de Química - Disponível em: http://qnint.sbq.org.br).

Apesar de alguns autores o considerarem um AINE, na prática clínica o paracetamol possui pequena atividade anti-inflamatória (CHANDRASEKHARAN *et al.*, 2002). Para mais, seu mecanismo de ação desvia-se do mecanismo clássico dos fármacos dessa classe pois a inibição da enzima cicloxigenase (COX 1 e 2) não ocorre diretamente e sim pela redução de peroxinitritos, moléculas ativadoras da COX, em tecidos com baixa concentração de ácido araquidônico e peróxidos (AMINOSHARIAE & KHAN, 2015; ARONOFF *et al.*, 2006). Fala-se, ainda, de uma terceira isoenzima COX envolvida no mecanismo de ação do paracetamol, a COX-3. Enzima derivada da já conhecida COX-1, a COX-3 foi foco de estudos que demonstraram uma maior concentração da mesma ocorrendo em córtex cerebral, o que pode explicar a maior ação a nível de sistema nervoso central (SNC) de AINEs como o paracetamol, por exemplo (CHANDRASEKHARAN *et al.*, 2002; OKSUZ *et al.*, 2016).

A maior parte de moléculas do fármaco sofre biotransformação no fígado (90% do total absorvido) através da atuação do sistema citocromo oxidase P450 (CYP450) e excreção pelos rins, o paracetamol torna-se responsável por desencadear efeitos tóxicos bem conhecidos em humanos e roedores como a hepatotoxicidade e a nefrotoxicidade em casos de overdose ou uso crônico (MCGILL & JAESCHKE, 2013). Vários são os mecanismos sugeridos em estudos pelos quais o fármaco desencadeia reações fisiológicas tóxicas. O principal mecanismo estudado correlaciona a formação do metabólito bioativo N-acetil-p-benzo-quinonamina (NAPQI), e a redução dos níveis intracelulares de glutationa reduzida (GSH) com o dano ao DNA e outros eventos tóxicos sofrido pelas células alvo como hepatócitos, por exemplo (SIEMIONOW *et al.*, 2016). Cerca de 5% do total de moléculas de paracetamol absorvidas são convertidas em NAPQI que, em doses terapêuticas, são conjugadas pela GSH e inativadas para posterior eliminação. Porém, em casos de overdoses, os estoques de GSH esgotam-se, fazendo com que a concentração de NAPQI intracelular aumente, desencadeando uma série de efeitos tóxicos como o dano em DNA e RNA e oxidação de membranas lipídicas (NUNES *et al.*, 2014). Além disso, a biotransformação do paracetamol pode ser responsável pela geração de grande quantidade de espécies reativas de oxigênio (EROS), as quais por sua vez podem desempenhar papel tóxico no organismo quando não neutralizadas (MCGILL & JAESCHKE, 2013). Outros mecanismos de toxicidade como estresse oxidativo em retículos endoplasmáticos e redução da

concentração de enzimas antiapoptóticas Bcl-xL, também são relatados (KALINEC *et al.*, 2014; LORZ *et al.*, 2005; NAGY *et al.*, 2010).

2.2.1 PARACETAMOL E COMPARTIMENTOS AQUÁTICOS

Devido a seu alto consumo, o paracetamol é um dos fármacos mais frequentemente detectados em amostras ambientais (OSORIO *et al.*, 2016), como demonstrado na tabela 1. Ao entrar no meio ambiente, o paracetamol pode passar por processos de oxidação química, fotodegradação, adsorção, hidrólise e biotransformação, por exemplo (LIANG *et al.*, 2016). Essas transformações geram metabólitos que, por sua vez, podem ser bioativos ou não. Já é conhecido, por exemplo, um metabólito resultante da fotodegradação do paracetamol por luz ultravioleta (UV), identificado como 1-(2-amino-5-hidroxifenil)etanona. Em estudo, esse metabólito se mostrou mais tóxico do que o fármaco original em teste com bactérias luminescentes (KAWABATA *et al.*, 2012).

Tabela 1: Concentrações de paracetamol detectadas em efluentes.

Média pré tratamento	**Média pós tratamento**	**Local**	**Referência**
10 $\mu g.L^{-1}$	< 0.05 $\mu g.L^{-1}$	República Checa	(CHEN *et al.*, 2016)
31.97 $\mu g.L^{-1}$	Não detectado	Espanha	(BIEL-MAESO *et al.*, 2018)
13 $\mu g.L^{-1}$	Não dosado	Costa Rica	(SPONGBERG *et al.*, 2011)
159.22 $\mu g.L^{-1}$	1.72 $\mu g.L^{-1}$	Portugal	(PAÍGA *et al.*, 2016)
38.31 $\mu g.L^{-1}$	3.14 $\mu g.L^{-1}$	China	(WANG *et al.*, 2017)
1.18 $\mu g.L^{-1}$	2.53 $\mu g.L^{-1}$	Islândia e Groelândia	(HUBER *et al.*, 2016)
> 0.24 $\mu g.L^{-1}$	Não dosado	Brasil	(KRAMER *et al.*, 2015)

Por conta da incidência do paracetamol em compartimentos aquáticos, o entendimento de seus efeitos tóxicos agudos e crônicos em organismos de diferentes níveis tróficos é de extrema relevância. Nunes *et al.* (2014) demonstraram concentrações de paracetamol que induzem metade do efeito máximo (EC50) em diferentes espécies de organismos aquáticos: *Vibrio fischeri* (bactéria), *Pseudokirchneriella subcapitata* (microalga), *Cylindrospermopsis raciborskii* (cianobactéria), *Lemna minor* e *Lemna gibba* (plantas) e *Daphnia magna* e *Daphnia longispina* (microcrustáceos). Os resultados deste edtudo apontam para uma grande variedade de efeitos tóxicos, bastante específicos para a condição fisiológica de

cada espécie. Esse fator é um dos complicantes em estudos ecotoxicológicos, visto que impossibilita a extrapolação de dados para outras espécies (NUNES *et al.*, 2014). Outro exemplo de como as condições fisiológicas alteram o efeito tóxico desempenhado pelo paracetamol foi descrito por Correia *et al.* (2016) em estudo que expôs mariscos da espécie *Ruditapes philippinarum* às concentrações de 0.05, 0.5 e 5 $mg.L^{-1}$ de paracetamol em diferentes salinidades (14, 28 e 35 ^{o}/oo). Os resultados encontrados apresentam diferenças significativas na extensão do efeito tóxico causado pelo fármaco aos animais expostos dependente da salinidade. O estresse oxidativo causado pelo paracetamol foi, inclusive, reduzido em casos onde o aumento de salinidade permitiu ao animal suportar a substância tóxica.

O uso de peixes em pesquisas sobre efeitos tóxicos do paracetamol acaba não sendo tão comum quando comparado ao uso de invertebrados mariscos e ostras, por exemplo. Porém, a toxicidade do paracetamol em peixes já foi relatada. A hepatotoxicidade do paracetamol foi demonstrada em peixes adultos da espécie *Rhamdia quelen* expostos a 0.25 e 2.5 $\mu g.L^{-1}$ (GUILOSKI *et al.*, 2017). O potencial nefrotóxico da droga foi descrito em larvas de peixes da espécie *Danio rerio* por Gorgulho *et al.* (2017). No estudo, o paracetamol foi capaz de reduzir *clearance* renal, causar hipertrofia dos tubos proximais e gerar alterações em mitocôndrias como: edema mitocondrial, dismorfias e quebra das cristas mitocondriais. Outras alterações fisiológicas causadas pelo paracetamol em organismos aquáticos incluem extresse oxidativo (NUNES *et al.*, 2015) e desregulação endócrina em peixes (GUILOSKI *et al.*, 2017), por exemplo.

Apesar da alta incidência desse contaminante em compartimentos ambientais, os atuais tratamentos convencionais de efluentes são ineficazes na retirada completa dessas moléculas (SANJUAN-REYES *et al.*, 2015). Tecnologias alternativas vêm sendo estudadas e testadas em diferentes etapas do tratamento de efluentes: utilização de filtros de biochar (DALAHMEH *et al.*, 2018), ozonização catalítica (WANG, *et al.* 2017), uso de biorreatores de membranas anaeróbias (XIAO *et al.*, 2017), aumento no tempo de retenção hidráulica (EJHED *et al.*, 2018) e a combinação entre o tratamento convencional a biofiltros (MCKIE *et al.*, 2016) são exemplos.

2.3 MODELO DE ESTUDO E BIOMARCADORES

A compreensão da atuação de fármacos em peixes traz aspectos de grande relevância ecológica, visto que peixes são encontrados em grande parte dos compartimentos aquáticos desempenhando importante papel em teias alimentares ao transportarem energia de níveis tróficos baixos a mais elevados (OOST *et al.*, 2003). O peixe *Rhamdia quelen,* pertencente à ordem Siluriformes, família Pimelodidae (Figura 3) tornou-se umpeixe de grande importância econômica, visto que não apresenta espinhos intramosculares e possui uma carne saborosa, aumentando sua aceitação com o mercado consumidor (CARNEIRO & MIKOS, 2005). São animais onívoros, com altas taxas de fecundação e fácil manejo (CARNEIRO & MIKOS, 2005) sendo, essas características, interessantes para a utilização da espécie como modelo de estudo. em relação a agrotóxicos (GOLOMBIESKI *et al.*, 2016), metais pesados (PEREIRA *et al.*, 2016) e fármacos (GUILOSKI *et al.*, 2017), por exemplo.

Figura 3: Exemplar juvenil de *Rhamdia quelen* (Fonte: A autora, 2016).

Dentre as diversas ferramentas utilizadas para estimar o impacto de contaminantes emergentes em organismos aquáticos encontram-se os biomarcadores. Estes, por sua vez, consistem em respostas biológicas que facilitam a determinação da extensão do dano sofrido pelo organismo exposto (AMORIM, 2003) sendo mensurados por alterações bioquímicas e celulares, por exemplo. Ao mensurar efeitos tóxicos sofridos por organismos alvo, a utilização de biomarcadores que possam detectar alterações ainda em fase inicial é priorizada em relação aos que apontam apenas alterações mais tardias (OOST *et al.*, 2003)(Figura 4).

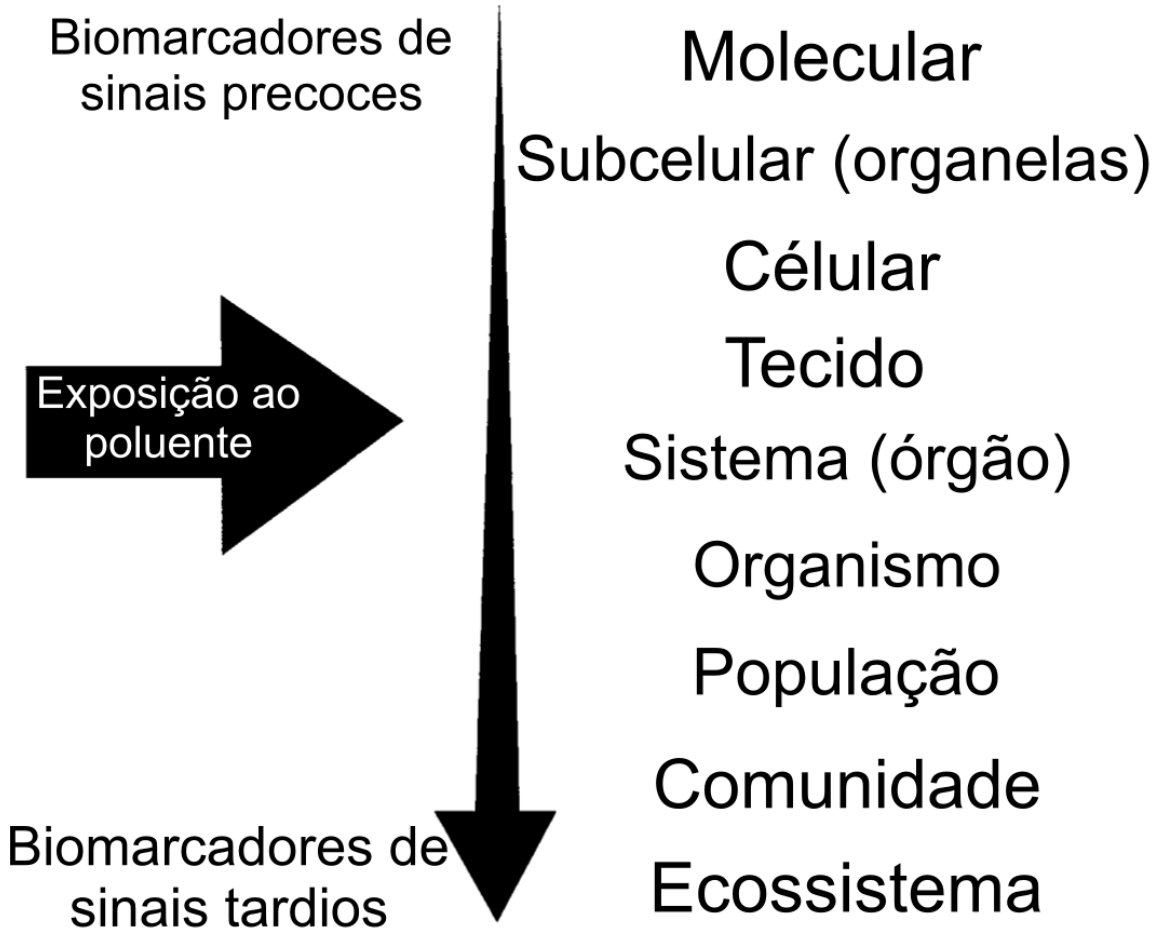


Figura 4: Sistema representativo da ordem de respostas biológicas em exposição a contaminantes. (Fonte: adaptado de Oost *et al.,* 2003).

Biomarcadores bioquímicos de estresse oxidativo, assim como biomarcadores hematológicos e imunológicos, fisiológicos, genéticos e morfológicos são amplamente utilizados, fornecendo dados importantes sobre os contaminantes estudados e contribuindo, também, com informações sobre o organismo exposto. Pamplona *et al.* (2011), por exemplo, demonstraram a capacidade hematotóxica do AINE dipirona em peixes *Rhamdia quelen* expostos as concentrações de 0.5, 5 e 50 $\mu g.L^{-1}$ do fármaco. No estudo, os organismos expostos sofreram redução da contagem de hemáceas e trombócitos, além da redução do hematócrito em todas as concentrações.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito tóxico da exposição dos peixes da espécie *Rhamdia quelen* a concentrações ambientalmente relevantes de paracetamol.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar possíveis alterações bioquímicas, hematológicas e osmorregulatórias através de biomarcadores em *Rhamdia quelen* após exposição subcrônica ao paracetamol;
- Avaliar possíveis danos em DNA através do ensaio cometa em rim posterior, sangue e brânquias de *Rhamdia quelen* expostos de forma subcrônica ao paracetamol;
- Avaliar possíveis danos teciduais em rim posterior e brânquias de *Rhamdia quelen* expostos de forma subcrônica ao paracetamol.

4 ARTIGO CIENTÍFICO

Toxic effects of environmentally relevant concentrations of Paracetamol in fish *Rhamdia quelen* (Jundiá).

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Abstract

Environmental contamination by pharmaceutical drugs is a worldwide problem, mainly because the treatment systems do not remove them entirely. The nonsteroidal anti-inflammatory drugs (NSAIDs) are amongst the most commonly detected classes of pharmaceuticals in effluent analysis and paracetamol is the the most frequently one. The aim of this study was to evaluate the possible toxic effects of environmentally relevant concentrations (0.25, 2.5 and 25 $\mu g.L^{-1}$) of paracetamol in *Rhamdia quelen* fish exposed for 14 days. Some biochemical, genetic, hematological, physiological and morphological biomarkers were evaluated. It was observed oxidative stress in posterior kidney and gills at all concentrations tested and lipoperoxidation in gills of animals exposed only to the concentration of 2.5 $\mu g.L^{-1}$. Genotoxicity was found in blood and gills at concentrations of 2.5 $\mu g.L^{-1}$ and in posterior kidney at 2.5 and 25 $\mu g.L^{-1}$. An osmoregulatory imbalance in plasma ions and a reduction in the activity of carbonic anhydrase in gills of the group exposed to 0.25 $\mu g.L^{-1}$ were observed. Tissue alterations occurred in gills of fish exposed to 25 $\mu g.L^{-1}$ and in posterior kidney at concentrations of 0.25 and 25 $\mu g.L^{-1}$ of paracetamol. These results demonstrated toxic effects of paracetamol on the fish, evidencing the need for monitoring the concentration of drugs released in the aquatic environment.

Keywords: Acetaminophen; oxidative stress; DNA damage; fish; nephrotoxicity.

1. Introduction

The presence of human and veterinary pharmaceutical drugs in the aquatic environment has been the focus of many ecotoxicological studies (Hoyett *et al.*, 2016; Papageorgiou *et al.*, 2016), and these drugs are viewed as emerging contaminants because of their high capacity of producing physiological effects in low doses (Ebele *et al.*, 2017). The presence of such substances in wastewater occurs most often through incorrect destination of the domestic, industrial and hospital sewage (Daouk *et al.*, 2016; Ding *et al.*, 2016; Liang *et al.*, 2016). Besides, sewage treatment plants do not have treatment that allow the total removal of these contaminants from water, in most of the cases (Trombini *et al.*, 2016).

The continuous release of these effluents can cause toxicity in aquatic organisms. Toxic effects range from acute to chronic levels (Al-Khazrajy and Boxall, 2016; Trombini *et al.*, 2016). It is estimated that about 12,000 different drug formulations are prescribed and distributed for human use (FDA, 2013). Considering the environmental contamination compartments, the most relevant classes are antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), hormones and β-blockers (Álvarez-Muñoz *et al.*, 2015). Paracetamol, a drug used as an analgesic and antipyretic, has been found in effluent analyzes around the world in varying concentrations (Biel-Maeso *et al.,* 2018; Chen *et al.,* 2016; Huber *et al.*, 2016; Kramer *et al.*, 2015; Paíga *et al.,* 2016; Spongberg *et al.*, 2011; Wang *et al.,* 2017). Mizukawa (2016) found in a study, that the concentration of paracetamol in different rivers of the Upper Iguaçu Basin (Paraná, Brazil) ranged from 0.01 $\mu g.L^{-1}$ to 94.68 $\mu g.L^{-1}$. In the aquatic organisms (fish and mollusks) the concentrations ranged from 4.9 to 490 $ng.g^{-1}$ (Vandermeersch *et al.*, 2015). Its mechanism of action involves the blockade of the synthesis of prostaglandins through the inhibition of the enzyme

cicloxigenase (COX) (Candido *et al.*, 2017; Kalani *et al.,* 2016).

Toxic effects have already been reported by exposure of aquatic organisms to paracetamol such as endocrine disruption (Álvarez-Muñoz *et al.*, 2015; Guiloski *et al.*, 2017b; You *et al.*, 2015), oxidative stress (Correia *et al.*, 2016), and biochemical and hepatic changes (Guiloski *et al.*, 2017a; Jyotsna, 2016; Nunes *et al.*, 2017). Nephrotoxicity is also an effect reported by paracetamol in mammals (Inoue *et al.*, 2017; Pérez-Villalva *et al.*, 2017; Yiang *et al.*, 2015). Gorgulho *et al.* (2017) demonstrated that paracetamol reduced renal *clearance*, increased mitochondrial changes as a dysmorphic shapes, loss of matrix granules and dilatation of proximal tubes in kidney of *Danio rerio* larvae. However, studies that demonstrate the same effect in juveniles or adult fish are scarce.

In this study our studied model was *Rhamdia quelen* fish, a fast-growing native fish species from Central and South America (Benaduce *et al.*, 2008). It has been used in *in vivo* studies with pollutants such as metals (Pereira *et al.*, 2016) pesticides (Sobjak *et al.*, 2017) and anti-inflammatory drugs (Guiloski *et al.*, 2017b). Since some hepatotoxic effects of paracetamol have already been described (Guiloski *et al.*, 2017a), this study aimed to analyze the toxic effects of paracetamol in other tissues. The blood, gills and posterior kidney of adult fish of the *Rhamdia quelen* species after 14 days of exposure to three concentrations of paracetamol were analyzed through hematological, genetic, biochemical, physiological and morphological biomarkers. Integrated biomarker analysis was also performed using the Integrated Biomarker Response (IBRv2) to provide an overview of the effects of the drug at each concentration tested.

2. Material and methods

2.1 Animals

The *Rhamdia quelen* fish, (N=80) measuring 14.02 ± 0.64 cm and weighing 25.2 g ± 3.1, were obtained from the State University of the West of Paraná (UNIOESTE) and transported to the Laboratory of Environmental Toxicology of the Pharmacology Department at Paraná Federal University (UFPR). The animals were acclimatized for 30 days in 100 L tanks containing filtered water, with constant aeration (dissolved O_2 7.5 ppm), temperature of 25 ± 2 ° C and photoperiod of 12 hours, being fed with commercial feed (MP 31 Brazil) once a day. The project was approved by the Ethics Committee for the Use of Animals (CEUA), of the Biological Sciences Sector of the Federal University of Parana (UFPR) under number 995.

2.2 Experimental design

After the acclimation period the animals were exposed via water for 14 days to three concentrations of paracetamol (Acetaminophen- A5000; CAS number 103-90-2; Sigma Aldrich, USA; powder; purity ≥ 98%; slightly soluble in water). The concentrations chosen (0.25, 2.5 and 25 $\mu g.L^{-1}$) were based on environmentally relevant concentrations found in the water. The concentrations of paracetamol already detected in water ranging from 0.01 $\mu g.L^{-1}$ (Togola and Budzinski, 2008) up to 94.68 $\mu g.L^{-1}$ (Mizukawa, 2016). Four groups were maintained: three test groups (0.25, 2.5 and 25 $\mu g.L^{-1}$) and one solvent control (acetone – 100 $\mu g.L^{-1}$). The experimente run in tree replicates per group (n=20 fish per group), fed once daily with commercial fish food (MP 31 Brazil). The degradation of paracetamol in water was analysed in a previous study in the lab. Based on this study half of the water of each experimental tanks was replaced with the contaminant each 24 hours. After 14 days of exposure the animals were anesthetized with 1% benzocaine, and the blood

was collected by the caudal vein. After the euthanasia by medullary section, posterior kidney and gills were collected and frozen at -80°C for further biomarkers evaluation.

2.3 Water chemical analysis

During the exposure period, water samples were collected from each group to quantify the total paracetamol concentration during the experiment. The analysis of the samples was performed by high performance liquid chromatography (HPLC), using a Metrohm chromatographic system model 882 Compact IC Plus equipped with an automatic sampler model 863 Compact and detector model 887 Professional UV/Vis. The samples were eluted in mobile phase (88% water, 11% acetonitrile and 1% acetic acid) at a flow rate of 1.0 $mL.min^{-1}$ and using a C18 column (150 x 4.6 mm, 5 µm, Phenomenex) at 25°C with detection at 250 nm. Quantifications were performed on external calibration using standard solution ofparacetamol, with the detection limit of 2.5 $\mu g.L^{-1}$. In order to detect and quantify the concentration of paracetamol in all groups, a known standard (10 $\mu g.L^{-1}$) was added, and the actual value of the sample was subtracted from the concentration of the standard.

2.4 Biochemical biomarkers

Gill and posterior kidney were homogenized in potassium phosphate buffer (0.1 M, pH 7.0) in a ratio of 1:10 (m/v) using a micro-homogenizer. The homogenate was centrifuged for 30 minutes at 15,000 x g at 4 ° C.

The total protein concentration was measured by the Bradford method (1976). Glutathione-S-transferase (GST) activity was measured according to the method described by Keen *et al.* (1976). The method established by Gao *et al.* (1998), was used to measured the activity of the enzyme Superoxide dismutase (SOD), and Catalase (CAT) activity according to the method of Aebi (1984). The activity of the

enzyme Glutathione peroxidase (GPx), was measured by the method described by Hafeman *et al.* (1974), and the concentration of reduced Glutathione (GSH) according to the method proposed by Sedlak and Lindsay (1968). The levels of lipoperoxidation (LPO) were analyzed through the evaluation of the hydroperoxide concentration by the FOX test (Jiang *et al.*, 1992).

The total ROS concentration was quantified using the 20-70-dichlorofluorescein-diacetate (DCFH-DA) assay as previously described by Driver *et al.*, (2000). The formation of dichlorofluorescein (DCF) was measured with a spectrofluorimeter at 485 and 528 nm for excitation and emission, respectively. The results are expressed as units of fluorescence.

2.5 Genotoxic biomarker

The DNA damage was analyzed by the comet assay with a technique described by Speit and Hartmann (1999), modified by Cestari *et al.* (2004) and Ferraro *et al.* (2004) for blood and Ramsdorf *et al.* (2009) for kidney and gills.

The assay was performed in a blind test of 100 nucleoids per slide under an epifluorescence microscope with a magnification of 400x. The nucleotide classification was performed according to Collins *et al.* (1997) through the length of the tail formed after electrophoresis, being classified from 0 (without apparent damage) to 4 (maximum damage).

2.6 Physiological biomarkers

2.6.1 Plasma magnesium, sodium and potassium concentration:

Plasma magnesium concentration was determined colorimetrically using a kit (Labtest, Brazil) in samples appropriately diluted in deionized water with absorbance

read at 505 nm. Sodium and potassium concentrations were determined through flame photometry in samples diluted in deionized water 1:100.

2.6.2 Carbonic anhydrase activity assay (CAA)

Tissue samples were weighed and homogenized at 10% (weight/ volume, in g/mL) with 10 mM phosphate buffer at pH 7.4, using an ultrasonic homogenizer. The homogenate was then centrifuged (2000x g for 5 min at room temperature), and the supernatant was aliquoted for the protein and CAA assays. CAA was assayed according to the method described by Vitale *et al.* (1999). The slope of the regression line of pH reduction along time(s) results in the catalyzed rate of activity of the carbonic anhydrase present in the sample (Henry, 1991; Vitale *et al.*, 1999). Total protein concentration in tissue homogenate was measured according to Bradford, (1976).

2.7 Hematological biomarker

Blood smears were stained with May Grünwald-Giemsa-Wright (MGGW). The total count of leukocytes and thrombocytes was performed under a microscope at 1000x, according to the methodology described by Tavares-Dias and Moraes (2006).

2.8 Histopathological biomarker

A fragment of the posterior kidney and gills were fixed in ALFAC solution (80% alcohol, formaldehyde and glacial acetic acid) for 16 hours. After that, they were dehydrated in alcohols (70%, 80%, 90% and 100%), diaphanized in xylol and included in Paraplast®. They were cut into 5 µm slices and stained with hematoxylin-eosin (HE). After the procedure, the sections were analyzed according to the injury index described by Bernet *et al.* (1999), where photoreceptors alterations were

classified in three severity factors (minimal, moderate and marked pathological importance). The injury indexes were obtained after the application of a mathematical equation established for each group of lesions:

$$IL = \sum_{pr} \sum_{alt} (a.w)$$

where *pr* is the reaction pattern; *alt* is the alteration; *a* is the score value; *w* is the importance factor. The damages considered to establish the index lesion in gills were: lifting of respiratory epithelium, cellular hyperplasia and vasodilation. In posterior kidney, the damages considered were: dimunition of mesangial cells, glomerular hypertrophy, presence of pyknotic cells, glomerular atrophy, glomerular degeneration, disappearance of the glomerular capsule and leukocyte infiltration.

2.9 Data analysis

Data normality was tested using the Kolmogorov-Smirnov test. For the parametric data, a one-way analysis of variance (ANOVA) was performed, followed by the Newman-Keuls test, with results expressed as mean ± standard error, and significance when $p < 0.05$. For the non-parametric data the Kruskal-Wallis test was performed, followed by the Dunn test and results were expressed in median. The results for biomarkers were applied to the Integrated Biomarker Responses Index (IBRv2) described by Beliaeff and Burgeot (2002) and modified by Sanchez *et al.* (2013) and expressed in star plot charts.

3. Results

3.1 Experimental conditions

No mortality was observed. The tank water parameters (expressed as mean ± standard error) were as follows: pH (6.7 ± 0.02 units), ammonia (0.46 ± 0.12 ppm),

nitrite (0.15 ± 0.07 ppm), dissolved O_2 (7.33 ± 0.25 ppm), hardness (53.33 ± 3.33 ppm) and temperature (20.4 ± 0.62 ° C). These values were not different among the groups.

3.2 Chemical analysis of water

The values found (mean ± standard error) for the nominal concentrations for paracetamol were: 0.65 ± 0.41 $\mu g.L^{-1}$ for the concentration of 0.25 $\mu g.L^{-1}$, 2.58 ± 0.60 $\mu g.L^{-1}$ for the concentration of 2.5 $\mu g.L^{-1}$ and 12.79 ± 1.64 $\mu g.L^{-1}$ for the concentration of 25 $\mu g.L^{-1}$.

3.3 Biochemical biomarkers

In the gills, paracetamol reduced GST activity (27% for 0.25 $\mu g.L^{-1}$, 19% for 2.5 $\mu g.L^{-1}$ and 25% for 25 $\mu g.L^{-1}$) and SOD activity (28% for 25 $\mu g.L^{-1}$) when compared to the control group (Table 1). The activity of CAT was not altered, whereas GPx presented an increase of 66% in concentration of 2.5 $\mu g.L^{-1}$ and 44% in the concentration of 25 $\mu g.L^{-1}$ when compared to the control group (Table 1). There was a reduction in GSH levels (69%, 59% and 48% for 0.25, 2.5 and 25 $\mu g.L^{-1}$ respectively), when compared to the control group (Table 1). LPO was significantly increased (47%) at the concentration of 2.5 $\mu g.L^{-1}$ when compared to the control group (Table 1). The concentration of ROS did not present statistical difference when compared to the control (Table 1).

In the posterior kidney, paracetamol increased the activities of GST (37% for 2.5 $\mu g.L^{-1}$), CAT (47% for 2.5 µg.L-1 and 51% for 25 µg. L^{-1}) and GPx (65% for 0.25 $\mu g.L^{-1}$, 137% for 2.5 µg.L-1 and 123 for 25 $\mu g.L^{-1}$) when compared to control group (Table 1). Paracetamol also increased GSH levels (164% for 0.25 $\mu g.L^{-1}$, 87% for 2.5

$\mu g.L^{-1}$ and 63% for 25 $\mu g.L^{-1}$) when compared to control group (Table 1). The SOD activity and LPO levels did not show a significant statistical difference compared to the control group (Table 1). ROS concentration was reduced (28% for 0.25 $\mu g.L^{-1}$) when compared to the control (Table 1).

Table 1: Biochemical biomarkers for gills and posterior kidney of *R. quelen* exposed to paracetamol.

Tissue/ Concentration	GST	SOD	CAT	GPx	GSH	LPO	ROS
GILLS							
Control	76.21 ± 3.53 ^{a}	256.90 ± 16.1 ^{a}	24.28 ± 1.05 ^{a}	27.44 ± 1.62 ^{a}	2.31 ± 0.34 ^{a}	3.00 ± 0.60^{a}	0.60 ± 0.98 ^{a}
0.25 µg.L^{-1}	56.30 ± 3.27 ^{b}	219.60 ± 14.53 ^{ab}	22.34 ± 1.51 ^{a}	29.55 ± 3.20 ^{a}	0.72 ± 0.14 ^{b}	3.82 ± 0.34 ^{ab}	1.21 ± 0.82 ^{a}
2.5 µg.L^{-1}	62.48 ± 2.05 ^{b}	211.90 ± 13.65 ^{ab}	23.93 ± 1.38 ^{a}	45.71 ± 3.02 ^{b}	0.97 ± 0.20 ^{b}	4.41 ± 0.33 ^{b}	0.78 ± 0.82 ^{a}
25 µg.L^{-1}	57.27 ± 5.33 ^{b}	187.00 ± 7.04 ^{b}	26.12 ± 0.99 ^{a}	39.66 ± 3.61 ^{b}	1.20 ± 0,24 ^{b}	3.50 ± 0.07 ^{ab}	1.21 ± 0.46 ^{a}
KIDNEY							
Control	87.59 ± 9.46 ^{a}	150.70 ± 12.62 ^{a}	37.34 ± 3.36 ^{a}	16.38 ± 1.04 ^{a}	2.77 ± 0.34 ^{a}	9.93 ± 1.09 ^{a}	2.71 ± 0.85 ^{a}
0.25 µg.L^{-1}	102.60 ± 8.60 ^{ab}	183.60 ± 9.16 ^{a}	48.68 ± 5.75 ^{ab}	27.12 ± 1.97 ^{b}	7.35 ± 1.06 ^{b}	12.09 ± 1.04 ^{a}	1.28 ± 0.63 ^{b}
2.5 µg.L^{-1}	120.40 ± 5.27 ^{b}	190.8 ± 11.69 ^{a}	45.89 ± 3.35 ^{b}	38.91 ± 2.65 ^{c}	5.21 ± 0.41 ^{c}	12.8 ± 1.41 ^{a}	2.35 ± 1.14 ^{a}
25 µg.L^{-1}	110.00 ± 7.79 ^{ab}	175.50 ± 10.03 ^{a}	56.64 ± 3.88 ^{b}	36.53 ± 1.79 ^{c}	4.53 ± 0.51 ^{c}	13.74 ± 1.04 ^{a}	2 ± 0 ^{ab}

Glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), lipoperoxidation (LPO). SOD (U/mg of protein), GST, CAT and GPx (nmol/min/mg of protein), GSH (µg/mg of protein) LPO (nmol of hydroperoxides/mg of protein). Values expressed as mean standard error. Different letters when p <0.05 (n=10).

3.4 Genotoxic biomarker

The DNA damage increased in the blood (314% for 25 $\mu g.L^{-1}$), in posterior kidney (76% for 2.5 $\mu g.L^{-1}$ and 70% for 25 $\mu g.L^{-1}$) and in gills (66% for 25 $\mu g.L^{-1}$), when compared to the control group (Figure 1).

3.5 Physiological biomarkers

The plasma levels were reduced for magnesium (24% for 0.25 $\mu g.L^{-1}$ and 35% for 2.5 $\mu g.L^{-1}$), potassium (39% for 0.25 $\mu g.L^{-1}$, 43% for 2.5 $\mu g.L^{-1}$ and 34% for 25 $\mu g.L^{-1}$) and sodium (7% for 0.25 $\mu g.L^{-1}$ and 9% to 2.5 $\mu g.L^{-1}$) when compared to the control (Figure 2). The activity of carbonic anhydrase in the posterior kidney was not altered. For the gills, the carbonic anhydrase activity decreased (20% to 0.25 $\mu g.L^{-1}$) when compared to the control (Figure 2).

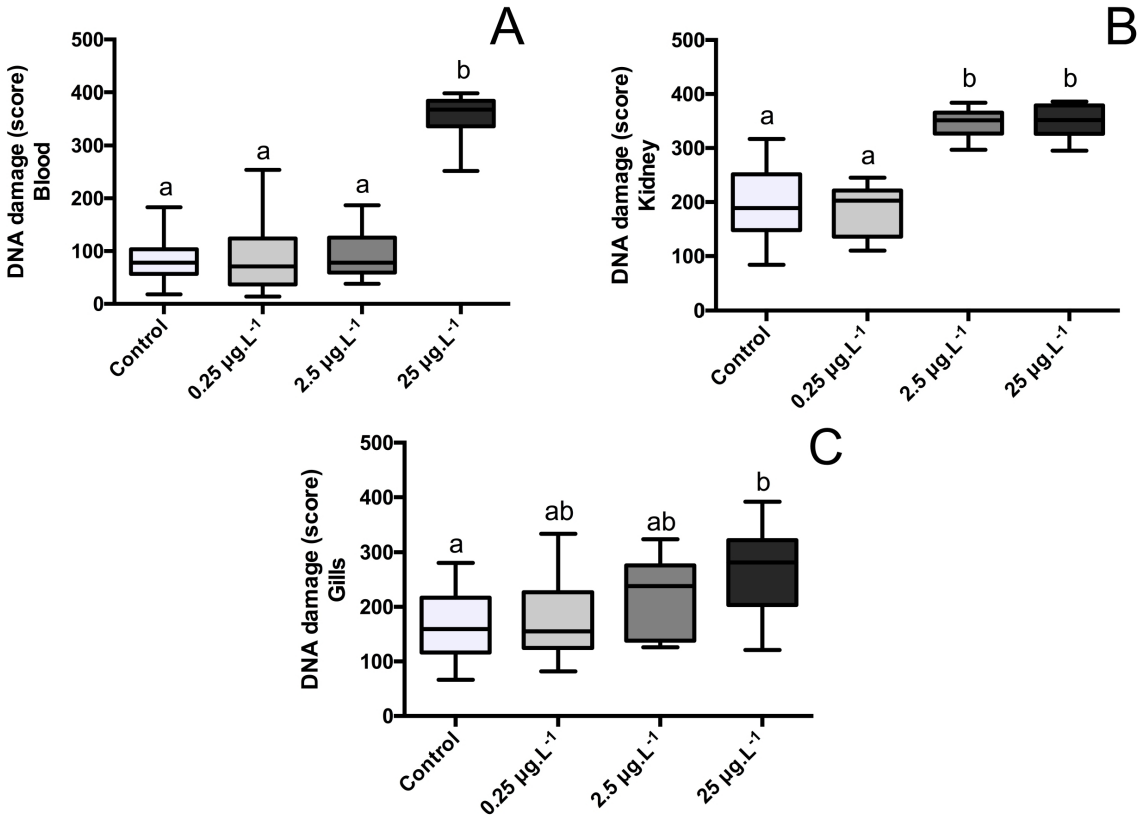

Figure 3: DNA damage of *R. quelen* exposed to 3 concentrations of paracetamol. Score of blood cells (A), posterior kidney (B) and gills (C). Results are expressed as median and interquartile range (25th and 75th percentiles). Different letters indicate $p < 0.05$.

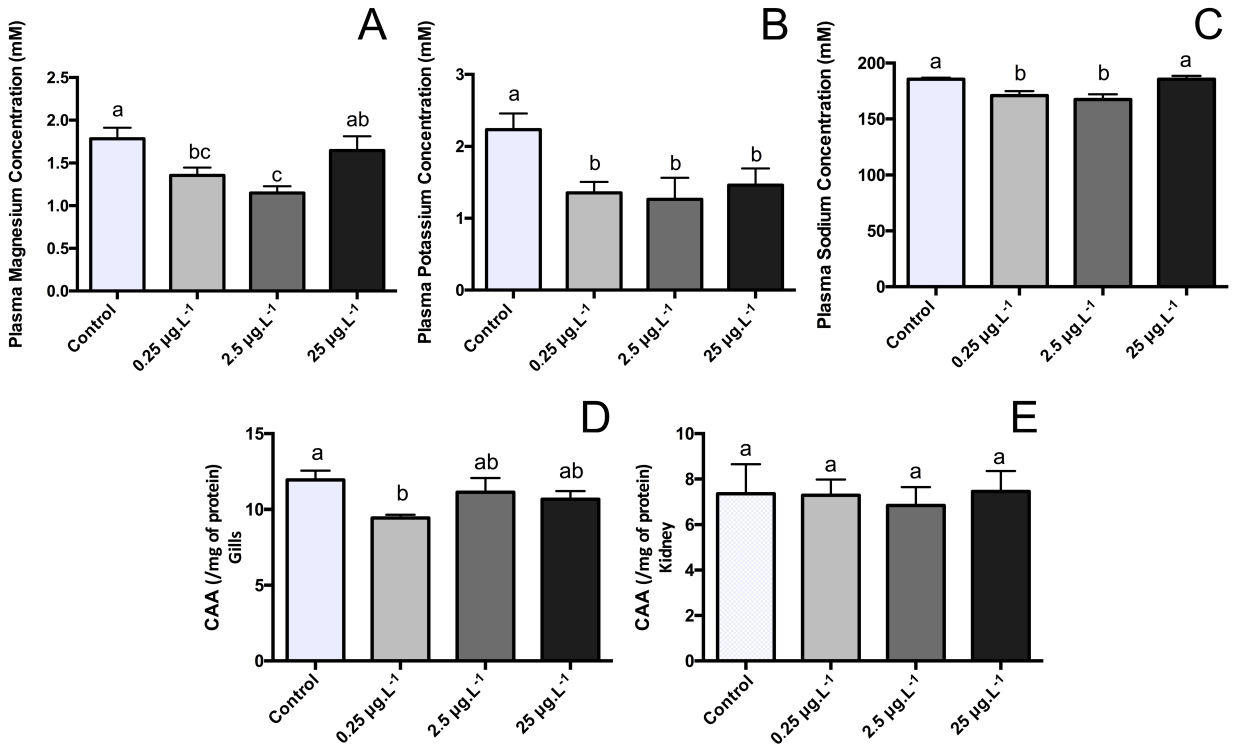

Figure 2: Plasma concentration of magnesium (A), potassium (B) and sodium (C) and carbonic anhydrase specific activity (CAA) in gills (D) and posterior kidney (E) of *R. quelen* exposed to 3 concentrations of paracetamol. Results are expressed as mean ± standard error. Different letters indicate $p < 0.05$.

3.6 Hematological biomarker

The total count of leukocytes and thrombocytes was reduced in the concentration of 25 µg.L^{-1} (44% and 41%, respectively) when compared to control group (Figure 3). No difference was found for the other concentrations.

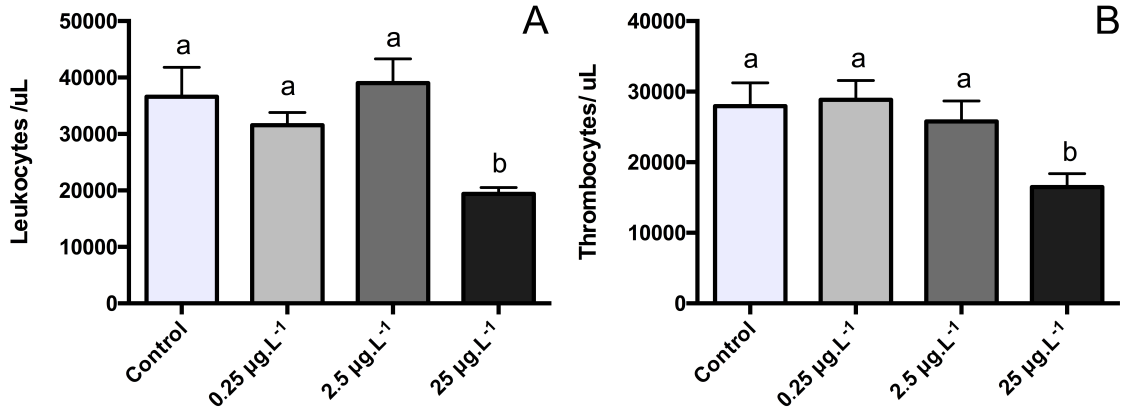

Figure 3: Total count of leukocytes (A) and thrombocytes (B) for *R. quelen* exposed to 3 concentrations of paracetamol. Results are expressed as mean ± standard error. Different letters indicate $p < 0.05$.

3.7 Histopathological biomarker

The renal tissue of the control group was homogeneous and unchanged (Figure 4A). The main changes observed in renal tissue were reduction of mesangial cells (Figure 4B) and glomerular hypertrophy (Figure 4C) at the concentration of 0.25 $\mu g.L^{-1}$ and glomerular degeneration (Figure 4D), leukocyte infiltration (Figure 4E), presence of pycnotic cells (Figure 4F) and infiltration of adipocytes (Figure 4G) at the concentration 25 $\mu g.L^{-1}$.

The gill tissue of the control group was homogeneous and unchanged (Figure 5A). The main alterations found in the analysis of the gill tissue were lifting of the respiratory epithelium (Figure 5B), cellular hyperplasia (Figure 5C) and vasodilatation (Figure 5D) at the concentration 25 $\mu g.L^{-1}$.

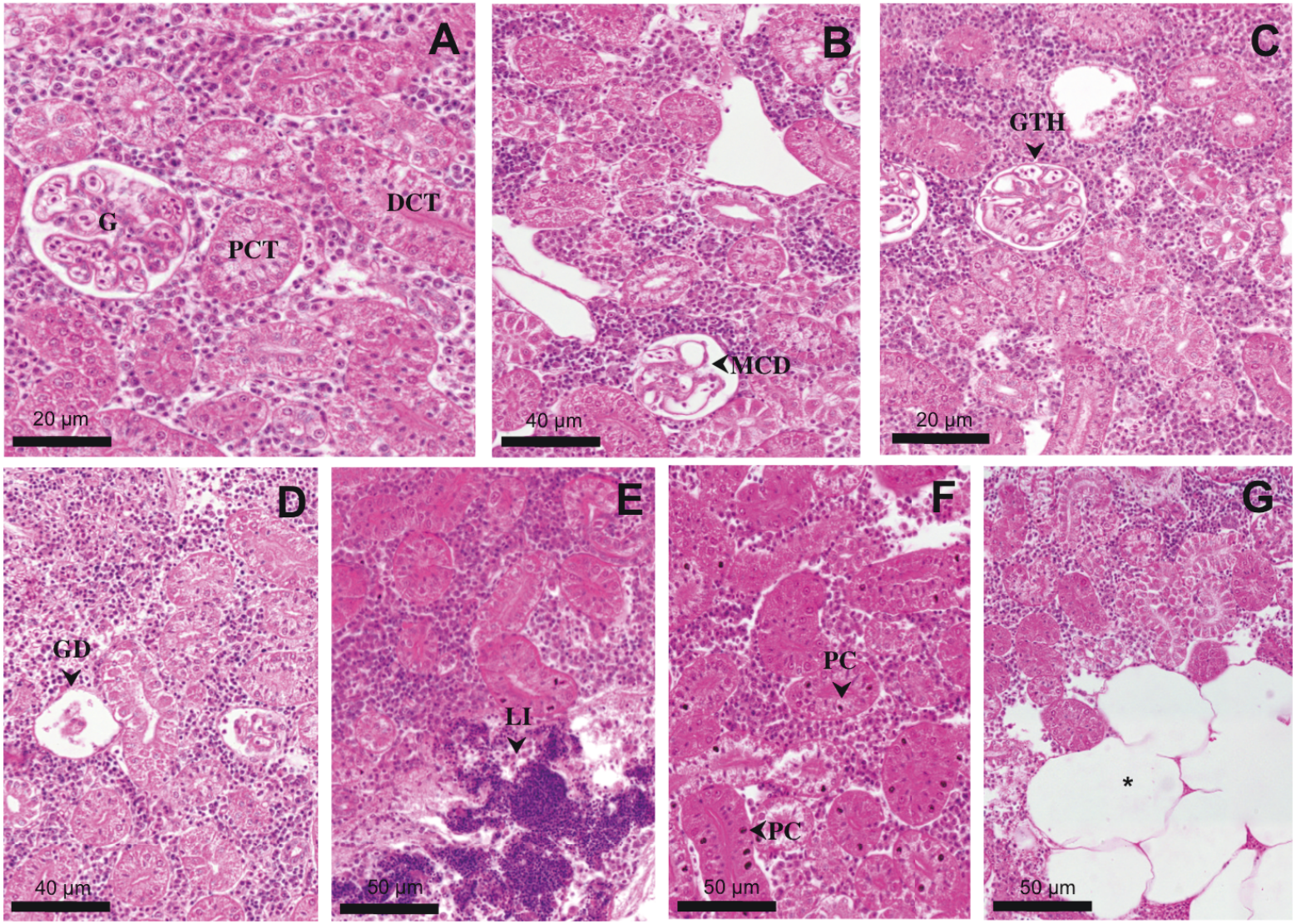

Figure 4: Cross-section of the posterior kidney of *R. quelen* exposed to 3 concentrations of paracetamol counterstained with hematoxylin/ eosin (HE). Glomerulus (G), proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) in control group (A). Mesangial cell decreased (MCD) (B) and glomerular tuft hipertrophy (GTH) (C) in group 0.25 µg.L^{-1}. (D-G) Glomerular degeneration (GD), leukocyte infiltration (LI), pyknotic cells (PC) and a large amount of adipocytes (*) in group 25 µg.L^{-1}, respectively.

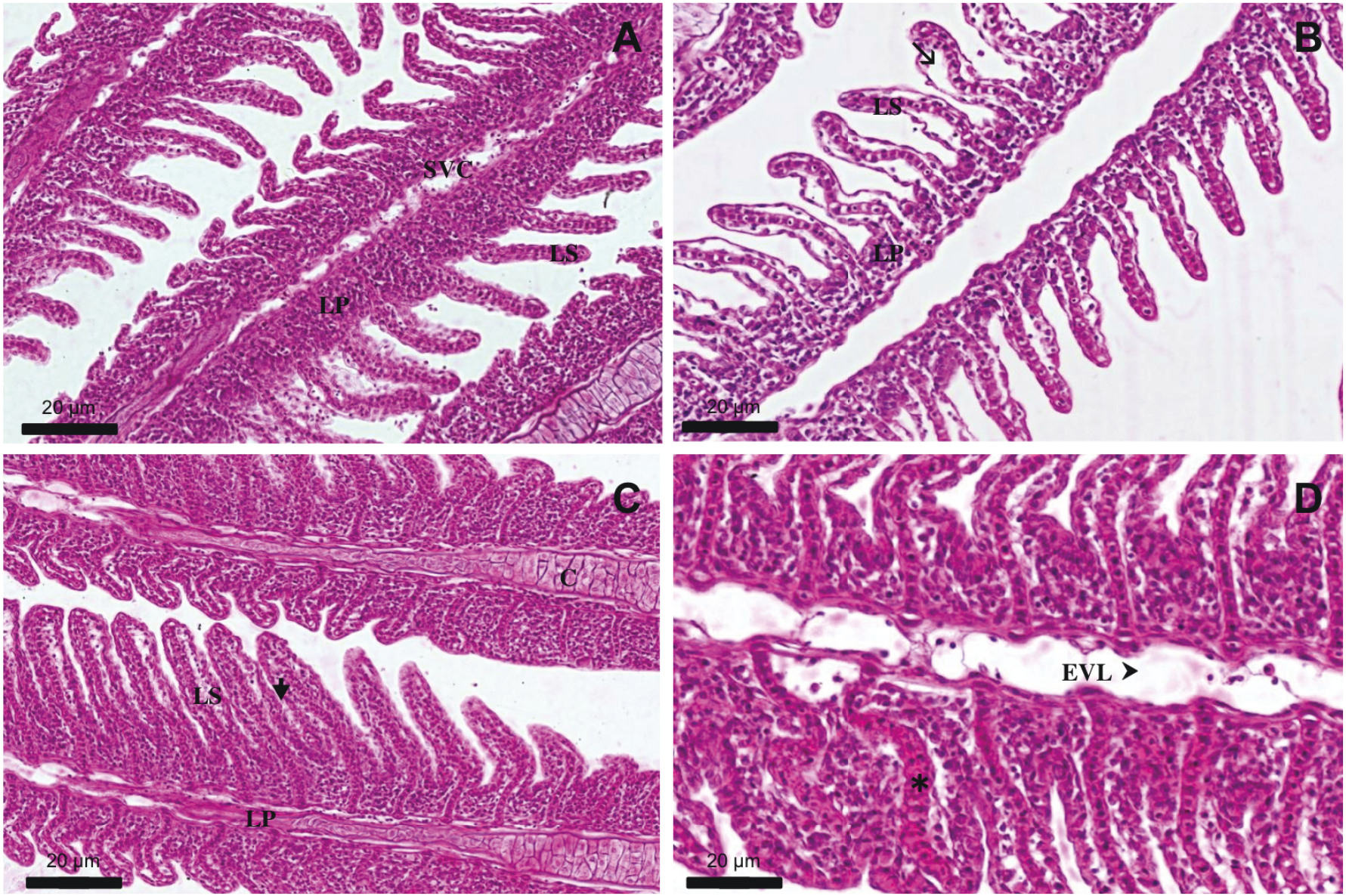

Figure 5: Cross-section of the gills of *R. quelen* counterstained with HE. Standard conformation of lamellae and their filaments. Primary lamella (LP), secondary lamella (LS), central venous sinus (SVC) in control group (A). Elevation of respiratory epithelium (↘), primary lamella (LP) and secondary lamella (LS) in group 25 µg.L^{-1} (B). Cellular hyperplasia with partial melting of secondary lamellae (⬇), primary lamella (LP), secondary lamella (LS) and cartilage (C) in group 25 µg.L^{-1}(C). Vasodilation (*) and vascular axis of lamella (evl) in group 25 µg.L^{-1} (D).

3.8 Integrated Biomarker Responses Index (IBRv2)

The values (mean ± standard deviation) found for the selected biomarkers of the control group in the posterior kidney and gills are shown in Table A.1. These values, considered basal, were used for the determination of IBRv2. The results obtained for the analysis of IBRv2 at each concentration are shown in Figure 7. The biochemical biomarkers for the concentrations of 0.25 and 2.5 $\mu g.L^{-1}$ were the most effective for paracetamol responses. For the concentration of 25 $\mu g.L^{-1}$, it was observed responses in biochemical, genotoxic and histopathological biomarker.

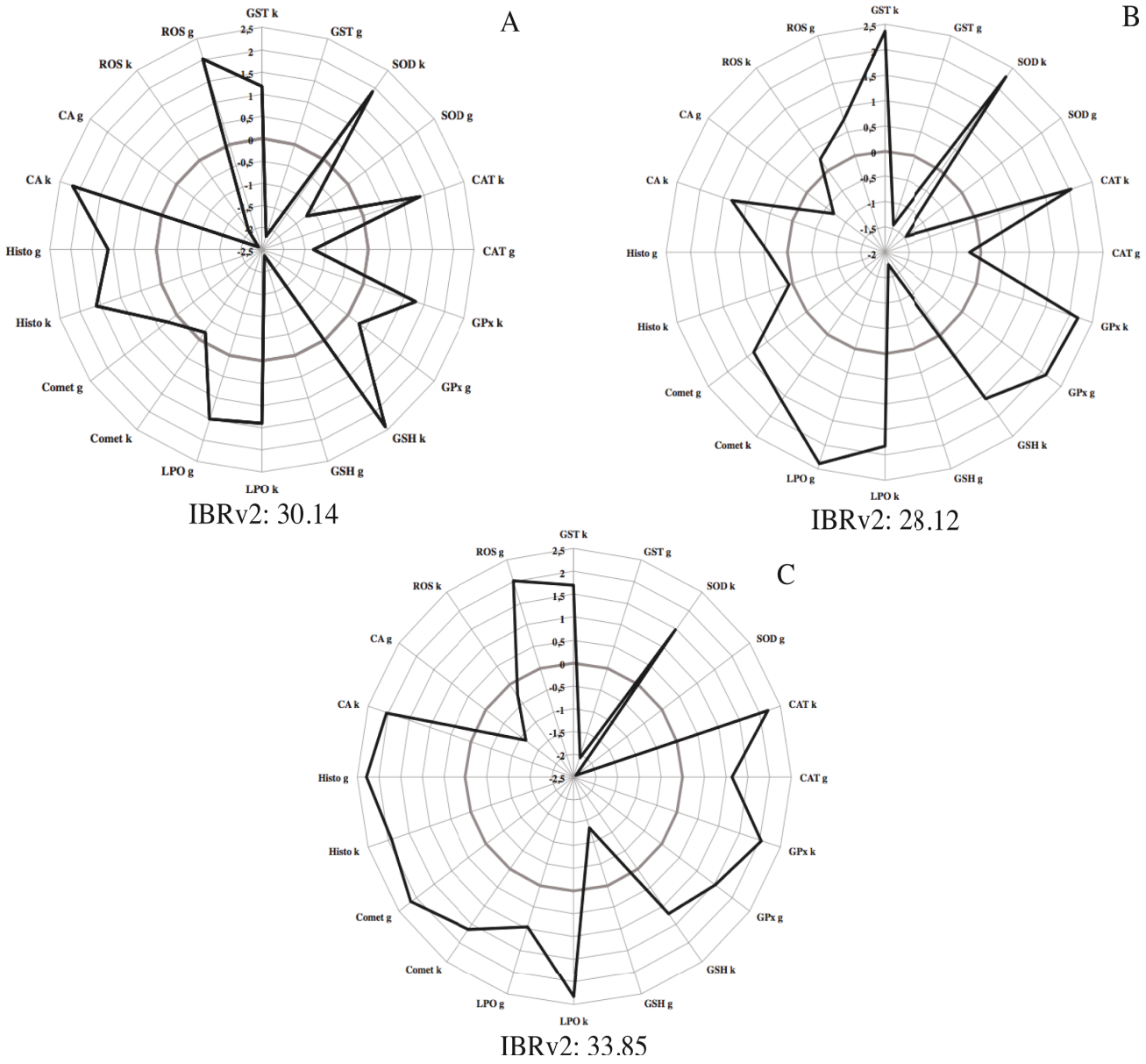

Figure 6: Integrated Biomarker Responses Index (IBRv2) for the concentrations of 0.25 (A), 2.5 (B) and 25 (C) µg.L^{-1} based on 10 biomarkers: glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), lipoperoxidation (LPO), comet assay (Comet), morphological biomarker (MB), carbonic anhydrase activity (CAA) and total reactive oxygen species (ROS) for gills (g) and posterior kidney (k). The area represented above 0 reflects the induction of the biomarker and below the reduction of the biomarker.

4. Discussion

This study presented some effects of toxicity in fish exposed to environmentally relevant concentrations of paracetamol. Different studies around the world detected the presence of paracetamol in environmental samples in a large scale of variance (Biel-Maeso *et al.*, 2018; Chen *et al.*, 2016; Huber *et al.*, 2016; Paíga *et al.*, 2016; Spongberg *et al.*, 2011; Wang *et al.*, 2017). In addition to this factor, the concentrations used in this study showed significant toxic effects, mainly the highest one.

For gills paracetamol was genotoxic at the concentration of 25 $\mu g.L^{-1}$, reduced the activity of GST, a phase II biostransformation enzyme, and changed the functioning of the antioxidant system reducing SOD activity and increasing GPx activity and GSH concentration at 2.5 and 25 $\mu g.L^{-1}$ groups. It also caused damage to membrane lipids at the concentration of 2.5 $\mu g.L^{-1}$. The reduction of enzymes activities may occur due to excess of substrate, but it is also a consequence of oxidative damage (Ge *et al.*, 2017; Lushchak, 2011). An effect in GST activity was observed in gills at all the concentrations and this change (reduction) has been reported by Gonzalez-Rey and Bebianno (2011) in a study using Ibuprofen, another NSAID.

SOD is an enzyme of the antioxidant system specialized in the conversion of superoxide (O_2^{+}) to H_2O_2 (Barbosa *et al.*, 2010). Reduction of SOD activity was observed only at the concentration of 25 $\mu g.L^{-1}$ in the gills, which could result in an increase of superoxides in the tissue. The increase of the enzyme activity of the antioxidant system, occurs as a way to neutralize ROS (Barbosa *et al.*, 2010). Although exposure to paracetamol did not lead to increased CAT activity in gills, exposure to the drug at concentrations of 2.5 and 25 $\mu g.L^{-1}$ increased the activity of GPx, the enzyme responsible for the reduction of peroxides using GSH as cofactor

(Davies, 2000). The increase in GPx activity was also observed by Nunes *et al.* (2017) in the acute exposure of *Ruditapes philippinarum*, a saltwater clam, to the concentration of 2.5 $\mu g.L^{-1}$ of paracetamol.

The metabolism of paracetamol generates a large amount of reactive oxygen species (ROS), by converting its original molecule to the N-acetyl-p-benzoquinone-imine (NAPQI) bioactive metabolite (McGill and Jaeschke, 2013). Reduction of GSH levels in the gill tissue is related to the increase of GPx activity. GSH also participates as a cofactor of the GST enzyme, in the process of converting NAPQI into less toxic matabolites, allowing excretion of the drug (McGill and Jaeschke, 2013). The GSH depletion leads to increased NAPQI levels, which may result in cytotoxic damage (Siemionow *et al.*, 2016; Weigt *et al.*, 2010).

The membrane damage observed in the gills through the increase of the LPO in the concentration of 2.5 $\mu g.L^{-1}$ may be the result of an accumulation of ROS in the tissue. Lipoperoxidation in gills of organisms exposed to paracetamol was also observed by Ramos *et al.* (2014). Although no statistical difference was observed among groups and control, a biological effect may be present based on the increase of ROS in gills at 0.25 $\mu g.L^{-1}$ and 25 $\mu g.L^{-1}$.

The activity of the enzyme carbonic anhydrase (CAA) was reduced in the gills in the concentration of 0.25 $\mu g.L^{-1}$. In freshwater fishes gills CAA is responsible by the reversible reaction of CO_2 hydration, which results in hydrogen ion (H_+) and bicarbonate (HCO_3^-). The cell HCO_3^- and H^+ are respectively exchanged by Cl^- or Na^+, or even pumped out (H^+) by the vacuolar apical H^+-ATPase, thus allowing for the active absorption of salt (Evans, 2005). Compatible with the CAA reduction at 0.25 $\mu g.L^{-1}$, plasma sodium (absorbed by the gill in freshwater) presented reduction after exposured to 0.25 $\mu g.L^{-1}$ and 2.5 $\mu g.L^{-1}$ of paracetamol, indicating reduction on

salt absorption by the gill epithelia. The reduction on CAA observed in the gills after exposed to 0.25 µg/L of paracetamol could be associated to its role in plasma sodium uptake (Evans, 2005; Prodocimo *et al.*, 2015). The CCA inhibition and loss of plasma ionic homeostasis was also observed in *R. quelen* exposed to 2 and 11 µg/L of copper (Mela *et al.*, 2013a). Oxidative stress lipoperoxidation and DNA damage in the gills contributes to tissue damage, and reduction of carbonic anhydrase activity and plasma sodium concentration may impair the control of ions in the organ (Lionetto *et al.*, 2012). Similar results were found in animals exposed to diclofenac (Saravanan *et al.*, 2011).

In the gills, the histopathological alterations observed were the elevation of respiratory epithelium, vasodilation and cellular hyperplasia. Tissue damage is a result of biochemical changes and genotoxicity, leading to changes of osmoregulation control by the tissue (Gröner *et al.*, 2017). Similar histopathological changes were found in gills of animals exposed to diclofenac (Gröner *et al.*, 2017; Triebskorn *et al.*, 2004) and to the antibiotic oxytetracycline (Rodrigues *et al.*, 2017).

In the kidney, paracetamol increased GST, CAT and GPx activities and GSH levels, but there was no change in SOD activity. Exposure to paracetamol did not generate lipoperoxidation in renal tissue. The alteration of the antioxidant system in the kidney is due to the high blood flow and role in the metabolism and excretion of drugs that this organ plays, being susceptible to the toxic effects of xenobiotics in fish (Gorgulho *et al.*, 2017). The increase in enzyme activity of the antioxidant system of the posterior kidney is in agreement with the reduction of ROS in the tissue, when the test groups were compared to the control group.

Parolini *et al.* (2010) found very similar results in a study that analyzed the acute toxic effect of paracetamol in *Dreissena polymorpha*, a small freshwater

mussel. The results indicated the increase in GST, CAT and GPx activities at concentrations of 0.75 and 1.51 µg.L-1. Alterations of the antioxidant system were also indicated by Mezzelani *et al.* (2016), Melvin (2016) and Gonzalez-Rey and Bebianno (2012) in aquatic organisms exposed to different NSAIDs.

Paracetamol was genotoxic in posterior kidney at concentrations of 2.5 and 25 $\mu g.L^{-1}$. The damage in DNA leads to the process of cell death, occurring process of apoptosis or tissue necrosis. Paracetamol was also shown to be genotoxic in an acute study with bivalves exposed to concentrations of 30 to 400 $\mu g.L^{-1}$ (Parolini *et al.*, 2009) and in renal cell culture at concentrations of 0.25, 2.5 and 25 $mg.mL^{-1}$ (Ribas *et al.*, 2014).

In the kidney, the alterations were mesangial cell reduction, hypertrophy and glomerular degeneration, adipocyte and leukocyte infiltration and the presence of pycnotic cells. The nephrotoxic potential of paracetamol is already well known in mammals (Majhi *et al.*, 2011; Mazer and Perrone, 2008). The presence of pycnotic cells, a result of the sum of oxidative stress and DNA damage, leads to the process of cellular migration of blood leukocytes to the renal tissue (Fischer *et al.*, 2006). The nephrotoxicity of paracetamol in fish has also been observed in other studies (Gorgulho *et al.*, 2017; Peng *et al.*, 2010). Compatible with histopathological changes observed, renal function was also affected, resulting in reduction on the rate of tubular magnesium reabsorption and consequently plasma magnesium reduction (Beyenbach, 2000). These results were similar to the concentrations found for *R. quelen* in previous studies and for freshwater teleosts (Beyenbach, 2000; Mela et al., 2013b, 2013a). Plasma potassium is maintained by ion uptake from ingested food into intestinal epithelia and also by renal tubular reabsorption using apical NKCC (Na+,K+,2Cl– co-transporter) (Marshall and Grosell, 2006). Potassium reduction

observed in all groups exposed to paracetamol may indicate reduction of potassium uptake by the intestinal epithelium, or reduction of its renal reabsorption (Evans, 2005; Marshall and Grosell, 2006). In contrast to its disturbing effect on gills CAA, paracetamol exposure did not affect this enzyme activity indicating maintenance of its renal function.

In this study, paracetamol also affected hematological parameters leading to a reduction in the total count of leukocytes and thrombocytes at 25 $\mu g.L^{-1}$, in addition, the drug demonstrated blood genotoxicity at the same concentration. In fish, as well as in mammals, leukocytes have a function in the body's defense mechanism. They are also capable of performing cell-mediated cytotoxicity against altered and foreign cells (Fischer *et al.*, 2006). In addition to leukocytes, thrombocytes are also functioning cells in the defense of the organism of these animals through phagocytosis (Nagasawa *et al.*, 2015). Studies have demonstrated the expression of the major histocompatibility complex (MHC II) molecule in thrombocytes, which reinforces the immune role of these cells (Ferdous and Scott, 2015) .

The reduction of thrombocytes was observed on exposure to other NSAIDs such as dipyrone, for example (Pamplona *et al.*, 2011). On the other hand, leukocyte reduction is hardly detected in subchronic studies. Some studies report the increase of leukocytes in aquatic organisms exposed to NSAIDs during subchronic and acute assay (Guiloski *et al.*, 2017a; Saravanan *et al.*, 2012, 2011).

The gills and the posterior kidney presented different responses after paracetamol exposure. These results can be a consequence of the difference in tissue function. The gills are responsible for the gas exchange and remain in direct contact with the external environment and contaminants (Machado, 1999). On the other hand, the fish posterior kidney has a non-specialized function of excretion and,

consequently, has more contact with metabolites of already biotransformed substances (D'Angelo et al., 2016).

Several biomarkers were used in this study and for an integrated visualization of the results o the use of IBRv2 as an indicator of environmental stress was used (Kim *et al.*, 2010; Sanchez *et al.*, 2013). The values found for IBRv2 demonstrated that the highest stress occurred at the concentration of 25 $\mu g.L^{-1}$. This finding allowed us to draw a parallel with the animal health, being important when considering environmental concentrations, since sites with higher concentrations of contaminants tend to trigger higher stress in the organisms.

5. Conclusion

Environmentally relevant concentrations of paracetamol affected the functioning of the antioxidant system of gills and posterior kidney in *Rhamdia quelen.* Paracetamol demonstrated non-concentration dependent effects, being genotoxic to blood, kidney and gills. It promoted osmoregulatory and tissue damage in the gills and posterior kidney, demonstrating its nephrotoxic effect. These results pointed to the need to control paracetamol concentrations found in aquatic compartments in order to avoid further ecological damage.

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Appendices

Table A.1: Integrated Biomarker Responses Index (IBRv2) of *R. quelen* exposed to paracetamol**.**

	GILLS	KIDNEY
Biomarker	mean ± SD	mean ± SD
GST	76.21 ± 10.60	87.59 ± 29.93
SOD	256.92 ± 52.84	150.73 ± 39.90
CAT	24.27 ± 3.32	37.33 ± 10.64

GPx	27.43 ± 5.12	16.37 ± 3.30
GSH	2.31 ± 1.02	2.77 ± 0.98
LPO	3.00 ± 1.02	9.93 ± 3.46
Comet assay	160.66 ± 59.99	197.14 ± 67.16
MB	0.60 ± 1.34	1.80 ± 2.57
CAA	11.93 ± 1.51	4.40 ± 1.40
ROS	0.60 ± 0.98	2.71 ± 0.85

Values related the results of biomarkers for the control group, used as baseline for IBRv2. Values are expressed by mean ± standard deviation (SD). Glutathione-S-transferase (GST), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), reduced Glutathione (GSH), Lipoperoxidation (LPO), Morphological biomarker (MB), Carbonic anhydrase activity (CAA) and Reactive Oxygen Species (ROS).

6 CONSIDERAÇÕES FINAIS

O presente estudo avaliou efeitos tóxicos de concentrações ambientalmente relevantes do fármaco paracetamol em sangue, rim posterior e brânquias de peixes da espécie *Rhamdia quelen*. Os resultados demonstram que em todas as concentrações o fármaco foi capaz de alterar parâmetros fisiológicos, tendo comportamento tóxico diferente entre os tecidos. Em brânquias, o paracetamol foi capaz de alterar o funcionamento do sistema antioxidante, provocar alterações teciduais e promover danos em DNA na maior concentração testada e, na menor concentração, reduziu atividade da enzima anidrase carbônica. Já no rim posterior, além de alterar o sistema antioxidante, o fármaco foi capaz de provocar danos em DNA e lesões teciduais. Dessa forma, comprovou-se o caráter nefrotóxico do paracetamol, além de sua capacidade genotóxica, produção de alterações no controle de íons, células sanguíneas brancas e morfologia de tecidos.

As informações contidas nesse trabalho contribuem para o conhecimento sobre o efeito de contaminantes emergentes em organismos aquáticos e podem auxiliar os órgãos governamentais na busca de mecanismos legais que exijam limites de detecção para fármacos em compartimentos aquáticos, assim como já ocorre para metais, por exemplo. Os resultados também reafirmam a necessidade de tecnologias aplicáveis em grande escala para a retirada desses contaminantes de efluentes.

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