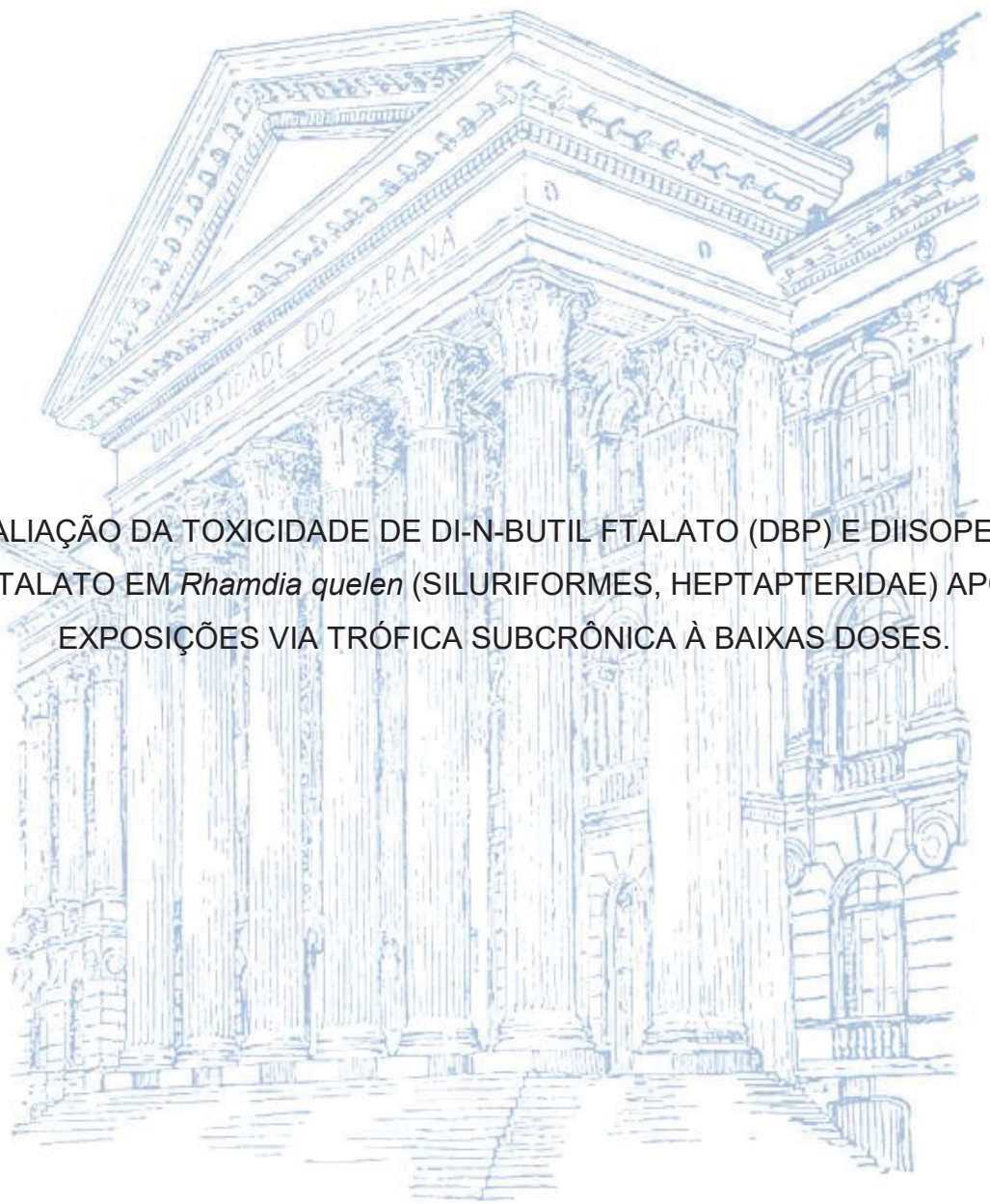
UNIVERSIDADE FEDERAL DO PARANÁ

LAÍS FERNANDA OYA SILVA



AVALIAÇÃO DA TOXICIDADE DE DI-N-BUTIL FTALATO (DBP) E DIISOPENTIL FTALATO EM *Rhamdia quelen* (SILURIFORMES, HEPTAPTERIDAE) APÓS EXPOSIÇÕES VIA TRÓFICA SUBCRÔNICA À BAIXAS DOSES.

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do grau de doutor em Ciências Biológicas, área de concentração Genética.

Orientadora: Prof.ª Dr.ª Marta Margarete Cestari

Coorientadoras: Profª. Drª. Daniela Morais Leme

Drª. Taynah Vicari

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LAÍS FERNANDA OYA SILVA** intitulada: **AVALIAÇÃO DA TOXICIDADE DE DI-N-BUTIL FTALATO (DBP) E DIISOPENTIL FTALATO EM Rhamdia quelen (SILURIFORMES, HEPTAPTERIDAE) APÓS EXPOSIÇÕES VIA TRÓFICA SUBCRÔNICA À BAIXAS DOSES.**, sob orientação da Profa. Dra. MARTA MARGARETE CESTARI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor 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.

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"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."

Albert Einstein

RESUMO

Ftalatos, ou ésteres de ácido ftálico, são moléculas orgânicas produzidas sinteticamente para uso industrial como plastificantes, umectante, fixadores, solventes em diversos ramos indústriais. O di-n-butil ftalato (DBP) é um dos ftalatos mais produzidos e utilizados no mundo, apesar de sua proibição em cosméticos e produtos de cuidado pessoal, ainda é amplamente utilizado em adesivos, tintas e vernizes, e principalmente na produção de policloreto de vinila (PVC). Assim como outros ftalatos, o DBP também é conhecido por ser desregulador endócrino e promover efeitos reprodutivos em animais. No Brasil, recentemente foi descoberta a exposição de humanos ao diisopentil ftalato (DiPeP) por meio da detecção de metabólitos na urina de gestantes e crianças. A produção do DiPeP no Brasil é alta devido à disponibilidade de álcool isoamílico advindo do processo de refino da cana de açúcar para a produção de etanol. Assim como o DBP, o DiPeP também é proibido em itens de cuidado pessoal, mas é utilizado pela indústria de maneiras semelhantes. Entretanto, pouco se sabe a respeito da toxicidade do DiPeP. Desta forma, o objetivo do presente estudo foi avaliar a potencial toxicidade dos ftalatos DBP e DiPeP em exposições subcrônicas em uma espécie nativa (*Rhamdia quelen*). Foi desenvolvido um desenho experimental em condições ambientalmente relevantes com exposição trófica e baixas doses (5 ng/g, 25 ng/g, 125 ng/g) administradas a cada 48 horas por 30 dias. Foram avaliados parâmetros hematológicos, formação de micronúcleos e alterações eritrocitárias nucleares, danos ao DNA de células sanguíneas, hepáticas, renais e cerebrais, atividade das enzimas glutationa S-transferase (GST), superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), concentração GSH e peroxidação lipídica (LPO) nos tecidos hepático e renal, além da atividade da acetilcolinesterase (AChE) muscular e cerebral. Os resultados mostraram que não houve efeito no tecido sanguíneo que pudesse ser identificado pelos biomarcadores hematológicos e genéticos. Também não observamos genotoxicidade nos tecidos cerebral e hepático. Entretanto, por meio dos biomaradores bioquímicos, observamos um desequilíbrio no sistema antioxidante de rim e fígado. No rim, ambos os ftalatos reduziram a concentração de GSH e a atividade da SOD e promoveram o aumento de danos ao DNA renal, porém, o DiPeP, para esses três biomarcadores, mostrou o efeito em todas as doses de forma semelhante, enquanto o DBP em apenas uma das doses administradas. Além disso, o DiPeP reduziu a atividade da GPx e a LPO, que não foi observada nos grupos tratados com DBP. No tecido hepático, apesar de não terem sido genotóxicos, ambos os ftalatos causaram aumento da atividade da GST. O tecido cerebral mostrou-se um potencial alvo ao aumentar a atividade da AChE tanto na presença de DBP quanto DiPeP, entretanto, o último promoveu a redução da atividade no músculo em todos os tratamentos. Assim, o presente estudo reforça a preocupação com a presença de ftalatos no ambiente, uma vez que esses mostraram-se potenciais agentes nefrotóxicos e neurotóxicos. O DiPeP mostrou-se tão tóxico quanto o DBP, evidenciando a importância de estudos de toxicidade e biomonitoramento do ecossistema aquático.

Palavras chave: rim, cérebro, genotoxicidade, sistema antioxidante, peixes, DiAP.

ABSTRACT

Phthalates, or phthalic acid esters, are organic molecules produced synthetically for industrial use as plasticizers, humectants, fixatives, solvents in various industrial sectors. Di-n-butyl phthalate (DBP) is one of the most produced and used phthalates globally, despite its prohibition in cosmetics and personal care products, it is still widely used in adhesives, paints and varnishes, and mainly in the production of polychloride vinyl (PVC). Like other phthalates, DBP is also known to be an endocrine disruptor and promote reproductive effects in animals. In Brazil, human exposure to diisopentyl phthalate (DiPeP) was recently discovered by detecting metabolites in the urine of pregnant women and children. The production of DiPeP in Brazil is high due to the availability of isoamyl alcohol resulting from the sugar cane refining process for the production of ethanol. Like DBP, DiPeP is also prohibited in personal care items, but it is used by the industry in similar ways. However, little is known about the toxicity of DiPeP. Thus, the objective of the present study was to evaluate the potential toxicity of phthalates DBP and DiPeP in subchronic exposures in a native species (*Rhamdia quelen*). An experimental design was developed under environmentally relevant conditions with trophic exposure and low doses (5 ng / g, 25 ng / g, 125 ng / g) administered every 48 hours for 30 days. Hematological parameters, micronucleus formation and nuclear erythrocyte alterations, damage to the DNA of blood, liver, kidney and brain cells, activity of the enzymes glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), GSH concentration and lipid peroxidation (OLP) in the hepatic and renal tissues, in addition to the muscle and brain acetylcholinesterase (AChE) activity were evaluated. The results showed no effect on blood tissue that could be identified by hematological and genetic biomarkers. We also did not observe genotoxicity in the brain and liver tissues. However, through biochemical biomarkers, we observed an imbalance in the kidney and liver antioxidant system. Both phthalates reduced the GSH concentration and SOD activity in the kidney and promoted increased damage to renal DNA. However, DiPeP, for these three biomarkers, showed the effect in all doses similarly, while DBP in only one of the doses administered. Besides that, DiPeP reduced the GPx activity and LPO, which was not seen in the groups treated with DBP. Although they were not genotoxic in the liver tissue, both phthalates caused an increase in GST activity. Brain tissue proved to be a potential target by increasing AChE activity both in the presence of DBP and DiPeP, however, the latter promoted a reduction in muscle activity in all treatments. Thus, the present study reinforces the concern with the presence of phthalates in the environment, since these have shown to be potential nephrotoxic and neurotoxic agents. DiPeP proved to be as toxic as DBP, showing the importance of studies on toxicity and biomonitoring of the aquatic ecosystem.

Keywords: kindey, brain, genotoxicity, antioxidant system, DiAP

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LISTA DE SIGLAS

ABIPLAST – Associação Brasileira da Indústria do Plástico

ACh – Acetilcolina

AChE – Acetilcolinesterase

ANVISA – Agencia Nacional de Vigilância Sanitária

BHT – *Butylated hydroxytoluene*

BSA – Albumina de soro bovino

CAT – Catalase

CEUA/BIO - UFPR – Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná

CFC – *Commercial food Control*

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico / *National Council for Scientific and Technological Development*

CONAMA – Conselho Nacional do Meio Ambiente

DBP – Di-n-butil ftalato

DEHP – Di (2-etil hexil) ftalato

DEP – Di-etil ftalato

DiAP – Diisoamil ftalato

DIDP – Di-iso-decilo Ftalato

DINP – Di-iso-nonilo ftalato

DiPeP – Diisopentil ftalato

DMP – DI-metil ftalato

DMEP – Bis (2-metoxietil) ftalato

DnPB – Di-n-pentil ftalato

DOP – *Di-n-octyl phthalate*

ENA – *Erythrocytic nuclear alterations*

ERO – Espécie reativa de oxigênio

FBS – *Fetal bovine serum*

FOX - *Ferrous Oxidation / Xylenol Orange*

FPG – *Formamidopyrimidine [fapy] -DNA glycosylase*

GPx – Glutationa peroxidase

GR – Glutationa redutase

GSH – Glutationa reduzida

GSSG – Glutationa dissulfeto

GST – Glutationa S-transferase

HMW – *High molecular weight*

H_2O_2 – Peróxido de hidrogênio

INMETRO – Instituto Nacional de Metrologia, Qualidade e Tecnologia

LATRAAC – Laboratório da Tecnologia da Reprodução de Animais Aquáticos / *Laboratory of Technology for the Reproduction of Aquatic Animals Cultivables*

LMW – *Low molecular weight*

LPO – Peroxidação lipídica

LSD – *Least significant difference*

MBP – *mono-n-butyl phthalate*

MiPeP – Mono-isopentil ftalato

MMS – *Methyl methanesulfonate*

MN – Micronúcleo / *Micronulei*

MPE – Mo*noalkyl phthalate esters*

NC – *Negative control*

NEA – *Nuclear erythrocyte alterations*

PAE – P*hthalic acid esters*

PC – *Positive control*

PETROM – Petroquímica Mogí das Cruzes SA

PL – Projeto de lei

PMT – *Piscine Micronucleus Test*

PP – Polipropileno

PVC – Policloreto de vinila

RDC – Resolução da Diretoria Colegiada

ROS – *Reactive oxygen species*

SCGE – *Single-Cell Gel Eletrophoresis*

SOD – Superóxido dismutase

U.S. EPA – *United States Environmental Protection Agency*

SUMÁRIO

1. INTRODUÇÃO

Ésteres de ácido ftálico, também conhecidos como ftalatos, são moléculas orgânicas que começaram a ser sintetizadas na década de 1920 (KIMBER; DEARMAN, 2010). Após 30 anos que a primeira molécula de ftalato foi sintetizada, a produção destes contaminantes emergentes aumentou significativamente devido ao desenvolvido do plástico policloreto de vinila (PVC) (KIMBER; DEARMAN, 2010).

Ftalatos de alto peso molecular, como di(2-etil hexil) ftalato (DEHP), di-iso-nonilo ftalato (DINP) e di-iso-decilo Ftalato (DIDP), são utilizados como "plastificantes" conferindo ao material maior flexibilidade, durabilidade e transparência, podendo representar até 60% do peso do produto final (HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; LIU et al., 2010). Esses materiais são destinados a produtos para a construção civil, peças automotivas, brinquedos, embalagens de alimentos, dispositivos medicos, entre outros (CHEON, 2020; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; WITORSCH; THOMAS, 2010; WITTASSEK et al., 2011).

Além dos ftalatos de alto peso molecular, existem os de baixo peso molecular que são utilizados como fixadores ou solventes industriais. Assim, os ftalatos como o di-metil ftalato (DMP), di-etil ftalato (DEP) e di-n-butil ftalato (DBP) são utilizados na formulação de esmaltes, tintas e vernizes, adesivos, explosivos, repelentes, hidratantes, perfumes, spray para cabelos e desodorantes (AL-SALEH et al., 2017; ERKEKOGLU; KOCER-GUMUSEL, 2014; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; LIU et al., 2010; ROMERO-FRANCO et al., 2011).

As moléculas de ftalatos não são ligadas de forma covalente às matrizes poliméricas dos plásticos ou aos aditivos de outros produtos e, por este motivo, podem ser dispersos para o ambiente em diferentes momentos (produção, utilização e descarte) por evaporação, migração ou lixiviação, e assim contaminam o ar, o solo, a água e alimentos (ABDEL DAIEM et al., 2012; CHENG et al., 2013; ROCHA et al., 2017; ZHENG; FENG; DAI, 2013). No ambiente, estudos já evidenciaram a presença de diferentes tipos de ftalatos em águas marinhas (TIWARI; SAHU; PANDIT, 2019), solo (LIU et al., 2010) e em rios e lagos (GAO et al., 2019; NAGORKA; KOSCHORRECK, 2020).

A degradação dos ftalatos pode ocorrer por hidrólise, fotodegradação e biodegradação, dessa forma o tempo de meia vida dos ftalatos no ambiente depende de fatores ambientais como a presença de oxidantes, densidade e espécies microbianas além da irradiação solar (NET et al., 2015; STAPLES et al., 1997a). Além disso, segundo Staples e colaboradores (1997), o tamanho do ftalato também é um fator importante em relação à sua permanência no ambiente, pois quanto maior a cadeia alcoólica desse, menor a taxa de hidrólise e, por consequência, maior sua meia vida. Assim, para um ftalato de baixo peso molecular como o DMP pode ter meia vida de aproximadamente 3 anos, enquanto o DEHP (de alto peso molecular) pode persistir no ambiente por até 2000 anos (STAPLES et al., 1997a). Entretanto, a degradação por microrganismos ou por fotólise pode reduzir consideravelmente os tempos de meia vida dos ftalatos para semanas e meses, respectivamente (NET et al., 2015).

Devido à sua característica hidrofófica, acreditava-se no grande potencial de bioacumulação dos ftalados em diversos organismos, entretanto os ftalatos sofrem uma diluição trófica e os vertebrados possuem maior eficiência na biotransformação dessas moléculas do que as espécies de invertebrados (GOUTTE et al., 2020). Em outras palavras, quanto maior o organismo e suas taxas metabólicas, maior será a capacidade de metabolização dos ftalatos e menor será o fator de acumulação (STAPLES et al., 1997b, 1997a). Apesar o fator de bioacumulação mostrar-se relevante em organismos com menor complexidade, como algas e moluscos, não foi evidenciada a biomagnificação na cadeia trófica, uma vez que peixes apresentam menor capacidade de bioacumulação do que algas e invertebrados (GOBAS; IKONOMOU, 2003; STAPLES et al., 1997a).

A toxicidade dos ftalatos está diretamente relacionada com tamanho de sua cadeia lateral. Assim, ftalatos compostos por até 4 carbonos na cadeia lateral (ftalatos de baixo peso molecular) são considerados mais tóxicos que suas versões de alto peso molecular, que possuem acima de 6 carbonos na cadeia lateral (ADAMS et al., 1995). Muitos estudos apresentam os ftalatos como desreguladores endócrinos (CHEN et al., 2014; MANKIDY et al., 2013; SWAN, 2008; WANG et al., 2012; WITTASSEK et al., 2011). Em exposições crônicas aos ftalatos podem comprometer o sistema imune, principalmente de organismos juvenis (SARAH; SHANE; LANGLOIS, 2016). Suspeita-se que ftalatos interferem nos processos biológicos celulares sendo potencialmente teratogênico, mutagênico e genotóxico, mesmo em baixas concentrações (GAO; WEN, 2016). Em mamíferos, o efeito da exposição aos

ftalatos é conhecido como “síndrome do ftalato”, que consiste em mudanças no desenvolvimento do sistema reprodutivo nos fetos, resultando em malformações do epidídimo, canais deferentes, vesícula seminal e próstata, criptorquidia, redução da espermatogênese e da fertilidade, além de apresentar sinais de feminização (SWAN, 2008; WANG et al., 2012; WITTASSEK et al., 2011). Ftalatos também são tóxicos para diversas espécies terrestres e aquáticas como algas, protozoários, anelídeos, moluscos, crustáceos,anfíbios e peixes (DU et al., 2015; RAMZI et al., 2020; SARAH; SHANE; LANGLOIS, 2016).

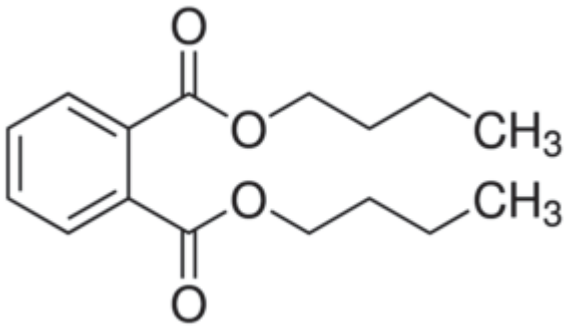
Além do efeito de desrregulção endócrina, ftalatos são conhecidos como proliferadores de peroxissomos. Estudos sugerem que efeitos como hipertrofia, hiperplasia e adenomas e carcinomas hepáticos sejam consequência do aumento de peroxissomos e mitocôndrias promovidos pela exposição aos ftalatos (DAVID et al., 1999; ERKEKOGLU; KOCER-GUMUSEL, 2014; RUSYN; PETERS; CUNNINGHAM, 2006; WANG et al., 2012).

A proliferação de peroxissomos está relacionada com a formação de espécies reativas de oxigênio (ERO), como o H_2O_2(DAVID et al., 1999; ERKEKOGLU; KOCER-GUMUSEL, 2014). Na presença de ERO, ocorre a ativação do metabolismo de enzimas e o aumento da atividade de enzimas peroxissomais como a catalase (CAT) (O’BRIEN; SPEAR; GLAUERT, 2005). Além disso, a superprodução de ERO, pela exposição à ftalatos, pode promover danos ao DNA e causar mutações e câncer (ANDERSON; YU; HINÇAL, 1999; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; KLEINSASSER et al., 2000a, 2001).

1.1 Di-n-butil Ftalato (DBP)

O DBP é um dos ftalatos presentes na lista de poluentes prioritários da U.S. EPA (*United States Environmental Protection Agency*) é classificado como químico desregulador endócrino, devido ao grande volume de produção e aplicação em diversos ramos industriais (FIGURA 1) (DU et al., 2015; U.S.EPA, 2015). Apesar de ser um ftalato de baixo peso molecular e utilizado em esmaltes para unhas, tintas, adesivos entre outros mencionados anterioremente, também é um dos principas plastificantes empregados na produção de PVC (AL-SALEH et al., 2017; LUZIA; SARROUH; LOFRANO, 2020; STAPLES et al., 1997a).

FIGURA 1 - ESTRUTURA MOLECULAR DO DBP



FONTE: www.sigmaaldrich.com

Um dos ftalatos mais abundantes no ambiente, presença de DBP é amplamente registrada em águas superficiais em diferentes países, destacando concentrações observadas de: 250 $ng.L^{-1}$ na Índia (SELVARAJ et al., 2014), 1.590 $ng.L^{-1}$ na China (GAO et al., 2019), até 3.100 $ng.L^{-1}$ na Holanda (VETHAAK et al., 2005), e entre 1.300 e 33.100 $ng.L^{-1}$ no Brasil (MONTAGNER et al., 2019; MONTAGNER; JARDIM, 2011). Apesar dos longos períodos de meia vida dos ftalatos, estimados anteriormente por Staples e colaboradores (1997b), a Agencia de Proteção Ambiental dos Estados Unidos (U.S.EPA, 2015) estima a meia vida do DBP na água em 2,9 dias, para condições aeróbicas e 14,4 dias para condições anaeróbicas. Essa grande divergência nos tempos de meia vida se deve pelas taxas de fotodegradação e hidrolização em condições naturais serem reduzidas (GAO; WEN, 2016).

Além da presença de DBP no ecossistema aquático (rios e mares), também foram encontrados em tecidos de peixes de diferentes níveis (ADENIYI; OKEDEYI; YUSUF, 2011; CHENG et al., 2013). Na China, em 20 espécies de peixes comercializadas para consumo, foram mensuradas concentrações de DBP variando entre 0,58 a 2,08 $\mu g.g^{-1}$ de peso seco, enquanto que na Nigéria as concentrações de DBP em três espécies de água doce foram significativamente maiores, variando de 686,67 a 1.716,67 $\mu g.g^{-1}$(ADENIYI; OKEDEYI; YUSUF, 2011; CHENG et al., 2013).

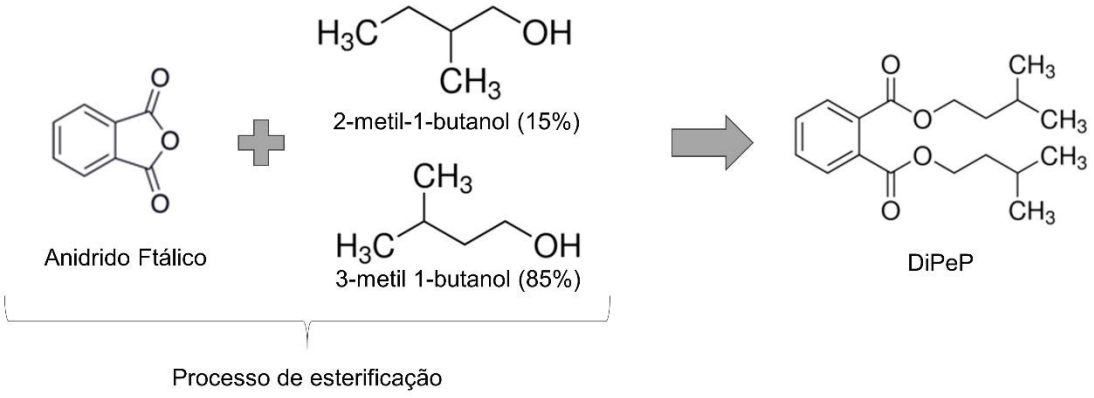
Em bioensaios, além dos efeitos no sistema reprodutivo e desregulação endócrina (AOKI et al., 2011; JARMOŁOWICZ; DEMSKA-ZAKĘŚ; ZAKĘŚ, 2013), o DBP apresentou efeitos de imuno e neurotoxicidade em embriões de *Danio rerio* (XU et al., 2013a, 2013b, 2015); estresse oxidativo e genotoxicidade em *Eisenia fétida* (anelídeo) e em peixes da espécie *Oreochromis niloticus* (BENLI; ERKMEN; ERKOC,

2016; ERKMEN et al., 2015; KHALIL; ELHAKIM; EL-MURR, 2016; WANG et al., 2019).

1.2 Diisopentil Ftalato (DiPeP)

O DiPeP, também conhecido como diisoamil ftalato (DiAP), é sintetizado a partir da esterificação do anidrido ftálico com álcool isoamílico (FIGURA 2). No Brasil, a produção de DiPeP é favorecida pela grande oferta de álcool isoamílico advindo do processo de refino da cana-de-açúcar para produção de etanol (BERTONCELLO SOUZA et al., 2018). A cada 1000 litros de etanol produzidos são gerados 2,5 litros de óleo fusel, um subproduto do processo de fermentação da cana-de-açúcar (BERTONCELLO SOUZA et al., 2018; PÉREZ; CARDOSO; FRANCO, 2001) Este óleo fúsel, dependendo da qualidade da matéria prima, possui entre 54 e 86,7% de álcool isoamílico, composto pelos isômeros 3-metil 1-butanol (85%) e 2-metil-1-butanol (15%) (FIGURA 2) (PÉREZ; CARDOSO; FRANCO, 2001; TEBAS et al., 2020).

FIGURA 2 - PROCESSO DE PRODUÇÃO DO DiPeP POR MEIO DA ESTERIFICAÇÃO DO ANIDRIDO FTÁLICO COM ÁLCOOL ISOAMÍLICO.



FONTE: A autora.

Segundo a descrição, divulgada nas páginas eletrônicas das empresas que produzem e comercializam ftalatos (Coremal e Brisco), o DiPeP é categorizado como plastificante, podendo ser utilizado em diversos tipos de borrachas e plásticos, principalmente o PVC, além de nitrocelulose, tintas, óleos, resinas sintéticas e naturais, adesivos entre outros.

Ainda no cenário brasileiro, o DiPeP já foi detectado em solo contaminados por resíduos de indústrias produtoras de plásticos em São Paulo e em material lixiviado de aterros no Rio Grande do Sul (DO NASCIMENTO FILHO et al., 2003; FERREIRA; MORITA, 2012). A exposição humana foi, também, evidenciada ao detectar, em urina de crianças e gestantes brasileiras, o metabólito primário do DiPeP, mono-isopentil ftalato (MiPeP), e os metabólitos secundários oxidados, 3OH - MiPeP e 4OH - MiPeP) (BERTONCELLO SOUZA et al., 2018; ROCHA et al., 2017). Não há, na literatura, registros internacionais de biomonitoramento humano que apresentem indícios de exposição ao DiPeP.

A literatura não dispõe de muitas publicações a respeito da toxicidade do DiPeP, entretanto, pela conhecida relação entre estrutura química e atividade, acredita-se no potencial antiandrogenico do DiPeP seja similar à outros ftalatos com 4 ou 5 carbonos na cadeia lateral, como o DBP (BERTONCELLO SOUZA et al., 2018). No estudo de Curi e colaboradores (2019), fêmeas de ratos Wistar foram expostas ao DiPeP (1, 10, 100 e 300 mg.kg.dia^{-1}) entre 0 10° dia de gestação e o 21° de lactação, os filhotes machos apresentaram efeitos reprodutivos típicos da síndrome do ftalato, como redução da distância urogenital, redução da vesícula seminal, além de alterações morfológicas e funcionais no testículo. Neubert da Silva e colaboradores (2019) observaram mudanças no comportamento relacionados ao acasalamento e motivação sexual em ratos machos Wistar, nascidos de fêmeas expostas às doses de 1 e 10 mg.kg.dia^{-1} de DiPeP durante o mesmo período do estudo anterior. Além disso, o testículo fetal de indivíduos cujas mães foram expostas ao DiPeP no período gestacional, apresentaram redução nos níveis de expressão gênica de importantes proteínas esteredoigênicas, redução nos níveis de transcrição do gene *Insl3* no testículo fetal e alteração na expressão gênica de receptores de hormônios esteroides (CURI et al., 2019).

1.3 Legislação no Brasil

Em 2016, foram rejeitados pelo Senado Federal quatro projetos de lei (PL), apensionados no PL nº 3.075/ 2011, que visavam a proibição de determinados ftalatos, entre eles o DBP, na produção de garrafas e copos descatáveis (PL 6388/2009), em embalagens para alimentos sólidos, bebidas e medicamentos (PL1197/2011), em insumos médicos como cateteres, bolsas de sangue, bolsas de soro e outros dispositivos (PL 3221/2012) e em brinquedos (PL 3222/2012). As justificativas para a rejeição dos projetos foram a incertezas científicas referentes ao impacto dos ftalatos sobre a saúde humana e a existência de normas determinadas pela Agencia Nacional de Vigilância Sanitária (ANVISA) e o Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO).

Para brinquedos, o INMETRO divulgou as Portarias nº369 de 27 de setembro de 2007, nº 217 de 18 de junho de 2020, determinando ensaios toxicológicos necessários e normas para o uso dos ftalatos BBP, DBP, DEHP, DiNP, DiDP, DNOP. Segundo a Portaria nª 369, a quantidade de ftalato nesse tipo de produto não pode ultrapassar 0,1% da massa de material vinílico do brinquedo.

Em 1998, a Secretaria de Vigilância Sanitária desenvolveu um regulamento técnico, Portaria nº 950/MS/SVS, sobre do uso de DEHP, ou outros químicos que venham a ser aprovados pela ANVISA, na produção de bolsas plásticas para coleta.

As Resoluções da Diretoria Colegiada (RDC) nº 105, de 19 de maio de 1999, RDC nº 17 de 12 de janeiro de 2008, RDC nº 51 de 26 de novembro de 2010 e RDC nº 56 de 16 de novembro de 2012 dispõem regulamentação técnica sobre embalagens e equipamentos plásticos que em sua aplicação mantenham contato com alimentos. Nessas resoluções estão listadas substâncias que podem ser utilizadas na produção desses itens plástico e determinados os limites de migração permitidos. No caso do DBP, segundo a ANVISA, o limite de migração específico (LME) é de 0,3mg/Kg e não pode ser utilizado em revestimentos que mantenham contato com alimentos gordurosos.

Em17 de junho de 2016, a ANVISA publicou a RDC nº 83 o qual contém uma lista de substâncias proibidas em produtos de higiene pessoal, cosméticos e perfumes. Entre as substâncias proibidas estão: DBP (Permitido apenas em produtos para unhas na concentração máxima de 15%), DEHP, bis (2-metoxietil) ftalato

(DMEP), di-n-pentil ftalato (DnPB), DiPeP e BBP. Essa é a única resolução que aborda o uso do DiPeP.

Em relação ao meio ambiente, o Conselho Nacional do Meio Ambinte (CONAMA) dispõe, na Resolução n° 420 de 2009, valores limites para a presença de DEHP (0,6 $mg.Kg^{-1}$ de solo), DMP (0,25 $mg.Kg^{-1}$ de solo) e DBP (0,7 $mg.Kg^{-1}$ de solo) no solo. Além disso, o mesmo documento apresenta as concentrações de DEHP (8 $\mu g.L^{-1}$) e DMP (14 $\mu g.L^{-1}$) em águas subterrâneas, acima das quais apresentes potenciais riscos à saúde humana. Entretanto, a resolução do CONAMA n°357 de 2005, a qual estabelece limites máximos de diferentes substâncias químicas em água doce, salina e salabro, não menciona em seus anexos os limites para nenhum ftalato.

1.4 Bioensaios com peixes

Bioensaios são importantes ferramentas em estudos de toxicologia pois permitem avaliar efeitos de contaminantes específicos em organismos teste, de forma isolada ou em misturas, em concentrações ou doses controladas, e reduzida influência a variáveis ambientais. Assim, é possível desenvolver estudos de especificidade relativa de efeitos com contaminantes avaliados (AKAISHI, 2003).

Organismos teste possibilitam diferentes vias de administração (hídrico, trófico e injeção intraperitoneal) e em períodos de exposição (aguda, subcrônica e crônica) da substância em estudo, permitindo a avaliação do efeito sob vias metabólicas distintas conforme a origem da exposição ao xenobionte, impactos em células somáticas e germinativas, tecidos e órgãos (PANDRANGI et al., 1995; RABELLO-GAY, RODRIGUES e MONTELEONE-NETO, 1991).

Peixes são muito utilizados tanto como organismos testes em bioensaios como bioindicadores em biomonitoramentos. Atuam em diferentes níveis da cadeia trófica, tem capacidade de bioacumulação, respondem à presença de contaminantes em baixas concentrações (GOKSØYR et al., 1991; MINISSI; CICCOTTI; RIZZONI, 1996).

Em peixes, os ftalatos podem ser absorvidos da água pelas brânquias durante a respiração, pela exposição dérmica ou pela ingestão de alimentos ou de sedimentos (BHATIA et al., 2014). Contaminantes emergentes, como ftalatos, podem demostrar baixa toxicidade em ensaios agudos, relativamente insignificantes em peixes. Entretanto, são capazes de causar efeitos no metabolismo de organismos aquáticos

em concentrações muito baixas (nanogramas ou microgramas por litro) quando expostos por longo período (MONTAGNER; JARDIM, 2011; RHODES et al., 1995).

Segundo Gobas e Ikonomou (2003), peixes ocupam níveis tróficos mais altos, e uma vez que organismos menos complexos, de níveis tróficos baixos, bioacumulam mais ftalatos, e que as concentrações biodisponíveis de ftalatos na água são muito baixas, torna a ingestão de alimento uma importante via de exposição.

1.5 *Rhamdia quelen*

A espécie neotropical *Rhamdia quelen*, popularmente conhecida como jundiá ou bagre, pertence à família Heptapteridae (NELSON; GRANDE; WILSON, 2016), habita lagos e poços fundos dos rios, de águas mais calmas com fundo de areia e lama, junto às margens e vegetação, do sul do México até o centro da Argentina (BOMBARDELLI et al., 2006).

É uma espécie nativa promissora para o cultivo, com rápido crescimento, hábito alimentar onívoro, fácil adaptação ao manejo, boa produtividade em açudes e alto potencial de comercialização (BOMBARDELLI et al., 2006; GOMES et al., 2000; MARCHIORO; BALDISSEROTTO, 1999).

Além da importância econômica, a espécie é utilizada como organismo teste em estudos de toxicologia, principalmente com exposições à baixas doses devido à sensibilidade da espécie (GHELFI et al., 2016; GHISI et al., 2011; GUILOSKI et al., 2017a; KLINGELFUS et al., 2017; PEREIRA et al., 2016; PERUSSOLO et al., 2019; PIANCINI et al., 2015a). Segundo Klingelfus e colaboradores (2015), a espécie endêmica da América do Sul, *Rhamdia quelen,* é adequada para bioensaios tróficos por ser onívorae facilmente aceitar a alimentação manual em contato com o tratador.

1.6 Biomarcadores

1.6.1 Parâmetros hematológicos

Segundo Nikinmaa (2020), é possível distinguir efeitos em células sanguíneas de peixes que fazem parte de seu ciclo natural, e causados por mudanças ambientais naturais, dos que são promovidos por substâncias tóxicas. Enquanto mudanças de estação e temperatura afetam a quantidade de eritrócitos e o volume de sangue,

exposições à contaminantes afetam a eritropoiese e estimulam a hemólise. Os parâmetros hematológicos de *Oreochromis niloticus* foram sensíveis à exposição ao DEP, sendo considerado um bom biomarcador para ftalatos (SEPPERUMAL; SAMINATHAN, 2013).

1.6.2 Biomarcadores bioquímicos

Considerados biomarcadores precoces, os biomarcadores bioquímicos detectam alterações, relacionadas ao estresse oxidativo e por contaminantes, em funções bioquímicas e fisiológicas antes da exposição ao xenobionte de gerar consequências em níveis populacionais ou da comunidade (BUCHELI; FENT, 1995; PAYNE et al., 1987). As enzimas envolvidas no processo de detoxificação de xenobiontes são um dos principais biomarcadores bioquímicos estudados (VAN DER OOST et al., 2003).

Existem relatos na literatura de alterações bioquímicas causadas pela exposição aos ftalatos, por exemplo a redução de acetilcolinesterase cerebral (AChE) e glutationa-S-transferase (GST), aumento de glutationa peroxidase (GPx), glutationa reduzida (GSH), glutationa redutase (GR) e lipoperoxidação (LPO), indicando possível estresse oxidativo (GHORPADE et al., 2002; KANG et al., 2010).

A glutationa S-transferase (GST) é uma enzima importante no mecanismo de biotransformação de fase II. Para facilitar a eliminação de moléculas lipofílicas fazem a conjugação da molécula com a glutationa reduzida (GSH) tornando-a hidrofílicas (MARIONNET; DESCHAUX; REYNAUD, 2006; VAN DER OOST; BEYER; VERMEULEN, 2003).

A superóxido dismutase (SOD) atua como uma primeira defesa contra EROs (CHO *et al,* 2006). É uma metaloenzima responsável por converter superóxidos reativos ($\bullet O_2^-$) em peróxido de hidrogênio (H_2O_2) e oxigênio (O_2) através da reação de dismutação. Apesar do produto gerado pela ação da SOD também causar danos celulares e alterar a atividade de enzimas importantes, o sistema antioxidante possui duas enzimas que atuam na degradação deste H_2O_2, essas são: catalase e glutationa peroxidase. (ATLI et al., 2006). Assim, enzimas como a catalase (CAT) e a glutationa peroxidase (GPx) são importantes na degradação deste H_2O_2(CHO et al., 2006).

Como mencionado no parágrafo anterior, a CAT atua na quebra do peróxido de hidrogênio em água e oxigênio. Localizadas nos peroxissomos na maioria das células,

atuam no metabolismo de ácidos graxos pincipalmente no fígado (STEGEMAN et al., 2018).

A glutationa peroxidase (GPx) além de degradar H_2O_2 também converte uma variedade de outros peróxidos (VAN DER OOST; BEYER; VERMEULEN, 2003). Em peixes, é a principal peroxidase, importante atuação na degradação de peróxidos lipídicos, impede a propagação de reações em cascata da peroxidação lipídica (LPO), evitando assim, danos de membrana. Para a atividade da GPx é essencial a presença da, molécula não enzimática, GSH que atua como seu cofator (VAN DER OOST; BEYER; VERMEULEN, 2003).

A peroxidação lipídica (LPO) é um importante biomarcador para detectar danos celulares promovidos por estresse oxidativo (HAI; VARGA; MATKOVICS, 1995; STEGEMAN et al., 2018; VAN DER OOST; BEYER; VERMEULEN, 2003). Devido à suscetibilidade dos lipídios de membrana reagirem com EROs, as sucessivas reações de oxidação afetam a função das membranas celulares, aumentando sua permeabilidade, e em casos extremos podem causar sua ruptura total, seguida de morte celular (GUTTERIDGE; HALLIWELL, 2000; MEAGHER; FITZGERALD, 2000; VAN DER OOST; BEYER; VERMEULEN, 2003).

A AChE é uma enzima que pode ser encontrada no cérebro, em eritrócitos e no músculo (KLEMZ; CRISTINA DA SILVA DE ASSIS, 2017). Sua função é realizar hidrólise da acetilcolina (ACh), presente nas sinapses, em colina e ácido acético (KAVITHA; RAO, 2007). Essa hidrólise previne um estimulo nervoso contínuo (VAN DER OOST; BEYER; VERMEULEN, 2003).

1.6.3 Biomarcadores genéticos

Assim como biomarcados bioquímicos, os genéticos também são considerados biomarcadores precoces pois permitem detectar efeitos iniciais causados por contaminantes (VAN DER OOST; BEYER; VERMEULEN, 2003). Essa classe de biomarcadores tem sido amplamente utilizadas como ferramentas para detecção de exposição e avaliação de possíveis aberrações cromossômicas, quebras no DNA, formação de micronúcleos e alterações morfológicas nucleares (BOMBAIL et al., 2001).

O teste do micronúcleo originalmente desenvolvido por Schmid (1975) para células de medula óssea de camundongos. Posteriormente, sofreu adaptações por

Hooftman e Raat (1982) para a aplicação em células sanguíneas de peixes mantidos em laboratórios. Essa técnica modificada passou a ser chamada de *Piscine Micronucleus Test* (Teste do Micronúcleo Písceo).

Micronúcleos são cromossomos, ou frações deles, resultantes de quebras e que, durante o processo de divisão celular, não foram incorporados ao núcleo da célula filha. Durante a divisão celular, também podem ocorrer anomalias celulares, quando determinada quantidade de material fica levemente atrasada na mitose, fazendo com que o núcleo resultante não seja oval, e apresente saliências de cromatina (BOMBAIL et al., 2001).

Os critérios para classificação de micronúcleo písceo são: apresentar-se visivelmente separado do núcleo principal, com as membranas distinguíveis e mesma refringência, e ter aproximadamente de 1/10 a 1/30 do tamanho núcleo principal (AL-SABTI; METCALFE, 1995; AYLLON; GARCIA-VAZQUEZ, 2000; GUSTAVINO et al., 2001).

Em peixes, os diferentes tipos de alterações eritrocitárias nucleares ainda não apresentam sua origem completamente compreendida. Carrasco; Tilbury; Myers (1990) descreveram e fotografaram as alterações morfológicas nucleares de eritrócitos de peixes e as classificaram como: *Blebbed* (Pequena evaginação na membrana nuclear); *Lobbed* (Grandes evaginações, geralmente apresentam várias estruturas dessas); Vacuolated (Apresentam uma pequena região nuclear destituída de material genético, como um vacúolo); *Notched* (corte ou invaginação na membrana nuclear).

Outro biomarcador genético muito utilizado em ensaios de toxicidade é o ensaio cometa, SCGE (*Single-Cell Gel Eletrophoresis*). O ensaio avalia danos no DNA em células individualizadas, por meio da medida da migração do DNA em gel de agarose após corrida eletroforética (FERRARO et al., 2004; SINGH et al., 1988). O nome cometa refere-se à característica que nucleóide apresenta após a passagem de corrente elétrica, quando apresença de muitos fragmentos de DNA que migram em direção ao polo positivo da cuba (BOMBAIL et al., 2001).

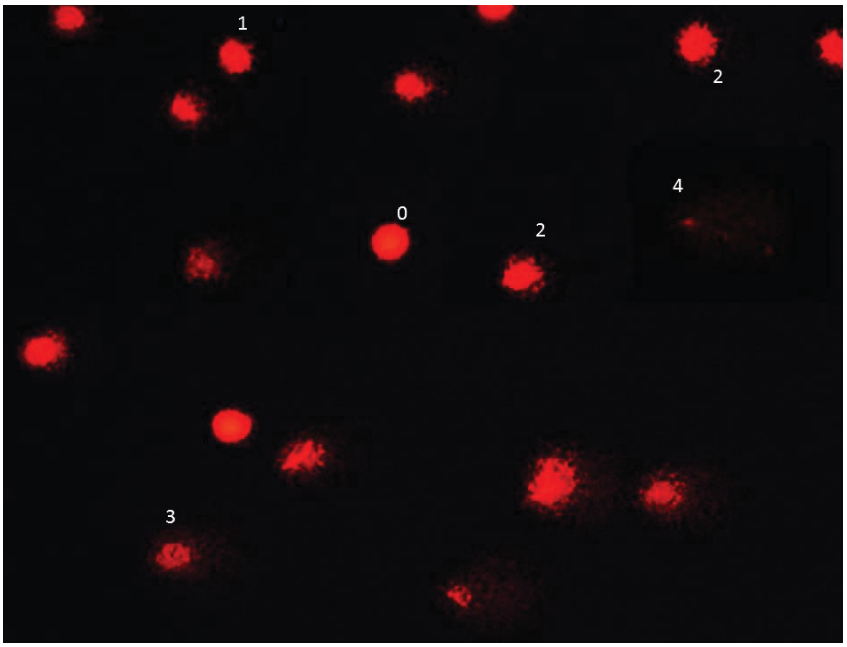
A técnica descrita por Singh et al. (1988) consiste em utilizar uma suspensão de células em agarose e depositadas em lâmina coberta com uma fina camada agarose. Posteriormente as células passam por um processo de lise para quebrar membranas e proteínas, restando apenas o material genético nuclear, o nucleóide. Esse passa pela etapa de desespirilização do DNA e, em seguida, pela corrida

eletroforética em tampão alcalino. Assim, é possível detectar eventos de quebras de fita simples ou duplas, sítios álcalo-lábeis e sítios de reparo tardio (KOPPEN et al., 2017).

O DNA da célula que não possuir dano migrará em conjunto durante a corrida eletroforética, apresentando-se como um círculo. Os nucleóides que sofrem danos em seu DNA apresentam fragmentos diversos. Fragmentos menores tendem a migrar com mais velocidade que fragmentos maiores. Ocorrendo danos intensos, muitos fragmentos de tamanhos variados serão formados, e migrarão em velocidades diferentes, formando então a figura típica de um cometa(OLIVE; BANÁTH; DURAND, 1990).

Para quantificar os danos observados, é a realizada de análise visual e a classificação de cada nucleóide de acordo com a relação entre o raio do núcleo e a extensão da “cauda” formada pelos fragmentos de DNA. Essa classificação varia entre classe 0, onde não ocorreu dano visível, até a classe 4, indicando a ocorrência de muitos danos ao DNA (FIGURA 3) (COLLINS et al., 1997).

FIGURA 3 - CLASSIFICAÇÃO DE NUCLEÓIDES SEGUNDO COLLINS ET AL. (1997).



FONTE: A AUTORA.

Uma das vantagens do ensaio cometa é que este não depende de proliferação celular para ser realizado, portanto pode ser utilizado com qualquer tipo celular nucleado. Além disso, é possível aumentar a sensibilidade do ensaio tratando as

lâminas com endonucleases, como FPG, ENDO III e ou hOGG1) que possibilitam a visualização de danos oxidativos, com a identificação de nucleotídeos oxidados. Por meio do ensaio cometa, é identificar possíveis tecidos-alvo, pela avaliação da genotoxicidade tecido-específica de um contaminante (PANDRANGI et al., 1995; ROJAS; LOPEZ; VALVERDE, 1999).

2. OBJETIVOS

2.1 Objetivo geral

O presente estudo teve como objetivo avaliar a toxicidade dos ftalatos di-n-butil ftalato (DBP) e diisopentil ftalato (DiPeP) em condições de exposição ambientalmente relevantes, de forma trófica e subcrônica em uma espécie de peixe nativa no Brasil (*Rhamdia quelen*.

2.2 Objetivos específicos

- Avaliar o potencial genotóxico do DBP e DiPeP em células sanguíneas, hepáticas, renais, cerebrais de *Rhamdia quelen*.
- Avaliar a influência do DBP e DiPeP na atividade de enzimas e moléculas não enzimáticas importantes no processo de desintoxicação (GST, GSH, SOD, CAT, GPx).
- Avaliar se o DBP e o DiPeP induzem a peroxidação lipídica nos tecidos analisados resultando em morte celular.
- Avaliar o potencial neurotóxico em tecido muscular e cerebral por meio da atividade da AChE.
- Identificar potenciais tecidos-alvo dos contaminantes estudados.

3. JUSTIFICATIVA

O ecossistema aquático é um dos mais impactados por ser o destino final de vários produtos gerados por atividades antrópicas. O despejo de esgoto industrial e doméstico, o uso de agrotóxicos, o descarte inapropriado de lixo, medicamentos, e outros produtos impactam diretamente nesse ecossistema.

As industrias utilizam em seus processos de uma vasta diversidade de produtos químicos potencialmente tóxicos, que podem gerar resíduos prejudiciais ao meio ambiente, como o caso dos ftalatos e a produção de plásticos. No Brasil, segundo a Associação Brasileira da Indústria do Plástico (ABIPLAST, 2019), existem 11.018 empresas do setor de transformados de plásticos. Em 2019, esse setor gerou, a partir de polímeros, mais de 7,6 milhões de toneladas em produtos plásticos como embalagens, acessórios para automóveis, brinquedos, etc. Entre as resinas poliméricas mais utilizadas por essas indústrias estão o polipropileno (PP) e o PVC correspondendo, respectivamente, a 21% e 13.6% do total de polímeros empregados nessa atividade (ABIPLAST, 2019).

Diante do volume da produção de plásticos, o uso de DBP e DiPeP na sua composição, torna-se preocupante as consequências que esses possam causar ao ambiente e aos organismos. Além disso, tanto DBP quanto DiPeP já foram detectados no ambiente e a exposição humana também foi evidenciada.

Apesar de existirem muitos estudos, com diferentes organismos e linhagens celulares, relatem os efeitos da toxicidade do DBP, poucos são os que avaliam exposições por longos períodos às concentrações ou doses baixas. Contudo, estudos de avaliação da toxicidade do DiPeP são escassos na literatura, mais precisamente existem apenas quatro artigos publicados a respeito da exposição humana e efeitos em roedores.

O processo que dá origem à grande disponibilidade de DiPeP está relacionado com questões políticas e econômicas do Brasil, a produção de etanol para substituição de combustíveis fósseis. Dessa forma, conhecer os possíveis efeitos promovidos pela exposição ao DiPeP é essencial para que possam ser desenvolvidas regulamentações do uso e destarte desse composto. Além disso, muitas pesquisas são desenvolvidas com espécies de peixes modelo como o *Danio rerio* ou de regiões temperadas. Nesse sentido, faz-se necessário estudo com espécies nativas, pois compostos químicos podem apresentar comportamentos diferentes em relação à

temperatura e aos organismos submetidos à estes xenobiontes, visto que esses apresentam comportamentos, metabolismo e sensibilidade diferentes entre regiões temperadas e tropicais (KWOK et al., 2007).

4. Impact of subchronic trophic exposure to low doses of DBP on *Rhamdia quelen*: evaluation by multiple biomarkers

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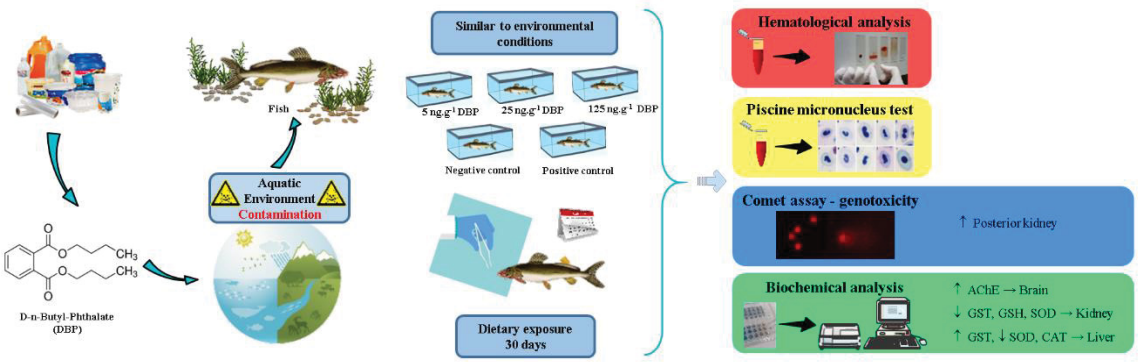
Graphical Abstract



Highlights

- Subchronic trophic exposure to DBP caused DNA damage in kidney cells.
- Renal and hepatic enzymes of the antioxidant system underwent significant changes.
- Anterior kidney showed more DNA damage when compared to posterior kidney.
- Low doses of DBP caused changes in the cerebral AChE of *Rhamdia quelen*.

Abstract

Di-n-butyl phthalate (DBP) is used in a range of industrialized products, including paints, cosmetics and plastics for building materials, medical devices, toys and packaging. Due the large volume of production and consumption of these products, DBP is one of the most abundant phthalates in water, soil, animal and human tissues. However, studies analyzing the effects of relevant environmental doses of DBP and for extensive periods of exposure are scarce. The aim of this study was to evaluate the effects of low doses of DBP after subchronic trophic exposure, using as biomarkers hematological, genetics (micronucleus test and comet assay), antioxidant system (glutathione S-transferase, reduced glutathione, superoxy dismutase, catalase, glutathione peroxidase and lipoperoxidation) as well as a neurotoxicological biomarker (acetylcholinesterase). Specimens of the Neotropical fish, *Rhamdia quelen*, were kept in individual aquariums and divided into six groups: commercial food control, negative control (coconut oil), 5 ng/g, 25 ng/g and 125 ng/g of DBP and positive control. Every other day, animals received food containing the respective dose for their treatment group. After 15 doses (30 days), the animals were anesthetized and euthanized. Tissues were collected: blood, anterior and posterior kidney, liver, brain and muscle. DBP did not cause changes in the hematological profile, frequency of micronucleus and nuclear abnormalities. An increase in DNA damage, was observed in the anterior kidney due to DBP exposure. In the posterior kidney, a reduction in GST, SOD, GPx activities, as well as a decrease in the concentration of GSH in groups exposed to DBP were observed. In hepatic tissue, DBP caused an increase in GST activity, but a reduction in the activities of SOD and CAT was verified. Acetylcholinesterase activity measured in *R. quelen* brain was increased in all groups treated with DBP. Our results demonstrate that low doses of DBP are able to promote toxic effects, mainly on hepatic and kidney antioxidant system, in addition presenting a neurotoxic potential on *R. quelen* brain.

Keywords: di-n-butyl phthalate, fish, oxidative stress, kidney, neurotoxicity, hematology.

4.1 Introduction

Phthalic acid esters (PAEs), known as phthalates, are synthetic organic compounds used in the production of several products, such as: plastics, personal care products, cosmetics, medical devices, toys, packaging, paints, construction materials, textile, pharmaceutical, foodstuffs products among others (CHEN et al., 2014; DU et al., 2015; GAO et al., 2019; NET et al., 2015; STAPLES et al., 1997a; TIWARI; SAHU; PANDIT, 2019; XU et al., 2013a). In plastics, such as polyvinyl chloride (PVC), for example, phthalate molecules are not chemically bound to polymeric matrices and consequently are transferred to the environment during industrial processes of production, use and effluent disposal (HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; NET et al., 2015; STAPLES et al., 1997a). One of the environments most affected by the presence of phthalates is the aquatic environment, as it is the final destination of several compounds used in products for human consumption (FRENZILLI; NIGRO; LYONS, 2009). Due to the increase in large-scale production of plastics and its most diverse industrial applications, phthalates have come to be considered a threat to both humans and aquatic biota (WANG et al., 2014). Initially, it was believed that the frequent non-detection of some phthalates in the aquatic environment would indicate that they would not pose a threat to organisms in this ecosystem (STAPLES; PARKERTON; PETERSON, 2000). However, several more studies have revealed the presence of different types of phthalates in different aquatic ecosystems (DOMÍNGUEZ-MORUECO; GONZÁLEZ-ALONSO; VALCÁRCEL, 2014; MONTAGNER et al., 2019; MONTAGNER; JARDIM, 2011; NET et al., 2015; PEIJNENBURG; STRUIJS, 2006; TIWARI; SAHU; PANDIT, 2019).

Among the phthalates detected in the aquatic environment, in countries such as Brazil, India and the Netherlands, Di-n-Butyl-Phthalate (DBP) is one of the most frequent in surface waters, with a concentration ranging from 66ng / L to 33,100ng / L, varying according to the intensity of anthropic activity developed in the collection region (MONTAGNER et al., 2019; MONTAGNER; JARDIM, 2011; SELVARAJ et al., 2014; VETHAAK et al., 2005). In fish of different trophic levels, caught in rivers and seas in China and Nigeria, 0.58 g and 1716.67 g of DBP per g of dry weight were measured, evidencing the exposure of these organisms to DBP (ADENIYI; OKEDEYI; YUSUF, 2011; CHENG et al., 2013). In fish bioassays, DBP has the potential to affect the reproductive system, to cause endocrine disruption (AOKI et al., 2011;

JARMOŁOWICZ; DEMSKA-ZAKĘŚ; ZAKĘŚ, 2013), immunological and neurotoxic effects (XU et al., 2013b, 2015). However, studies on early DBP effects, after long periods of exposure and low doses or concentrations are scarce in the literature.

Rhamdia quelen (Siluriforme, Heptapteridae) is a species of omnivorous freshwater fish, endemic to Neotropical regions with a wide distribution in South America (BOMBARDELLI et al., 2006; GOMES et al., 2000). Besides its economic importance (MARCHIORO; BALDISSEROTTO, 1999), the species has been widely used in studies of environmental toxicology as a test organism in trophic exposures (KLINGELFUS et al., 2015), mainly at low concentrations of contaminants of different classes (GHELFI et al., 2016; GHISI et al., 2011; GUILOSKI et al., 2017a, 2017b; MATHIAS et al., 2018; PEREIRA et al., 2016; PERUSSOLO et al., 2019; PIANCINI et al., 2015a).

Late and reproductive effects of phthalate exposure in fish are described in the literature (AOKI et al., 2011; JARMOŁOWICZ; DEMSKA-ZAKĘŚ; ZAKĘŚ, 2013; RHODES et al., 1995). However, the early effects of subchronic exposure to DPB in fish are poorly described in the literature. Thus, the experimental model used in our study aimed to reproduce, in a controlled aquatic environment, the exposure of specimens of *Rhamdia quelen* to prolonged trophic contamination at doses of environmentally relevant DBP. As a source of evidence, we use hematological, genetic and biochemical biomarkers and assess which tissues are potential targets for this compound to infer which of the tested biomarkers may be affected in higher periods of exposure to these compounds. To date, this is the first report of a subchronic trophic assay, with constant exposure to low doses of DBP, in a species of Neotropical fish.

4.2 Material and Methods

4.2.1 Animals

The study was approved by the Ethics Committee on the Use of Animals of the Biological Sciences Sector of the Federal University of Paraná (CEUA / BIO - UFPR) under number 1180. The Laboratory of Technology for the Reproduction of Aquatic Animals Cultivables (LATRAAC), from the State University of Western Paraná (UNIOESTE), provided the ninety juvenile specimens of *Rhamdia quelen* used in this study. Upon arriving at the Animal Cytogenetics and Environmental Mutagenesis

Laboratory at the Federal University of Paraná (UFPR), the animals were acclimatized for 60 days in a 2000 L tank, at 27ºC, constant aeration and a 12 h light / 12 h dark photoperiod. During the acclimatization period, fish were fed commercial food (Supra® 32% protein) *ad libitum* once a day.

4.2.2 DBP

Di-n-butyl-phthalate (DnBP 99%, CAS n° 84742, Sigma-Aldrich®, Darmstadt, Germany, Product number 524980) was diluted in commercial organic extra virgin coconut oil (COPRA®) in concentrations of 5 mg/L, 25 mg/L and 125 mg/L. The solutions remained for five minutes in an ultrasonic water bath (Schuster®, model: L-100) at a frequency of 42 kHz for complete homogenization. The concentrations were calculated to correspond to the respective doses of 5 ng/g, 25 ng/g and 125 ng/g (JARMOŁOWICZ; DEMSKA-ZAKĘŚ; ZAKĘŚ, 2013) when administered in the proportion of 1 μL of the phthalate solution per gram of fish (For example, a 60 g fish received 60 μL of the DBP solution, in the concentration of the respective treatment group, in each dose administered). Coconut oil was selected as a solvent because different from other organic solvents, such as DMSO and ethanol, it is a natural product commercialized mainly for food purposes. Besides, this oil solidifies at temperatures below 21°C (MURSALIN et al., 2016), ensuring the administration of the correct dose of DBP at the moment of supply to the fish.

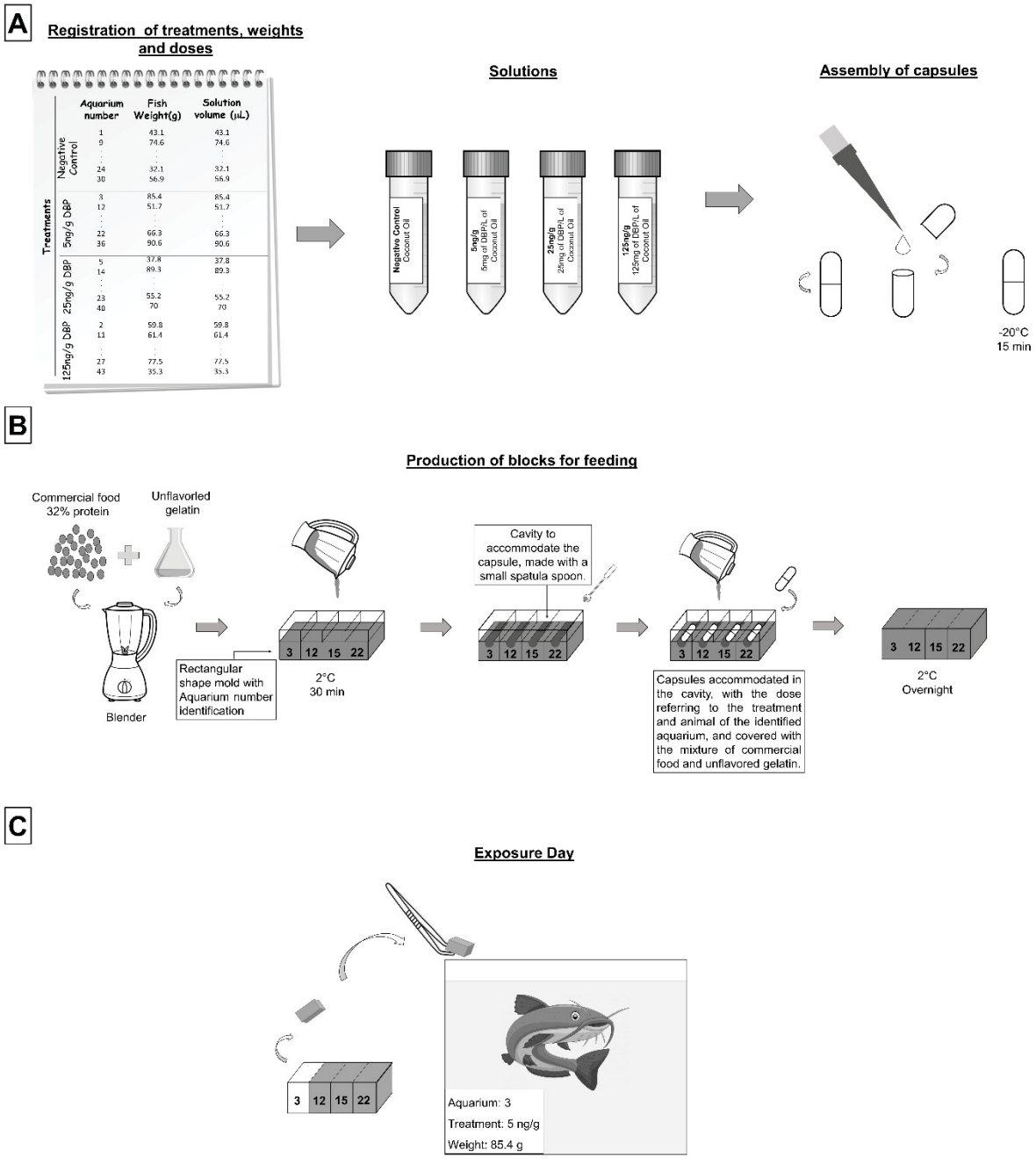
4.2.3 Animal training

After the acclimatization period, the animals were weighed (mean weight 76.40 ± 25.44 g) and transferred to individual 20 L aquariums, with a temperature of 26.4 ± 2.7 ° C, constant aeration and 12 h light / 12 h dark photoperiod. Fifteen days previous to the exposure, the animals were trained to accept the food and eat it entirely, at the time of the offer. During training, every 48 h, a mixture of ration and unflavored gelatin in block format was offered (Figure 1).

4.2.4 Trophic exposure

The aquariums were identified and linked numerically to the weight of animal and the treatment to be received. The doses of DBP were calculated according to the weight of each specimen of *Rhamdia quelen* and to the treatment, and inserted in gelatinous commercial capsules (Mawin®), which were immediately frozen (Figure 1). Rectangular shaped molds were used to make the blocks of food that were the vehicles of the exposure. The mold shapes were identified with the number assigned to each aquarium, these were filled 50% of their volume with a mixture of commercial food (Supra® 32% protein) and unflavored gelatin. After 30 minutes under refrigeration, cavities were made to accommodate the capsules with the corresponding doses. Immediately after the allocation of the doses, the forms were filled with the mixture of commercial food and unflavored gelatin until the total volume was completed. The mold shapes were kept under refrigeration overnight, to ensure complete solidification of the unflavored gelatin and coconut oil. In the mornings following the production of these food blocks, the exposures happened.

Figure 1 - Preparation mode of the food blocks applied for the trophic exposure of *Rhamdia quelen*. A: The registry of treatments, weights and doses linked the information of each animal to a numbered aquarium. Four solutions for trophic exposure were used: Negative Control (Cononut oil), 5 ng/g (5 mg/L), 25 ng/g (25 mg/L) and 125 ng/g (125 mg/L). According to the animal weight and treatment, the dose was determined in the proportion of 1μL of solution per gram of fish. This volume was inserted into gelatin capsules and immediately transferred to a freezer -20 ° C for 15 minutes. B: A mixture of commercial food and unflavored gelatin was deposited in rectangular mold forms with the identification of each aquarium. After 30 minutes at temperature of 2 °C, a cavity was produced to accommodate the capsule with the capsule corresponding to the treatment of the aquarium identified in the mold shapes. When receiving the capsules with the DBP doses, the forms were completely filled, with the mixture of commercial food and unflavored gelatin, and kept at 2 ° C overnight. C: On the day of the exposure, the solidified food block was offered to the animal present in the aquarium corresponding to the mold shape identification.



The animals were divided into six groups (n = 15 / group): Commercial food Control (CFC), Negative Control (NC), 5 ng DBP/g fish, 25 ng DBP/g fish and 125 ng DBP/g fish and Positive Control (PC). The blocks containing the doses of phthalates were offered at intervals of 48 h, totaling 15 doses (30 days). The CFC and PC groups received only commercial food *ad libitum*, in the same time interval. The animals belonging to the PC group received, 24 hours before tissue collection, a single dose of 5 μg of methyl methanesulfonate (MMS – Sigma Aldrich® - CAT nº: 66-27-3) per g of fish, by intraperitoneal injection (PIANCINI et al., 2015b). The PC of this experiment was used only as a control of the comet assay technique.

After 30 days of exposure, the animals were anesthetized in benzocaine (Merck®, Darmstadt, Germany) diluted in 10% ethanol, and before use, diluted in water at a concentration of 10 mg/L (GONTIJO et al., 2003). Blood was collected with a heparinized syringe by puncture of the caudal artery and used to evaluate genotoxicity (micronucleus piscine and comet assay), as well as for the analysis of hematological parameters. Then, the animals were euthanized by the spinal cord section and tissues were collected. The organs collected for the comet assay (liver, anterior kidney, posterior kidney and brain) were kept in 500 μL of fetal bovine serum (FBS), previously refrigerated and in the absence of light. The tissues (liver, posterior kidney, brain and muscle) intended for biochemical tests were immediately frozen in liquid nitrogen and subsequently stored in a freezer -80°C until processing.

4.2.5 Hematological biomarkers

The erythrocytes count was performed by diluting the blood (10 μL) in 2 mL of formaldehyde-citrate in a Neubauer chamber (OLIVEIRA-JÚNIOR; TAVARES-DIAS; MARCON, 2009), and analyzed in optical microscope.

For the leukocyte count, a blood extension was performed and stained with Instant Prov (New Prov®). The total count of leukocytes and thrombocytes was performed under an optical microscope according to the methodology described by Tavares-dias and Moraes (2006).

4.2.6 Genetic biomarkers

Piscine Micronucleus Test (PMT)

The Piscine Micronucleus Test (PMT) was performed as described by Heddle (1973) and Schmid (1975), with modifications by Ferraro et al. (2004), to assess the frequency of micronuclei in peripheral erythrocytes. Immediately after blood collection, a drop was deposited on a clean slide and blood extension was performed. After overnight drying, the slides were fixed for 30 minutes in ethanol P.A. (Merck®, CAT nº: 107017) and stained with 10% Giemsa solution (Merck®, CAT nº: 109204). In this test, in addition to the micronucleus, the frequencies of other nuclear erythrocytes alterations were assessed, as described by Carrasco et al. (1990) and Çavaş and Ergene-Gözükara (2005). One slide was made per fish and 2,000 erythrocytes were analyzed in each slide under an optical microscope.

Comet Assay

For the performance of the comet assay in blood cells, about 10 μL of blood was added in 1000 μL in fetal bovine serum previously refrigerated, and the analysis was performed according to the methodology described by Speit and Hartmann (1999) with alterations by Cestari et al. (2004) and Ferraro et al. (2004). The solid tissues (liver, anterior kidney, posterior kidney and brain) were mechanically homogenized in 500 μL of refrigerated fetal bovine serum, to individualize the cells. To make the slides, cell suspensions (10 μL for blood and 20 μL for solid tissues) were dissolved in 120 μL of low melting point agarose (0.5%) (KLINGELFUS et al., 2017; RAMSDORF et al., 2009).

The slides were immersed in lysis solution (Stock solution of lysis: NaCl, 2.5 M; EDTA, 100 mM; Tris, 10 mM; NaOH, 0.8%; N-sodium lauryl sarcosinate; 1%; Lysis working solution: 1 mL Triton X - 100; 10 mL DMSO; and 89 mL of lysis stock solution) for 48 hours. After this period, they were placed in a buffer tank of electrophoresis submerged in an alkaline buffer (NaOH and 200mM EDTA, pH> 13) for 25 minutes, and then submitted to electrophoresis at 300mA, 25V for 25 min. The slides received three baths of 5 minutes each, with neutralization buffer (0.4 M Tris, pH 7.5). Then, when the slides were completely dried, they were fixed in ethanol for 5 min.

In addition to the alkaline version of the comet assay, carried out with all the tissues mentioned above, duplicated liver slides were produced for the oxidative version. The slides destined to the oxidative comet assay went through the same processes previously described for the the alkaline version. However, after the lysis stage, the slides were subjected to treatment with the enzyme (formamidopyrimidine [fapy] -DNA glycosylase) - Fpg (New England Biolabs® - CAT n°: M0240), according to the protocol first described by Collins et al. (1993) and described, step by step, by Reeves et al. (2008). The positive control for the oxidative version was performed using hydrogen peroxide (H_2O_2 - 50 µM - Sigma-Aldrich®-CAT nº: H1009), according to Tice et al. (2000).

The slides were stained with 20 µL of ethidium bromide (10 µL/mL) and analyzed using a Leica® epifluorescence microscope (400 x magnification - model DM2500). One hundred nucleoids were analyzed visually and classified according to the degree of damage (damage 0, damage 1, damage 2, damage 3 and damage 4) and the amount of damage, as described in Koppen et al. (2017) and Azqueta et al. (2011).

4.2.7 Biochemical bimarkers

The liver and posterior kidney samples were homogenized in phosphate buffer 0.1 M (pH 7.0). The homogenate was centrifuged for 30 minutes at 15,000g at 4ºC. The supernatant of the samples was divided into aliquots for the analysis of: glutathione S-transferase (GST), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid lipoperoxidation (LPO) and protein concentration.

The GST activity was measured according to the method described by Keen et al. (1976). The supernatants of liver and posterior kidney were diluted in potassium phosphate buffer (0.1M, pH 6.5). In microplates, 20 µL of the sample of each fish and tissue was added, followed by 180 µL of reaction solution [GSH (3mM), CDNB (3 mM)]. The readings were performed at 340 nm in a spectrophotometer every 30 seconds for 3 minutes.

The reduced glutathione concentration was measured by the method proposed by Sedlak and Lindsay (1968), using a GSH standard curve. For the protein precipitation, 50 µL of trichloroacetic acid 50% (TCA) was added to each 200 µL of

supernatant. The samples were then centrifuged at 10,000 x g for 10 min at 4 °C. For readings, 50 µL of the sample supernatant and 230 µL of buffer (400 mM Tris-base, pH 8.9) were added in a microplate. As a white, 230 µL of 400 mM Tris-base buffer plus 50 µL of TCA 10% was used. Finally, 20 µL of 2.5 mM DTNB (5.5'-dithio-bis-2-nitrobenzoic acid) (in methanol 25%, 400 mM Tris-base buffer, pH 8.9) was added. The readings were performed at 415 nm in a spectrophotometer.

For the analysis of SOD activity, the method proposed by Gao et al. (1998) was used and it is based on the ability of SOD to inhibit the auto-oxidation of pyrogallol. The supernatant was diluted 1:10 (or 10% V / V) in potassium phosphate buffer at 0.1M, pH 7.0. In a microtube, 885 µL of buffer (Tris-base,1 M/EDTA, 5 mM, pH 8.0) was added to 40 µL of the sample. After vortexing, 50 µL of pyrogallol (15 mM) was added and the solution was incubated for 30 minutes. The reaction was stopped with 25 µL of HCl (1N) added to the solution. In a microplate, 300 µL of the solution was added per well and the reading was performed on a spectrophotometer at 440 nm. Concomitantly, a sample control was performed with the addition of reagents, but without incubation, and this value was considered equal to 100%. The amount of SOD that inhibited the reaction by 50% (IC50) is equivalent to 1 unit (U) of SOD.

Applying the method described by Aebi (1984), the catalase kinetics was measured by the gradual reduction in absorbance caused by the breakdown of hydrogen peroxide in water and oxygen. The supernatant was diluted 1: 5 (v / v) in phosphate buffer (0.1 M, pH 7.0). In a microplate, 5 µL of the sample was added in 295 µL of the reaction solution (Tris buffer - base (1 M) / EDTA (5 mM, pH 8.0), hydrogen peroxide (20 mM) and Milli-Q® water). The readings were performed in a spectrophotometer at 240 nm every 15 seconds for 1 minute.

The GPx activity was analyzed by the reduction of oxidized glutathione (GSSG), catalyzed by glutathione reductase (GR), in the presence of NADPH (PAGLIA; VALENTINE, 1967a). In a microplate, 10 µL of supernatant and 130 µL of reaction medium (0.1 M sodium phosphate buffer, pH 7.0; 3 mM sodium azide; 0.3 mM β-NADPH, 1.54 U.ml-1 of GR and 3 mM were added GSH). After 2 minutes, 60 µL of hydrogen peroxide (1.5 mM) was added. The readings were performed in a spectrophotometer at 340 nm every 30 seconds for 2 minutes.

The lipid peroxidation, or lipoperoxidation, was analyzed using the FOX method (Ferrous Oxidation / Xylenol Orange Method), which is based on the rapid oxidation of Fe^{+2} mediated by peroxide under acidic conditions and subsequent formation of the

Fe^{+3} - orange of xylenol complex in the presence of the butylated hydroxytoluene stabilizer (BHT), which absorbs light at 550-570 nm (JIANG; HUNT; WOLFF, 1992). The supernatant was resuspended in 1: 2 (v / v) methanol and centrifuged for 10 minutes at 10,000 x g at 4ºC. Then 100 µL of the supernatant was added in microtubes and incubated with 900 µL of the reaction solution (100 µM xylenol orange, 25 mM H_2SO_4, 4 mM BHT, 250 µM ammoniacal ferrous sulfate, 90% methanol) for 30 min. The readings were performed at 570 nm in a spectrophotometer.

The quantification of total proteins in the samples was performed by the method of Bradford (1976), with a standard curve of bovine serum albumin (BSA). In a microplate, 10 µL of the diluted supernatant 1:20 (v / v) was added with 250 µL of Bradford's Reagent (Biorad®). The readings were performed in a spectrophotometer at 595 nm.

4.2.8 Neurotoxicity biomarkers

The brain and muscle samples were homogenized in 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged for 20 minutes at 10,000g at 4ºC. The supernatant was used to measure the activity of the enzyme acetylcholinesterase (AChE), according to the method of Ellman et al. (1961). In a microplate, 50 µL of the supernatant, 200 µL of DTNB (5.5 dithiobis-2-nitrobenzoate - 0.75 mM) and 50 µL of acetylthiocholine (10 mM) were added. The readings were performed in a spectrophotometer at 405 nm at intervals of 30 seconds for 3 minutes.

4.2.9 Statistical analysis

First, the normality of the data was tested using the Kolmogorov-Smirnov test. For data with normal distribution, the ANOVA test (1 criterion) was then applied, followed by the post-test t (LSD - Least significant difference). For data that did not have a normal distribution or showed uneven variances, the Kruskal-Wallis non-parametric test was used, with Student-Newman-Keuls post-test. Statistical significance adopted for all tests was 0.05 (95% of confidence). All analyzes were performed using the BioEstat 5.0 and GraphPad® Prism 6.0 programs.

4.3 Results

There was no mortality during the period of the bioassay. The following water quality parameters were measured: pH (6.2 ± 0.04), ammonia (<0.1 mg/L), dissolved oxygen (8.6 ± 0.4 mg/L), hardness (36.3 ± 1.7 mg/L) and temperature (26.4 ± 0.6ºC). NC and CFC groups did not show statistically significant differences between themselves for any of the biomarkers used in this study (Supplementary material). For this reason, the NC was used for comparison with the other treatments.

4.3.1 Hematological parameters

DBP did not cause significant changes in the number of erythrocytes, leukocytes and thrombocytes in the treatments when compared to the NC group (Table 1).

Table 1 - Hematological parameters of *Rhamdia quelen* after 30 days of trophic exposure to DBP.

Parameters	Treatments			
	NC	5ng/g	25ng/g	125ng/g
Erythrocytes10^6 /mL	2.64 ±0.64	2.06±0.75	2.08±0.98	2.19±0.97
Leukocytes /mL	27.22±14.19	20.37±14.15	22.92±10.96	22.96±17.21
Thrombocytes /mL	60.44±28.07	47.40±31.49	42.26±24.70	38.56±25.89

Values are presented as mean ± standard deviation. NC - negative control.
There was no statistical difference between treatments and negative control (p> 0.05)

4.3.2 Evaluation of genetic damage

Micronuclei were found at low frequencies, with no statistical differences between treatments and NC.

The nuclear erythrocyte alterations (NEA) as blebbed (evagination), notched (invagination) and vacuole types were observed in *Rhamdia quelen* erythrocytes. However, exposure to DBP did not cause changes in the frequencies of the aforementioned morphological changes or the total morphological changes (Table 2).

Table 2 - Frequency of micronuclei (MN) and nuclear erythrocyte alterations (NEA) in *Rhamdia quelen*, discriminate in blebbed, lobed, notched, vacuolated and Total NEA (MN + NEA).

Treatments	MN	*Blebbed*	*Notched*	*Vacuolated*	Total NEA
NC	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)	2 (1 / 6)	3 (2 / 8)

5ng/g	0 (0 / 0)	0 (0 / 0)	0 (0 / 1)	5 (3 / 10)	5 (3 / 11)
25ng/g	0 (0 / 0)	0 (0 / 0)	0 (0 / 1)	3 (2 / 9)	4 (3 / 9)
125ng/g	0 (0 / 0)	0 (0 / 0)	0 (0 / 1)	4.5 (2 / 5.25)	5 (4 / 8)

Values are expressed as median and interquartile (1st Q / 3rd Q).
NC - negative control.
NEA – nuclear erythrocyte alterations
There was no statistical difference between treatments and NC (*p*> 0.05)

The positive controls of the alkaline and oxidative comet assay, of all analyzed tissues, showed significant differences (p <0.02) when compared to the negative controls, indicating that the MMS (alkaline version) and H_2O_2 (oxidative version) caused increased damage to the genetic material of the cells of the analyzed tissues and, consequently, that the execution of the test was efficient in detecting the different types of damage (Figure 2).

In the alkaline version of the comet assay, there was no increased damage to the DNA of blood, liver, brain and posterior kidney tissues in groups exposed to DBP (p> 0.05) (Figure 2 A, B, D, E).

The comet assay in the oxidative version did not show increased damage to DNA, from liver cells, in the groups treated with DBP in relation to the NC (P> 0.05) (Figure 2 C).

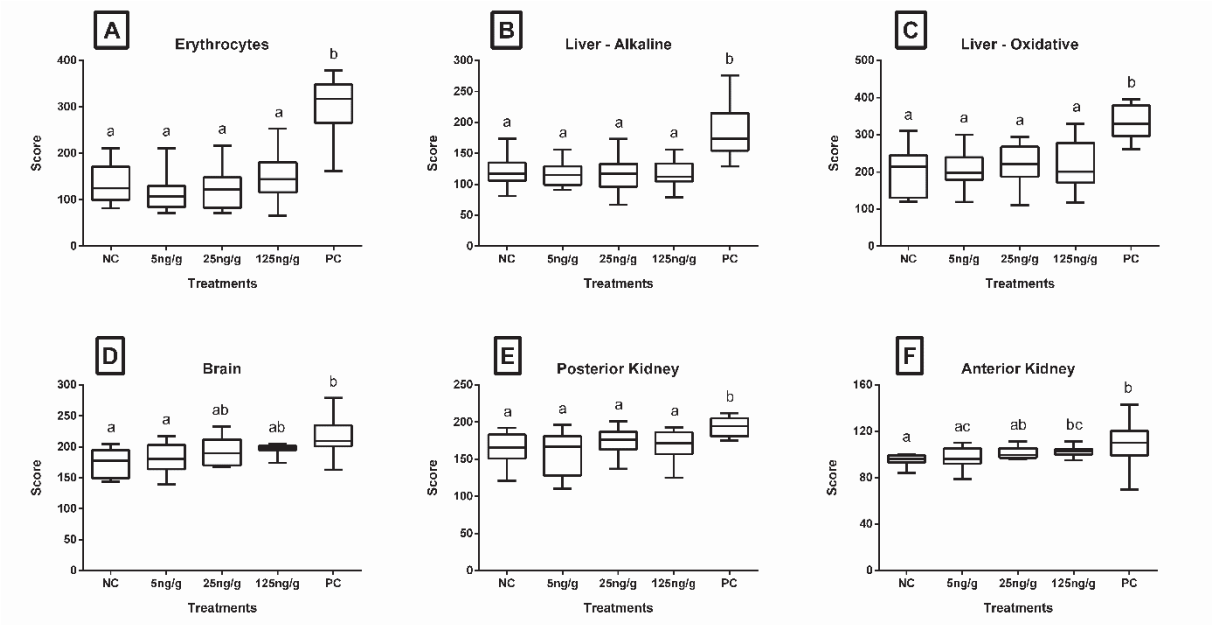
In the anterior kidney, the group treated with 125 ng / g of DBP showed a significant increase in DNA damage compared to the negative control (p = 0.0084). The groups 5 and 25 ng / g of DBP did not differ significantly in relation to the amount of DNA damage from the NC (p> 0.05) (Figure 2 F).

Figure 2 - DNA damage of *Rhamdia quelen* detected by the comet assay after subchronic exposure, by trophic route, to DBP. The data are represented by medians and quartiles. Different lowercase letters indicate statistical differences between groups (p <0.05).

4.3.3 Biochemicals biomarkers

Liver

GST activity in liver tissue, after exposure to DBP, suggests a dose-response curve. Although the 5 ng/g dose is not significantly different from NC, a significant gradual increase in GST activity was observed in treatments of 25 ng/g and 125 ng/g when compared to the control (p = 0.0411 and p <0.001, respectively). There was also a difference between responses to doses of 5 ng/g and 125 ng/g of DBP (p = 0.0012), and between doses of 25 ng/g and 125 ng/g of DBP (p = 0.0133) (Figure 3 A).

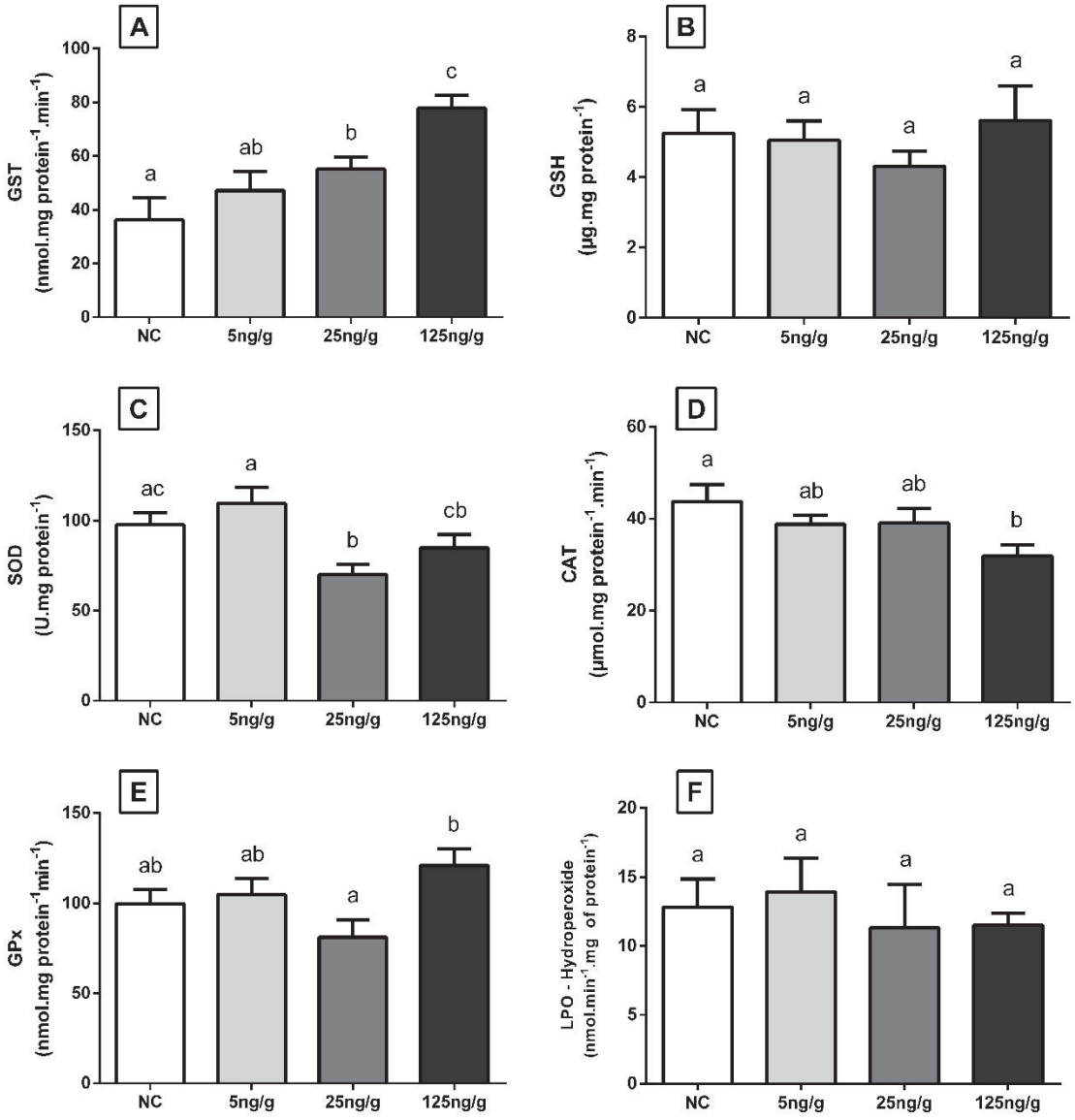


The DBP did not cause changes in hepatic GSH levels when treatments were compared to NC (p> 0.05) (Figure 3 B).

Regarding the SOD enzyme, there was a reduction in its activity in the 25 ng/g group of DBP when compared to the NC (p = 0.0179) (Figure 3 C). Catalase also exhibited reduced activity, however, in the group treated with 125 ng / g of DBP (p = 0.0021) (Figure 3 D).

Exposure to DBP did not induce changes in GPx activity or cause lipoperoxidation in the liver cells of the treated groups compared to the NC ($p > 0.05$) (Figure 3 E, F).

Figure 3 - Biochemical biomarkers evaluated in the liver of the species *Rhamdia quelen*, after subchronic trophic exposure to DBP. The data are presented by means and standard deviation. Different lowercase letters indicate statistical differences between groups (p <0.05).



Posterior kidney

In the posterior kidney, the treatments with the two highest doses of DBP (25 and 125 ng/g), reduced GST activity in relation to the NC ($p = 0.0195$ and $p = 0.0223$, respectively). (Figure 4 A).

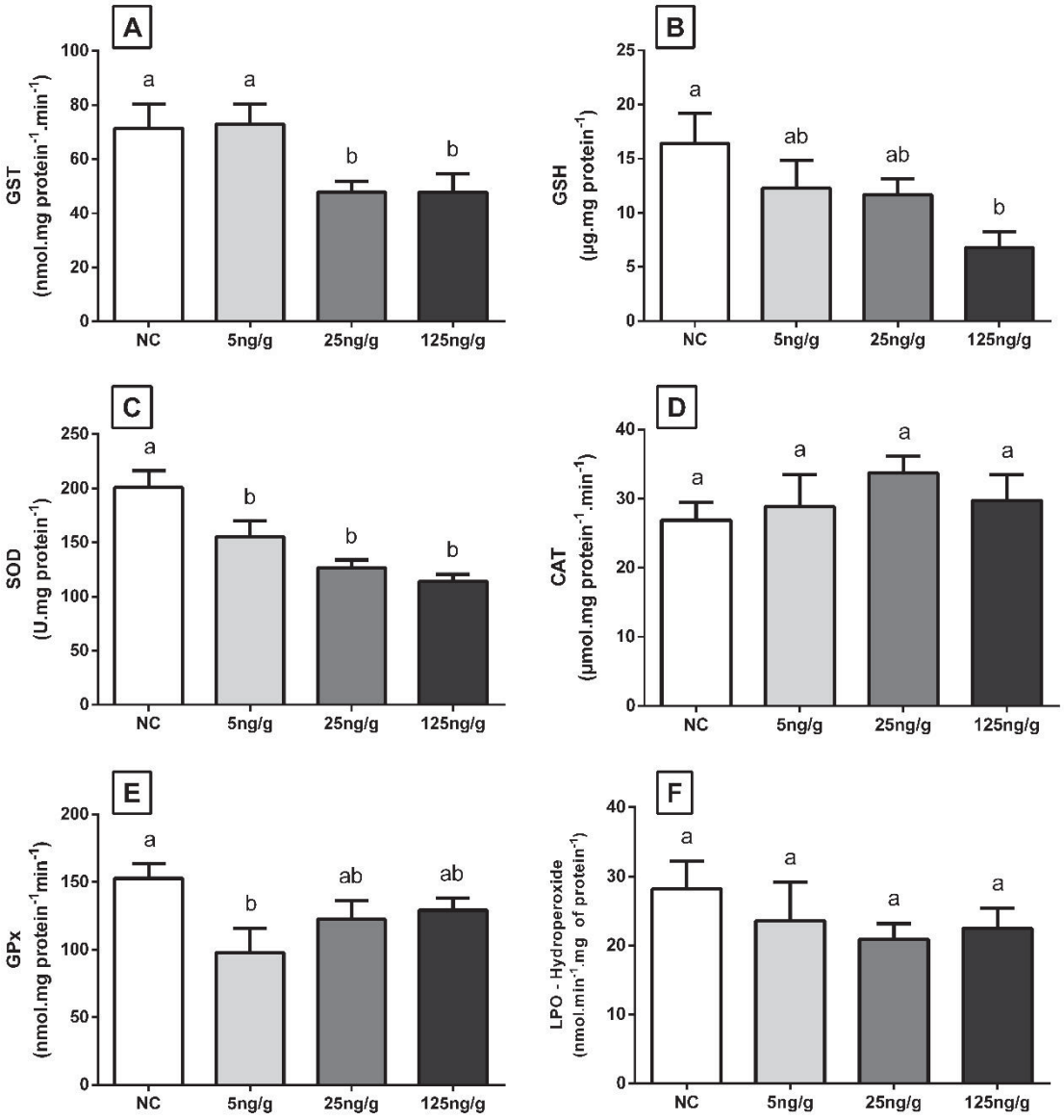
The concentration of GSH suggests a dose-response curve, but only treatment with the highest dose (125 ng/g) caused a significant reduction compared to the negative control ($p = 0.0031$) (Figure 4 B).

The three doses of DBP caused a significant reduction in SOD activity compared to the negative control ($p =0.0429$, $p=0.0005$ and $p<0.0001$). The reduction occurred according to the DBP dose, also suggesting a dose-response curve, but there was no significant difference between doses (Figure 4 C).

The exposure to DBP did not change the CAT enzyme activity in relation to the negative control ($p> 0.05$) (Figure 4 D) and GPx had a significant reduction only in the dose of 5 ng/g in relation to the NC ($p = 0.0022$) (Figure 4 E).

There was no evidence of lipoperoxidation in renal cells after exposure to DBP ($p> 0.05$) (Figure 4 F).

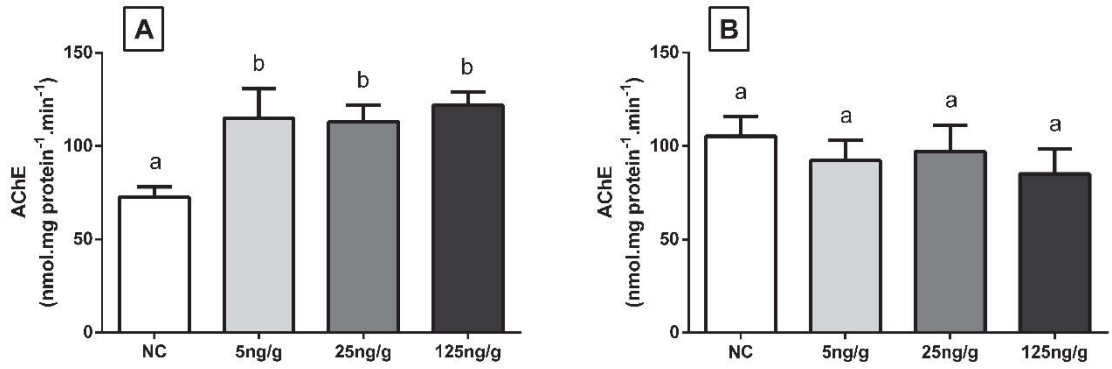
Figure 4 - Biochemical biomarkers evaluated in the posterior kidney of the species *Rhamdia quelen*, after subchronic trophic exposure to DBP. The data are presented by means and standard deviation. Different lowercase letters indicate statistical differences between groups (p <0.05).



4.3.4 Neurotoxicity evaluation

DBP increased the cerebral AChE activity in treatments of 5 ng/g (p = 0.0079), 25 ng/g (p = 0.0078) and 125 ng/g (p = 0.0005) compared to the NC (Figure 5 A). However, in muscle tissue, the exposure to DBP did not alter AChE activity in relation to the NC (Figure 5 B).

Figure 5 - Cerebral (A) and muscular (B) acetylcholinesterase (AChE) activities quantified in the fish species *Rhamdia quelen*, after subchronic trophic exposure to DBP. Data are presented as means and standard deviations. Different lowercase letters indicate statistical differences between groups (p <0.05).



4.4 Discussion

Considering the increasing use of phthalates in the most diverse industrial processes, questions arise concerning their presence in terrestrial and aquatic environments (ADENIYI; OKEDEYI; YUSUF, 2011; MONTAGNER; JARDIM, 2011; PEIJNENBURG; STRUIJS, 2006; SUN et al., 2013), its effects and risks in human exposure (CHENG et al., 2013; KLEINSASSER et al., 2001; KOO; LEE, 2010; SCHETTLER, 2006; WITORSCH; THOMAS, 2010; WITTASSEK et al., 2011), as well as the effects on wildlife (DU et al., 2015; SHEN et al., 2019; WANG et al., 2019; XU et al., 2015). Most published studies have identified the effects of endocrine disruption and, consequently, effects on the reproductive system of several species and stages of life, as the major cause for concern regarding these compounds (BOTELHO et al., 2009; CHEN et al., 2014; MARTINO-ANDRADE et al., 2009; MARTINO-ANDRADE; CHAHOUD, 2010; WITORSCH; THOMAS, 2010). Still, phthalates can affect several tissues simultaneously, triggering the action of mechanisms by different routes, causing adverse effects not only on the reproductive system of organisms but also on other physiological systems. Thus, the evaluation by the biomarkers applied in our study is essential because they are able to detect early effects at molecular and cellular levels in diverse systems of organisms.

We found no evidence of significant changes in the hematological profile, genotoxic and/or mutagenic effects in the blood samples of animals exposed to the tested doses. Although the literature reports changes in the hematological profile in

Cyprinus carpio (POOPAL et al., 2017) and an increase in the frequency of piscine micronucleus and nuclear erythrocyte changes in *Oreochromis niloticus* (BENLI; ERKMEN; ERKOC, 2016), both studies used hydric exposure in their models and concentrations of DBP in the magnitude of mg/L. The increased damage to blood cell DNA, promoted by exposure to DBP, is widely reported for humans (AL-SALEH et al., 2017; ANDERSON; YU; HINÇAL, 1999; KLEINSASSER et al., 2000b, 2001), but in *Rhamdia quelen* did not induce DNA damage to blood cells. Although Jian Ge et al. (2012) found the metabolism of DBP in *Ctenopharyngdon idellus* blood tissue is faster than other tissues, we believe that the absence of effects on the blood genetic material and the hematological profile of *Rhamdia quelen*, in this study, may be related to the bioavailability of DBP in the body due to trophic exposure and the dose administered.

Although phthalate excretion mechanism has not yet been fully elucidated, Hu et al. (2016) suggest that kidneys and gills are the main metabolic pathways for the elimination of phthalates and their respective metabolites, the monoalkyl phthalate esters (MPEs), and the less hydrophobic or short-chain, such as DBP and its metabolite, mono-n-butyl phthalate (MBP). These, quantified in *Ctenopharyngdon idellus* urine, suggest that DBP molecules are hydrolyzed, forming MBP, and later conjugated with molecules endogenous to the organism (JIAN GE et al., 2012). These two processes make the molecule more hydrophilic, facilitating its excretion.

In fish, one of the organs responsible for excretion is the kidney, specifically its posterior portion. In our study, the effects of DBP exposure, concerning the antioxidant system of the posterior kidney (GST, SOD, GPx and GSH), showed a behavior similar to that described in the review by Asghari et al. (2015) in which the phthalates exposure reduced the activity of the afore mentioned enzymes and also the concentration of GSH. The concomitant reduction in GST activity and in GSH concentration in the group exposed to a 125ng/g dose of DBP, also corroborates the results obtained by Latif et al. (2020). Latif et al. (2020), after 21 days of hydric exposure to Diethyl phthalate (DEP) with the fish species *Labeo rohita* had observed a reduction in GSH levels in the kidney, and consequently also observed an effect on GST activity, since GST performs the conjugation of GSH with the exogenous molecule to make it more hydrophilic.

Data about the effect, promoted by exposure to DBP, on the activity of the enzymes SOD, CAT and GPx in the renal tissue of fish are scarce in the literature. These enzymes perform a fundamental role in the antioxidant system of cells, SOD is

responsible for catalyzing the dismutation of superoxide anions in H_2O_2, and this peroxide can be removed by the CAT enzyme or other peroxidases, such as GPx (VAN DER OOST; BEYER; VERMEULEN, 2003).

We observed a reduction in SOD activity in all groups exposed to DBP. Different effects on SOD activity are reported in the literature, due to exposure to phthalates, varying according to the tissue analyzed, time and concentration of exposure, besides the species and stage of life (DU et al., 2015; KHALIL; ELHAKIM; EL-MURR, 2016; QU et al., 2014; SHEN et al., 2019; WANG et al., 2018; XU et al., 2013a; YANG et al., 2018). During a similar period of exposure to our study, DBP (100 mg/kg) caused a reduction in SOD activity in earthworms of the species *Eisenia fetida* (DU et al., 2015). In long periods of exposure to DBP, the decrease in the SOD enzyme activity may be due to the saturation of natural antioxidant defenses, and thus generate an imbalance in the formation and removal of superoxides, compromising the organism (DU et al., 2015). Another possible explanation for the reduction in renal SOD activity is the previously described ability of other enzymes in the antioxidant system, such as CAT and GPx, to be inactivated by the excess of their own substrate(ZHENG; FENG; DAI, 2013). That is, in the SOD case, it may have occurred due to the presence of excess H_2O_2.

The H_2O_2, formed by SOD activity or other pathways, is catabolized by the enzymes CAT and GPx. In our study, renal CAT showed no changes in its activity in any group exposed to DBP. However, the GPx enzyme showed a reduction in its activity only in the group exposed to 5ng/g of DBP. For CAT and GPx activity in fish kidney tissue, Kang et al. (2010) also did not observe changes in the activities of these enzymes after four days of exposure to the three different doses (100, 300 and 900mg/Kg) of DEP administered intraperitoneally in the fish species *Paralichthys olivaceus*. However, Zheng et al. (2013), after ten days of exposure to 10mg/kg of DBP via intraperitoneal injection in *Carassius auratus*, observed a reduction in CAT and GPx activity. The maintenance of CAT activity in groups treated with DBP may be related to the reduction of SOD activity. Since SOD is not producing an excess of H_2O_2, CAT will not have enhanced activity due to elimination of H_2O_2. GPx activity depends on GSH as a cofactor, but even though there was a reduction of available renal GSH in the group treated with 125 ng/g of DBP, GPx activity did not exhibited a significant alteration in the same treatment group. Thus, the statistically significant attenuation of

GPx activity, observed in the group treated with 5 ng/g of DBP, may have other causes, such as its inactivation due to substrate excess, as previously suggested for SOD.

In the liver, the outcomes in the antioxidant system were different from those observed in the posterior kidney. GST activity, in the groups treated with 25 and 125 ng/g of DBP, increased significantly. In the literature, different effects are reported to those reported in our study (LATIF; FAHEEM; ASMATULLAH, 2020; SHEN et al., 2019). The increase in hepatic GST activity may indicate that the liver is the main organ responsible for the biotransformation of DBP in a hydrophilic molecule.

In our study, no changes in hepatic GSH concentration were observed in any group exposed to DBP. Our results corroborate those obtained by Erkmen et al. (2015) with the species *Oreochromis niloticus*, whose hepatic GSH levels did not change after 24 and 96 hours of water exposure to 10 mg/L of DBP. According to the authors, due to the conservation of the adaptive response of hepatic detoxification ability, the maintenance of hepatic GSH concentration, after exposure to DBP, may indicate a higher potential of the liver as a detoxification organ.

Resembling that observed in the posterior kidney, SOD activity in the liver showed a reduction, but only in the group treated with 25 ng/g of DBP. While the CAT had its activity reduced in the liver, only at the highest dose (125 ng/g). In the study by Qu et al. (2014), a reduction in liver SOD activity was observed after intraperitoneal exposures to 10 mg/kg of DBP in the fish species *Carassius auratus*, fifteen days after the beginning of the exposure. In that same study, there was no change in CAT activity. In addition, another study by Jiao et al. (2020), they also observed a reduction in the activities of the enzymes SOD and CAT, in the liver of *Danio rerio*, after 96 hours of exposure to 10 mg/L of MBP, DBP metabolite. These enzymes activities reduction may be related to the imbalance of the liver antioxidant system. Besides that, the reduction in CAT can delay the H_2O_2 removal process and, thus, promote the accumulation of this reactive species which can, consequently, inhibit its own activity and the activity of other enzymes, such as SOD.

Although some studies presented reports of reduced GPx activity in *Carassius auratus* liver, after 10 (ZHENG; FENG; DAI, 2013) and 15 days (QU et al., 2014) of exposure to 10 mg/kg of DBP, in our study, we did not observe any change in its activity. Exposures to low doses administered in this study were not sufficient to cause changes in GPx activity and GSH concentration. Thus, we believe that both liver GPx and GSH are not pathways susceptible to the effects caused by low doses of DBP.

Some studies demonstrate that exposure to DBP, by different exposure routes, can cause increased lipid peroxidation in microcrustacean species, such as Daphnia magna (neonatal/adult), fish species such as zebrafish (embryo) and Carassius auratus, and annelids, as in the species Eisenia fetida (DU et al., 2015; KHALIL; ELHAKIM; EL-MURR, 2016; QU et al., 2014; SHEN et al., 2019; XU et al., 2015). Probably, the increase in lipid peroxidation occurs due to an imbalance of antioxidant enzymes and the excess of reactive oxygen species (ROS) produced, which consequently are responsible for damage to cell membranes (ERKMEN et al., 2015; VAN DER OOST; BEYER; VERMEULEN, 2003). In our study, lipid peroxidation effects were not observed in the posterior kidney and liver. Similarly, the species Oreochromis niloticus, after 96 h of exposure to 10 mg/L of DBP, despite the data showing a small increase in lipid peroxidation in the liver, this alteration was also not significant (ERKMEN et al., 2015). The results, found in our study, may be related to the ability of organisms to combat the oxidative stress caused by DBP through the antioxidant system. Also, it is possible that low doses of DBP, by trophic administration for 30 days, did not produce enough reactive oxygen species to cause damage in the kidney and liver cells membranes of Rhamdia quelen.

In addition to lipid peroxidation, phthalate genotoxicity is also associated with its ability to generate ROS (ERKEKOĞLU et al., 2010; ERKEKOGLU; KOCER-GUMUSEL, 2014). In the review by Erkekoglu and Kocer-Gumusel (2014), the authors cite several studies that reported increased DNA damage resulting from exposure to phthalates, mainly for human cells. However, in fish, by the alkaline comet assay, we did not evidence of increased damage in the genetic material of blood, liver and brain cells of *Rhamdia quelen* exposed to DBP. Although the relationship between genotoxicity and reactive oxygen species has been described in the literature, the oxidative version of the comet assay in liver tissue has not shown an increase in DNA damage caused by ROS. Thus, the low doses of DBP administered continuously for 30 days did not cause a greater intensity of DNA damage, either directly or indirectly through ROS produced by the processes of DBP metabolization.

As mentioned earlier, the fish kidney is divided into two portions: anterior and posterior. Despite exercising different functions, both share essential roles in the animal's immune system (BROMAGE et al., 2004; GRASSI MILANO; BASARI; CHIMENTI, 1997; MARTINS et al., 2016; ZAPATA, 1979; ZWOLLO et al., 2005). According to Martins et al. (2016), portions of the kidney respond differently to the

presence of DBP, with the anterior kidney being more sensitive to exposure to this phthalate. When evaluating the expression of the genes involved in the hematopoiesis and immune system processes, in the anterior and posterior kidneys, Martins et al. (2016) found that gene expression in the anterior portion was more affected when compared to the posterior kidney. In our study, we found a greater intensity of DNA damage, only in animals treated with 125 ng/g of DBP. The posterior kidney showed no differences in the DNA damage assessed in this study in relation to the groups exposed to DBP. This difference in effects, caused by DBP and evidenced in the DNA of the anterior and posterior kidneys, is compatible with the results described by Martins et al. (2016). In addition, the low doses of DBP administered in our study were probably metabolized efficiently by the organism, thus the reactive oxygen species (ROS) produced in this process were not able to cause a significant increase in DNA damage in blood, liver, brain and posterior kidney cells.

Although exposure to DBP did not induce DNA damage in brain tissue, our study showed changes in the nervous system of *Rhamdia quelen* through the activity of the enzyme AChE, present in different tissues in fish and widely used as a biomarker of neurotoxicity (VAN DER OOST; BEYER; VERMEULEN, 2003). Recent studies with zebrafish embryos have observed neurotoxicity caused by exposure to DBP (XU et al., 2013b, 2020). In the fish species *Pseudobagrus fulvidraco*, after eight weeks of trophic exposure to doses of 100, 500 and 1000 mg/Kg of DBP, the AChE activity of both brain and muscle was reduced (JEE et al., 2009). However, in our study, low doses of DBP did not change the activity of muscle AChE. Besides, it was possible to observe an increase in cerebral AChE activity in all groups treated with DBP. According to (ZHANG; GREENBERG, 2012), one of the causes for the increase in AChE activity may be related to apoptotic processes. AChE is a signaling molecule for the onset of apoptosis, which is closely associated to this process, so in studies, *in vitro* (ZHANG et al., 2002) and *in vivo* (HU et al., 2009), AChE can be used as a biomarker of cell death (ZHANG; GREENBERG, 2012).

Many published studies demonstrate the ability of chemical molecules cause endocrine-disrupting effects and cell damage, such as DBP, in nanometric concentrations or doses (VANDENBERG et al., 2012). Commonly, the dose-response curves generated after exposure to these molecules are non-monotonic. Thus, for application in environmental regulation, dose-response curves obtained from exposures to high doses or concentrations of these contaminants are not ideal for

predicting or extrapolating possible effects or safe limits of exposures (SWAN, 2008; VANDENBERG et al., 2012). For this reason, we emphasize the importance of studies like ours, in which low doses of DBP were administered by subchronic trophic exposure in *Rhamdia quelen*, showing toxic potential for different organs, such as kidneys, liver and brain. The liver and posterior kidney responded differently to this exposure, probably due to the DBP molecule, which when initially hydrolyzed, mainly by hepatic GST, subsequently produces metabolites and ROS, causing a distribution to other organs, such as kidneys. Consequently, as the genotoxicity was only observed in the anterior kidney, the other tissues may have been preserved by the efficiency of the antioxidant system.

4.5 Conclusion

In our study, we demonstrated, for the first time, evidence that prolonged intake at low doses of DBP can lead to changes in the activity of enzymes and non-enzymatic molecules of the antioxidant system of Rhamdia quelen. These changes indirectly suggest that the metabolism of DBP may result in MPEs and ROS, responsible for unbalancing the antioxidant protection system. Our results reinforce that the route of exposure can be directly correlated to the effects observed in different tissues, as already reported in other studies. However, in our study, we showed that environmentally relevant doses of DBP can trigger harmful effects on individuals exposed to more extended periods than those usually tested. Future studies with exposures to low doses or concentrations of DBP are necessary for an attempt to elucidate possible metabolic pathways, according to the route of administration, as well as effects that have not yet been described and/or effects on other organs (e.g. gonads). In addition, it is necessary to carry out studies with phthalate metabolites, since data in the literature show a high toxic potential of these. Thus, the construction of a vast database, about the effects promoted by phthalates in lower doses and/or concentrations, is essential to determine safe exposure limits for the aquatic ecosystem.

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5. Evidence of genotoxicity, neurotoxicity and antioxidant imbalance in silver catfish *Rhamdia quelen* after subchronic exposure to diisopentyl phthalate

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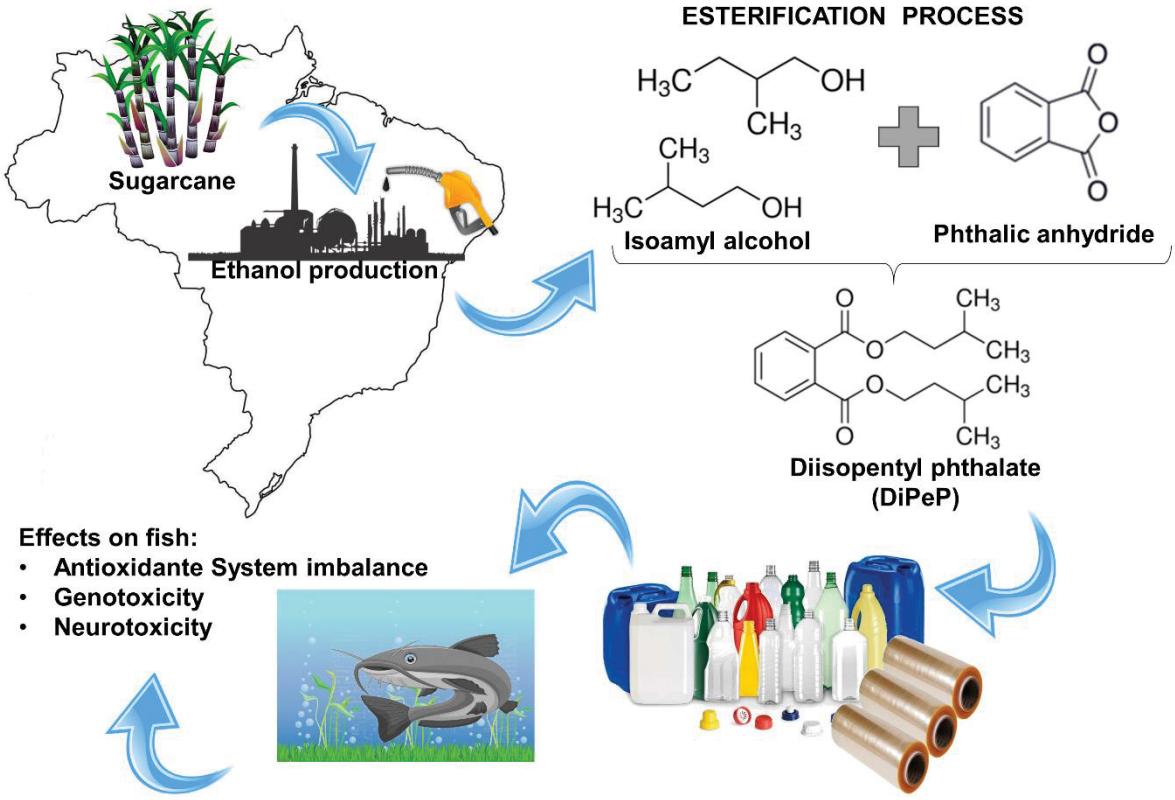
Graphical Abstract



Highlights

- The DiPeP exposure caused increased of DNA damage in head kidney cells.
- Renal antioxidant system was significantly impacted by DiPeP.
- In liver 125ng/g of DiPeP promoted changes in GST activity and GSH concentration.
- DiPeP caused significant changes in muscle and brain AChE.

Abstract

Due to sugar cane refining process in Brazil, the diisopentyl phthalate (DiPeP) is a plasticizer with significant offer and application in local industries. We developed an experimental design of trophic exposure to environmentally relevant doses (5, 25 and 125ng / g of DiPeP) to evaluate possible target tissues and toxic effects promoted by subchronic exposure to DiPeP in a Neotropical fish species, *Rhamdia quelen*. After thirty days of exposure, blood, liver, kidney, brain and muscle were collected and submitted to hematological, genetics and biochemical biomarkers: The kidney was the organ most affected, in the anterior portion, genotoxicity was evidenced in all groups exposed to DiPeP. In addition, posterior kidney showed a reduction in the superoxide dismutase and glutathione peroxidase activities and reduced glutathione concentration. In the liver, 125ng/g of DiPeP caused an increase in glutathione S-transferase activity and a reduction in reduced glutathione. In muscle, acetylcholinesterase had reduced activity in all DiPeP treatments, however, the brain presented an increase in the lowest doses and a significant reduction in the highest dose. In this study, DiPeP proved to be a xenobiotic of environmental concern, since we have evidenced its nephrotoxic and neurotoxic potential even in low doses.

Keywords – Kidney, fish, antioxidant system, diisoamyl phthalate, comet assay, micronucleus, acetylcholinesterase

5.1 Introduction

Phthalates, or phthalic acid esters (PAEs), are synthetic organic compounds widely used by various industry branches. For providing flexibility, durability and transparency to plastic products, they are known as plasticizers (HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007). High molecular weight phthalates (HMW), such as di- (2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DOP), are generally used in building materials, car products, clothing, food packaging, children's products, medical devices, among others (CHEON, 2020; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007). However, low molecular weight phthalates (LMW), such as diethyl phthalate (DEP), Di-n-butyl phthalate (DBP) and dimethyl phthalate (DMP), have other attractive features for the cosmetics, personal care, resins, dyes and adhesives industry, acting as a solvent and / or fixative in their compositions (AL-SALEH et al., 2017; CHEON, 2020; HEUDORF; MERSCH-SUNDERMANN; ANGERER, 2007; LIU et al., 2010). The amount of phthalates in their final composition of plastics can reach up to 60% of their total weight (LIU et al., 2010).

The main concern about phthalates, in addition to the volume of production and consumption as a raw material or final product, is the fact that these molecules are not covalently linked to the polymer matrix, and thus are transported to the environmental matrices directly or by processes of leaching, abrasion or migration (CHENG et al., 2013; ROCHA et al., 2017). In the environment, phthalates can bioaccumulate in plants, invertebrates and vertebrates of different trophic levels, but bioaccumulation is greater in organisms with less biotransformation capacity of this xenobiotic (BORAN; TERZI, 2019; MANKIDY et al., 2013; STAPLES et al., 1997a). In fish, in addition to the potential to cause reproductive effects (AOKI et al., 2011; BHATIA et al., 2013; JARMOŁOWICZ; DEMSKA-ZAKĘŚ; ZAKĘŚ, 2013), histological damage to gills, liver and kidney (LATIF; FAHEEM; ASMATULLAH, 2020) has also been reported genotoxicity (KHALIL; ELHAKIM; EL-MURR, 2016) and immunotoxicity (MARTINS et al., 2015, 2016; XU et al., 2015). According to Rhodes et al. (1995), phthalates may not cause severe damage in acute exposures, but prolonged periods of exposure to low concentrations can be harmful to aquatic organisms.

Diisopentyl phthalate (DiPeP), also known as diisoamyl phthalate (DiAP), has shown great concern in the Brazilian scenario. Recent studies have shown the exposure of the Brazilian population to DiPeP, due to the presence of its metabolites,

mono-isopentyl phthalate (MiPeP), mono-3OH- (3-methylbutyl) phthalate (3OH-MiPeP) and mono 4OH- (30methylbutyl) phthalate (4OH-MiPeP), in the urine of Brazilian pregnant women and children (BERTONCELLO SOUZA et al., 2018; ROCHA et al., 2017). In no other biomonitoring population with a published study, of the same nature, showed the presence of these metabolites. This exhibition is associated with the origin of DiPeP in Brazil and its production. Due to the large amount of sugar cane that is refined for ethanol production, a by-product known as fuel oil, rich in isoamyl alcohol, is generated in this process. The large supply of isoamyl alcohol favors the production of DiPeP, through the esterification of alcohol with phthalic anhydride (BERTONCELLO SOUZA et al., 2018). DiPeP is used as a plasticizer, polyvinyl-chloride (PVC), in electronics and explosives (CHEON, 2020).

In the environment, in two different states in Brazil, DiPeP was detected in the soil near to the plasticizer industry (1,038mg / Kg) and in landfill leachate material (DO NASCIMENTO FILHO et al., 2003; FERREIRA; MORITA, 2012). However, there is no record in the literature on the presence of DiPeP in the aquatic ecosystem. The absence of this evidence does not mean that DiPeP is not present, but probably that this phthalate has not been studied by any research group even now.

The species *Rhamdia quelen* (Siluriformes, Heptapteridae) is Neotropical and endemic in South America (BOMBARDELLI et al., 2006; GOMES et al., 2000). A freshwater fish, omnivorous habit, has great economic importance due to its high growth rate and ease of cultivation, being an important food resource for the population (BARCELLOS et al., 2004; BOMBARDELLI et al., 2006). Besides, the species is a good test organism for bioassays with a focus on environmental toxicology, showing sensitivity to respond to exposures to low doses and/or concentrations of xenobiotics through genetic and biochemical biomarkers(FERREIRA et al., 2010; GHELFI et al., 2016; GUILOSKI et al., 2017b, 2017a; KLINGELFUS et al., 2015; MATHIAS et al., 2018; PEREIRA et al., 2016; PIANCINI et al., 2015a)

Once human exposure to DiPeP is recognized, in addition to its presence in soil and landfill leachate, it is necessary to understand the effects promoted by exposure to DiPeP to aquatic organisms. In this way, we developed an experimental design with doses, period and route of exposure that are environmentally relevant. Different tissues were subjected to hematological, genetic and biochemical biomarkers in order to identify potential target tissues and which early effects can be detected after

subchronic exposure to DiPeP. This study is the first report of a bioassay of trophic exposure to DiPeP in fish.

5.2 Material and Methods

5.2.1 Animals

The *Rhamdia quelen* fish (n=90) were provided by the Laboratory of Technology for the Reproduction of Aquatic Animals Cultivables (LATRAAC), from the State University of Western Paraná (UNIOESTE). The animals were acclimatized at the Animal Cytogenetics and Environmental Mutagenesis Laboratory at the Federal University of Paraná (UFPR), for 60 days in a 2000 L tank, with a temperature of 27ºC, constant aeration and a 12 h light / 12 h dark photoperiod. In the acclimatization period, they were fed once a day *ad libitum* with commercial food (Supra® 32% protein). This study was previously approved by the Ethics Committee on the Use of Animals of the Biological Sciences Sector of the Federal University of Paraná (CEUA / BIO - UFPR) under number 1180.

5.2.2 DiPeP

The plasticizer industry, Petroquímica Mogí das Cruzes SA (PETROM, Brazil), provided the Diisopentyl phthalate (DiPeP, CAS n° 84777-06-0, purity of 99%). As a solvent of DiPeP, we selected a commercial organic extra virgin coconut oil (COPRA®). The reason for the choice was that, in addition to being a natural product and produced mainly for food purposes, when below of temperatures at 21°C this oil solidifies (Mursalin et al. 2016), different from other natural oils, as canola and soy oil, or organic solvents as DMSO and ethanol. This characteristic was important from our design experiment to ensure the administration of the correct dose of DiPeP to the fish. Three different concentrations of DiPeP were calculated to produce solutions to be equivalent to doses 5 ng/g (5 mg/L), 25 ng/g (25 mg/L) and 125 ng/g (125 mg/L), when administrated in the proportion of 1 μL for each g of fish. The DiPeP was dissolved in coconut oil and homogenized for five minutes in an ultrasonic water bath (Schuster®, model: L-100) at a frequency of 42 kHz.

5.2.3 Design Experimental, Training and exposure

We developed a passive trophic exposure methodology, prioritizing animal well-being, avoiding the stress caused by handling and, at the same time, reproducing environmentally relevant dose, period and exposure conditions (Oya-Silva et al., *in preparation*). Fifteen days before the exposure, to transfer the specimens of *Rhamdia quelen* to individual aquariums of 20 L, with a temperature of 26.4 ± 2.7 ° C, photoperiod 12 h light / 12 h dark and constant aeration. In this process, the animals were weighed (mean weight 74.85 ± 23.73 g) and in their respective aquarium was assigned with a number identification number, which was associated with their weight and treatment to be received. The next stage, 24 h before the transfer process, we started the training animals to accept the food immediately after the offer and ingest it in its entirety, without breaks. As a food, the animals received a mixture of commercial food and unflavored gelatin in block format, every other day.

In our model of exposure, we used as an exposure vehicle blocks of food make by commercial food and unflavored gelatin (Oya-Silva et al., in preparation). The DiPeP doses were previously inserted in gelatinous commercial capsules (Mawin®), which were immediately frozen. Rectangular shaped molds were used to mold the food blocks. These mold shapes were identified with the number assigned to each aquarium and filled 50% of their volume with a mixture of commercial food (Supra® 32% protein) and unflavored gelatin. After the solidification under refrigeration, cavities were made to accommodate the frozen capsules with the corresponding doses. The mold shapes were kept under refrigeration overnight to ensure complete solidification of the unflavored gelatin. In the mornings following the production of these food blocks, the exposures happened. In total, fifteen food blocks were produced for each animal.

We divided, randomly, the animals into six groups (n = 15 / group): Commercial food Control (CFC), Negative Control (NC), 5 ng DiPeP/g fish, 25 ng DiPeP /g fish and 125 ng DiPeP /g fish and Positive Control (PC). The food blocks containing the doses of DiPeP were offered at intervals of 48 h, totaling 15 doses (30 days). The CFC and PC groups received only commercial food *ad libitum*, at the same time interval. The PC group was used only as a control of the alkaline version of the comet assay technique. Thus, the PC group animals received, 24 h before tissue collection, a single dose of 5 μg of methyl methanesulfonate (MMS – Sigma Aldrich® - CAT nº: 66-27-3) per g of fish, by intraperitoneal injection (PIANCINI et al., 2015a).

After 24 hours of the last exposure, the animals were anesthetized in benzocaine (Merck®, Darmstadt, Germany) solution at a concentration of 10mg/L (GONTIJO et al., 2003). Blood was collected with a heparinized syringe by puncture of the caudal artery and used to evaluate genotoxicity (micronucleus piscine and comet assay), as well as for the analysis of hematological parameters. Then, the animals were euthanized by the spinal cord section and tissues were collected. The organs collected for the comet assay (liver, anterior kidney, posterior kidney and brain) were kept in 500 μL of fetal bovine serum (FBS), previously refrigerated and in the absence of light. The tissues (liver, posterior kidney, brain and muscle) intended for biochemical tests were immediately frozen in liquid nitrogen and subsequently stored in a freezer -80°C until processing.

5.2.4 Hematological parameters

The erythrocytes count was performed by diluting the blood (10 μL) in 2 mL of formaldehyde-citrate in a Neubauer chamber (OLIVEIRA-JÚNIOR; TAVARES-DIAS; MARCON, 2009), and analyzed in optical microscope.

The total count of leukocytes and thrombocytes was performed a blood extension and stained with Instant Prov (New Prov®), under an optical microscope according to the methodology described by Tavares-dias and Moraes (2006).

5.2.5 Evaluation of effects on genetic material

Piscine Micronucleus Test (PMT) and Erythrocytic nuclear alterations (ENA)

The Piscine Micronucleus Test (PMT) was performed as described by Heddle (1973) and Schmid (1975), with modifications by Ferraro et al. (2004), to assess the frequency of micronuclei in peripheral erythrocytes. In addition to the micronucleus, the frequencies of other nuclear erythrocytes alterations were assessed, as described by Carrasco et al. (1990) and Çavaş and Ergene-Gözükara (2005). For each fish, 2,000 erythrocytes were analyzed in each slide under an optical microscope (1000x magnification).

Comet Assay

For the performance of the comet assay in blood cells, about 10 μL of peripheral blood was added in 1000 μL in fetal bovine serum previously refrigerated, and the analysis was performed according to the methodology described by Speit and Hartmann (1999) with alterations by Cestari et al. (2004) and Ferraro et al. (2004). The solid tissues (liver, anterior kidney, posterior kidney and brain) were mechanically homogenized in 500 μL of refrigerated fetal bovine serum, to individualize the cells. To make the slides, cell suspensions (10 μL for blood and 20 μL for solid tissues) were dissolved in 120 μL of low melting point agarose (0.5%) (KLINGELFUS et al., 2017; RAMSDORF et al., 2009). The liver samples, of DiPeP groups, were used to made duplicate slides, because we performed the alkaline and oxidative versions of the comet assay. And, the liver samples of NC group were used to made triplicate slides, two slides to NC of alkaline and oxidative comet assay, and the third to made a positive control of the oxidative version.

The slides destined to the oxidative comet assay went through the same processes previously described for the alkaline version. However, after the lysis stage, the slides were subjected to treatment with the enzyme (formamidopyrimidine [fapy] - DNA glycosylase) - Fpg (New England Biolabs® - CAT n°: M0240), according to the protocol first described by Collins et al. (1993) and described, step by step, by Reeves et al. (2008). The positive control for the oxidative version was performed using hydrogen peroxide (H_2O_2 - 50 μM - Sigma-Aldrich®-CAT nº: H1009), according to Tice et al. (2000).

The slides were stained with 20 μL of ethidium bromide (10 μL/mL) and analyzed using a Leica® epifluorescence microscope (400 x magnification - model DM2500). One hundred nucleoids were analyzed visually and classified according to the degree of damage (damage 0, damage 1, damage 2, damage 3 and damage 4) and the amount of damage, as described in Koppen et al. (2017) and Azqueta et al. (2011).

Assessment of oxidative stress and antioxidant system effects

A homogenate of the liver and posterior kidney were made in phosphate buffer 0.1M (pH 7.0). This samples were centrifuged for 30 minutes at 15,000g at 4ºC, and the supernatant of the was divided into aliquots for the analysis of: glutathione S-transferase (GST), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation (LPO) and protein concentration.

The GST activity was measured according to the method described by Keen et al. (1976), at 340 nm in a spectrophotometer every 30 seconds for 3 minutes. The reduced glutathione (GSH) concentration was measured by the method proposed by Sedlak and Lindsay (1968), using a GSH standard curve and readings were performed at 415 nm in a spectrophotometer. The analysis of SOD enzyme was performed according to Gao et al. (1998), were the amount of SOD that inhibited the reaction by 50% (IC_{50}) is equivalent to 1 unit (U) of SOD. Applying the method described by Aebi (1984), the catalase (CAT) activity was measured by the gradual reduction in absorbance caused by the breakdown of hydrogen peroxide in water and oxygen. The GPx activity was analyzed by the reduction of oxidized glutathione (GSSG), catalyzed by glutathione reductase (GR), in the presence of NADPH (PAGLIA; VALENTINE, 1967b). The lipid peroxidation (LPO) was analyzed using the FOX method (Ferrous Oxidation / Xylenol Orange Method), which is based on the rapid oxidation of Fe^{+2} mediated by peroxide under acidic conditions and subsequent formation of the Fe^{+3} - orange of xylenol complex in the presence of the butylated hydroxytoluene stabilizer (BHT), which absorbs light at 550-570 nm (JIANG; HUNT; WOLFF, 1992).

The Bradford's method was performed for quantification of total proteins in the samples (BRADFORD, 1976), with a standard curve of bovine serum albumin (BSA). The readings were performed in a spectrophotometer at 595 nm.

Neurotoxicity evaluation

The brain and muscle samples were homogenized in 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged for 20 minutes at 10,000g at 4ºC. The supernatant was used to measure the activity of the enzyme acetylcholinesterase (AChE), according to the method of Ellman et al. (1961), in a spectrophotometer at 405 nm at intervals of 30 seconds for 3 minutes.

Statistical analysis

First, the normality of the data was tested using the Kolmogorov-Smirnov test. For data with normal distribution, the ANOVA test (1 criterion) was then applied, followed by the post-test t (LSD - Least significant difference). For data that did not have a normal distribution or showed uneven variances, the Kruskal-Wallis non-parametric test was used, with Student-Newman-Keuls post-test. Statistical significance adopted for all tests was 0.05 (95% of confidence). All analyzes were performed using the BioEstat 5.0 and GraphPad® Prism 6.0 programs.

5.3 Results

During the exposure period, water quality was monitored, the controlled parameters are shown in Table 3. During the 30 days of the bioassay, no mortality was observed.

Table 3 - Water quality parameters during the exposure period.

Parameters	Values
pH	6.2 ± 0.04
Ammonia	<0.1 $mg.L^{-1}$
Dissolved oxygen	8.6 ± 0.4 $mg.L^{-1}$
Hardness	36.3 ± 1.7 $mg.L^{-1}$
Temperature	26.4 ± 0.6 °C

Values represents the mean ± SD.

5.3.1 Hematological evaluation

There were no changes in the hematological profile of *Rhamdia quelen*, compared to the control group, according to the parameters of the number of erythrocytes, leukocytes and thrombocytes (Table 4).

Table 4 - Hematological parameters of Rhamdia quelen after 30 days of trophic exposure to DiPeP.

Parameters	Treatments			
	NC	5 $ng.g^{-1}$	25 $ng.g^{-1}$	125 $ng.g^{-1}$
Erythrocytes $10^6.mL^{-1}$	2.64±0.64	2.97±0.70	2.45±0.75	2.65±0.63
Leukocytes.mL^{-1}	33.36±16.59	35.25±17.98	27.47±14.26	30.69±18.85
Thrombocytes.mL^{-1}	60.43±28.07	52.84±39.65	61.84±38.26	65.98±40.97

Values represents the mean ± SD.
There was no statistical difference between treatments and the negative control group (p> 0.05)

5.3.2 Evaluation of effects on genetic material

Micronuclei were observed in some animals treated with DiPeP. However, the frequency of their formation was low and no statistically significant when compared to the negative control.

The erythrocytic nuclear alterations observed were: blebbed (evagination), notched (invagination) and vacuole. Despite the high frequency of vacuoles in all groups, there was no statistically significant difference between treated and negative control groups. There was no changes in the blebbed and notched frequencies or total erythrocytic nuclear alterations (Table 5).

Table 5 - Frequency of micronucleus and erythrocytic nuclear alterations (ENA) in *Rhamdia quelen,* separated by blebbed, notched, vacuolated and total nuclear morphological alterations.

Treatment	Micronucleo	Blebbed	Notched	Vacuolated	Total ENA
NC	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)	2 (1 / 6)	3 (2 / 8)
5 $ng.g^{-1}$	0 (0 / 0.25)	0 (0 / 1)	1 (0 / 2)	7.5 (3.7 / 14)	9.5 (4 / 16)
25 $ng.g^{-1}$	0 (0 / 0)	0 (0 / 0)	0 (0 / 1)	3 (2 / 9)	6 (2 / 10)
125 $ng.g^{-1}$	0 (0 / 0)	0 (0 / 0)	0 (0 / 1)	4 (2 /15)	5 (2 / 16)

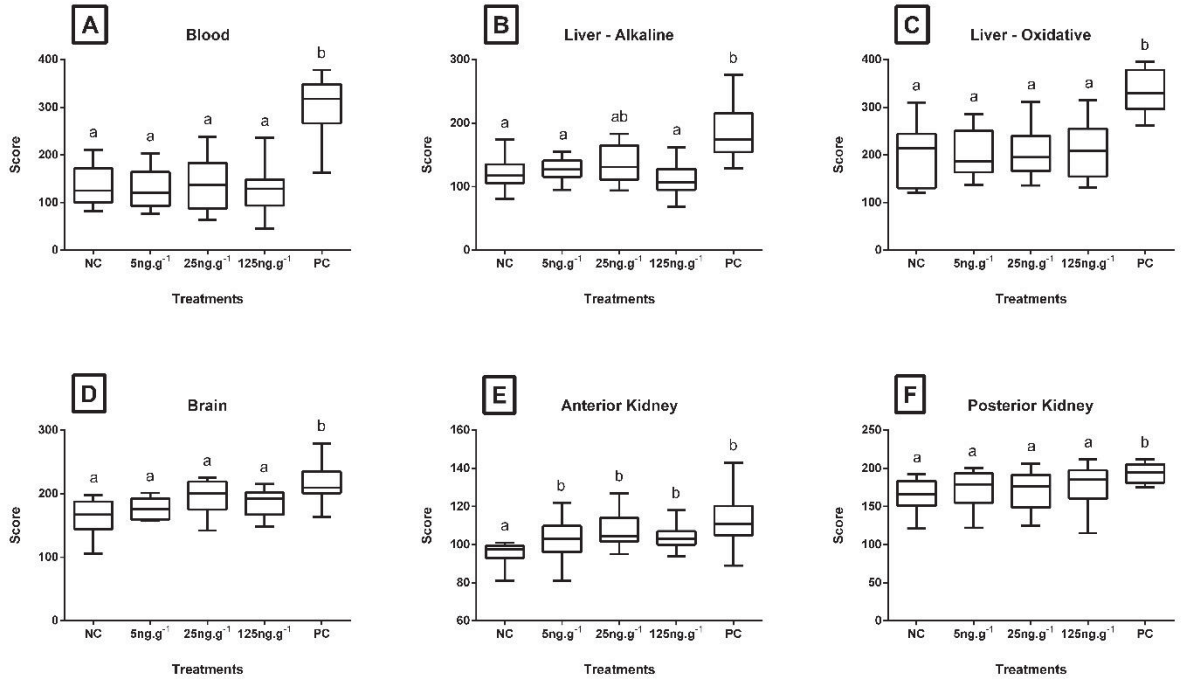
Data are expressed as median and quartiles. (1ºQ/3ºQ).
NC – Negative control.
There was no statistical difference between treatments and the negative control group (p> 0.05).

MMS and H_2O_2 proved to be effective positive controls of the comet assay technique in the alkaline and oxidative versions, respectively, by inducing a significant increase (p> 0.05) of damage to the genetic material of the tissue cells obtained compared to their controlled controls. The alkaline version of the comet assay did

not show increased damage to genetic material (p> 0.05), nucleated blood cells, hepatic, cerebral and posterior renal cells, in animals exposed to DiPeP (Figure 6 A, B, D, F). However, the groups treated with com 5ng.g^{-1} (p=0.0144), 25 ng.g^{-1} (p<0.001) and 125 ng.g^{-1} (p=0.0018) of DiPeP had significantly higher scores on the anterior kidney compared to the negative control (Figure 6 E).

In addition to the alkaline version, hepatic cells were subjected to the comet assay's oxidative version. However, when compared to the negative control, no increases in oxidative damage to liver DNA were observed in any group treated with DiPeP (Figure 6 C).

Figure 6 - Damage to the DNA of *Rhamdia quelen*, after trophic exposure to DiPeP, detected by the alkaline and oxidative comet assay. NC: Negative control; PC: Positive Control. Data are represented as median and quartiles. Different lower case indicates statistics significantly different between groups (*p*<0.05).



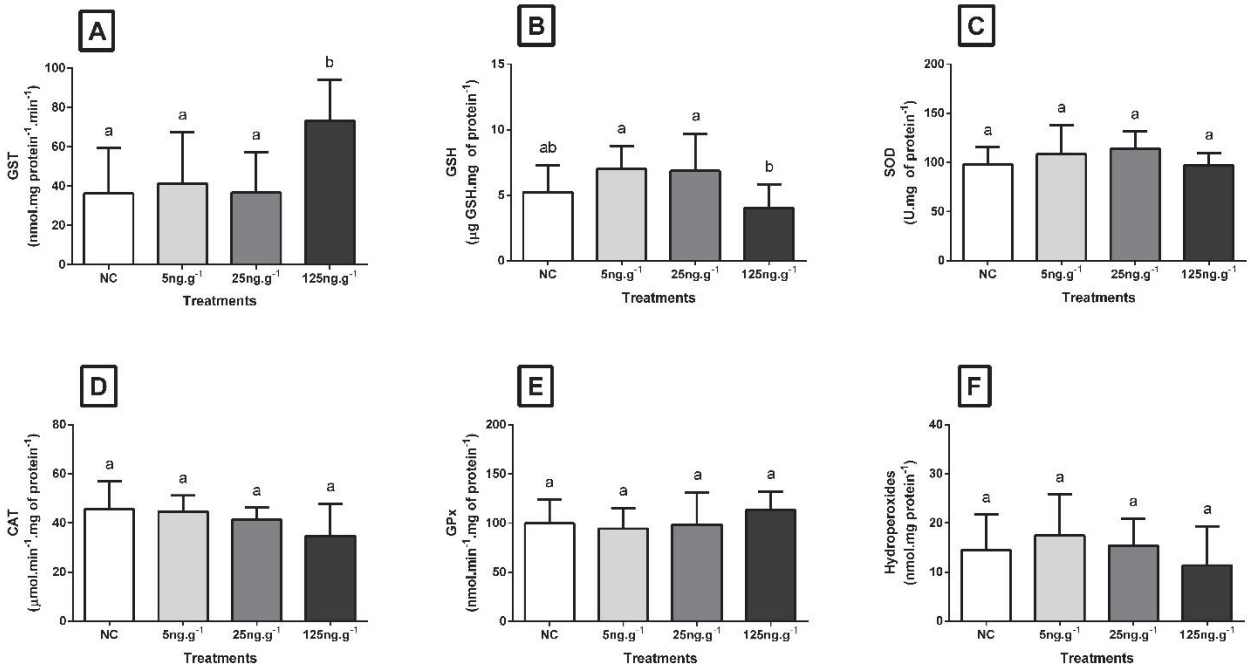
5.3.3 Assessment of antioxidant system and oxidative stress

In liver, the GST enzyme showed a significant increase in its activity in the group exposed to doses of 125ng.g^{-1} of DiPeP in relation to the negative control (p = 0.0017) (Figure 7A).

The SOD, CAT and GPx activities and GSH concentration did not show significant changes, compared to the negative control, promoted by exposure to DiPeP

(p> 0.05) (Figure 7 B, C, D and E). Likewise, there was no evidence of membrane lipoperoxidation in liver cells after DiPeP exposure (p> 0.05) (Figure 7 F).

Figure 7 - Antioxidant system and oxidative stress biomarkers in the liver of *Rhamdia quelen* after trophic exposure to DiPeP. NC: Negative control. Data are represented as mean ± S.E.M. (n=15). Different lower case indicates statistics significantly different between groups (p<0.05).

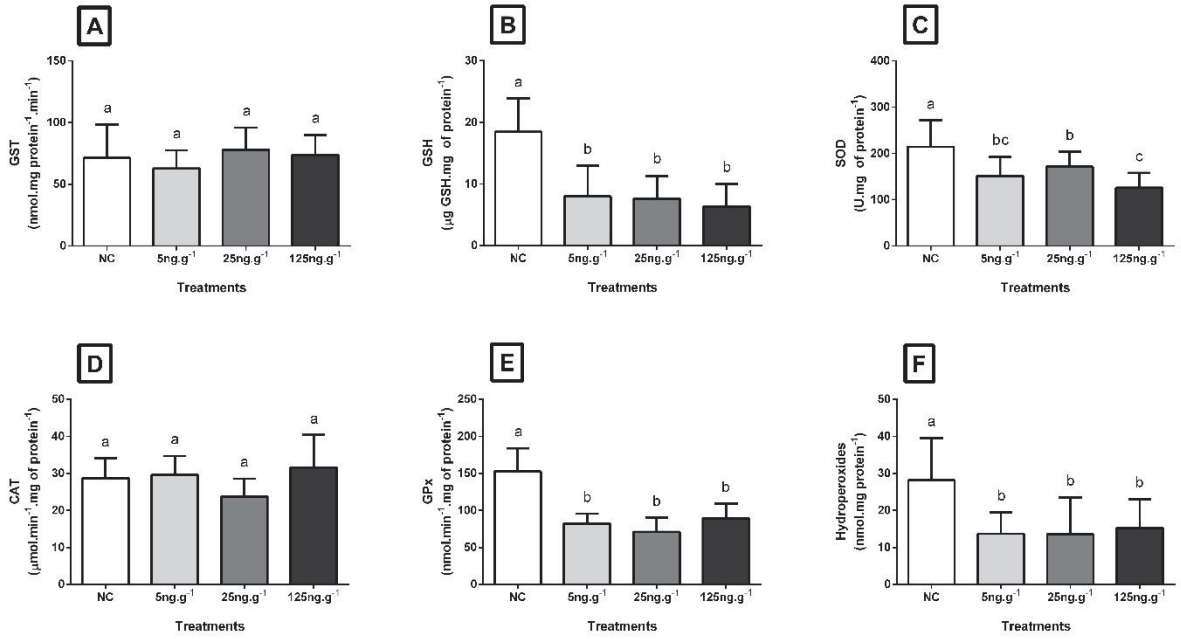


In the posterior kidney, the activities of the GST and CAT enzymes were not different from the negative control (Figure 8A and D). However, the GSH concentration was significantly reduced in all treatments with DiPeP compared to the control group (p = 0.0136, p = 0.0094 and p = 0.0028) (Figure 8B).

DiPeP also promoted a reduction in renal SOD activity in the three exposed groups, differing significantly from the control (p = 0.0024, p = 0.0326, p <0.001) (Figure 8C). Fig.4E shows the significant reduction in the GPx activity in all treatments with phthalate (p <0.001 in all groups) compared with the control.

The LPO biomarker also showed a reduction in all groups exposed to DiPeP (p = 0.002, p = 0.0015 and p = 0.0042) (Figure 8F).

Figure 8 - Antioxidant system and oxidative stress biomarkers in the posterior kidney of *Rhamdia quelen* after trophic exposure to DiPeP. NC: Negative control. Data are represented as mean ± S.E.M. (n=15). Different lower case indicates statistics significantly different betw between groups ($p<0.05$).

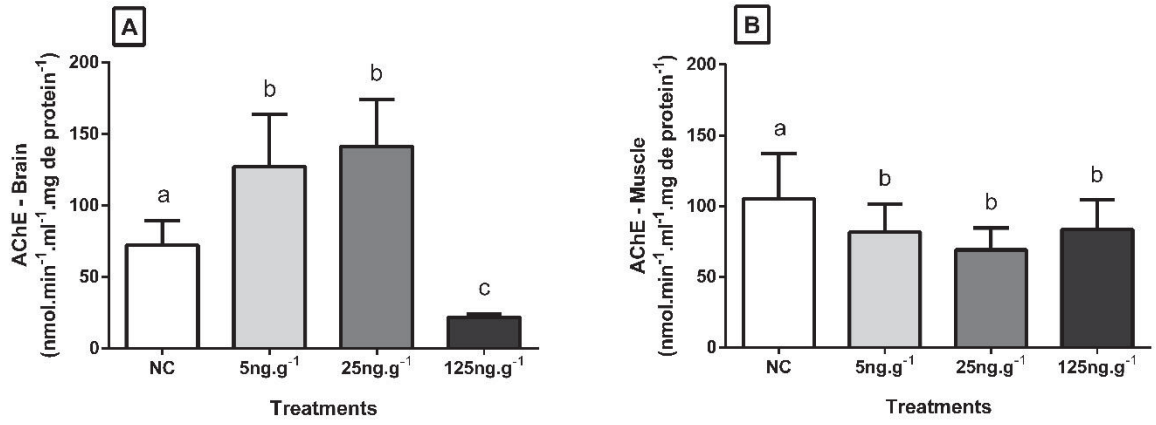


5.3.4 Neurotoxicity evaluation

The brain AChE activity was significantly increased in the groups treated with 5 $ng.g^{-1}$ ($p < 0.001$) and 25 $ng.g^{-1}$ ($p < 0.001$) of DiPeP. However, in the highest dose, 125$ng.g^{-1}$, activity decreased significantly compared to the negative control group ($p < 0.001$),(Figure 9A).

DiPeP caused a reduction in muscle AChE activity in all exposed groups compared to the negative control group ($p = 0.0298$, $p = 0.0013$ and $p = 0.0469$) (Figure 9 B).

Figure 9 - Acetylcholinesterase (AChE) activities quantified in Cerebral (A) and muscular (B) tissues of the fish species *Rhamdia quelen*, after subchronic trophic exposure to DBP. Data are presented as means and standard deviations. Different lowercase letters indicate statistical differences between groups (p <0.05).



5.4 Discussion

Some studies, with different species of fish, report that water exposure to phthalates, such as di-n-butyl phthalate (DBP) and di-ethyl phthalate (DEP), promote changes in the hematological profile of these animals after 96 hours (SEPPERUMAL; SAMINATHAN, 2013) and 30 days (POOPAL et al., 2017). In addition, the increases in the frequencies of micronuclei and nuclear changes of the notched type were also observed in *Oreochromis niloticus* after 24 hours of water exposure to DBP (BENLI; ERKMEN; ERKOC, 2016). However, in our study, in the groups treated with DiPeP, there were no changes in the hematological profile or the frequency of micronuclei or nuclear erythrocyte changes. Besides, exposure to DiPeP did not demonstrate a genotoxic potential for *Rhamdia quelen* blood cells. Our research group found the same results for exposure to DBP, for the three biomarkers, in a study with the same experimental design (Oya-Silva et al. in preparation).

According to Asghari et al. (2015), phthalates reduce the antioxidant capacity of organisms, which may decrease the activity of enzymes such as GST, SOD, GPx, in addition to the GSH concentration, and at the same time can cause an increase in the CAT activity. However, we found studies with different species of fish subjected to water (LATIF; FAHEEM; ASMATULLAH, 2020) and intraperitoneal (KANG et al., 2010) exposures to DEP that observed an increase in GSH. Furthermore, Latif et al. (2020) showed a reduction in hepatic GST activity after 21 days of DEP exposure. The GST

and GSH have a fundamental role in the biotransformation of hydrophobic molecules into hydrophilic ones, an important characteristic that facilitates elimination (VAN DER OOST; BEYER; VERMEULEN, 2003). In our study, in the liver, only the highest dose (125ng.g^{-1}) of DiPeP caused an increase in GST activity and reduced the GSH concentration. Based on our results, we believe that the intense hepatic GST activity, in the group treated with 125ng.g^{-1}de DiPeP, may have generated a depletion of GSH in the liver.

After 30 days of exposure to DiPeP, the liver enzymes SOD, CAT and GPx, did not change. In the literature, we found results similar to ours for intraperitoneal exposure to DBP, the SOD (ZHENG; FENG; DAI, 2013) and CAT activities (QU et al., 2014) did not changes, but for GPx activity was reduced in both studies. In a water exposure to DEP, Latif et al. (2020) found no effects in GPx activity, but the CAT was reduced after 21 days of exposure. Since the routes and periods of exposure between these studies and ours are different, we believe that these are important factors to be considered in the difference in the responses of the enzymes.

Despite of increased lipoperoxidation in *Danio rerio* larvae (XU et al., 2013a) in the microcrustaceans *Daphnia magna* (SHEN et al., 2019; WANG et al., 2019) and *Oreochromis niloticus* (KHALIL; ELHAKIM; EL-MURR, 2016), after exposure to DBP, in our study we found no changes in the lipoperoxidation of liver cells in the groups treated with DiPeP. However, the literature also presents results similar to those observed in water exposure to DBP for 96 hours (ERKMEN et al., 2015) and intraperitoneally after 15 days (QU et al., 2014). Both authors associate the maintenance of basal lipoperoxidation with the liver's detoxification efficiency, showing an adaptive response when exposed to a xenobiotic.

The literature associates the potential for genotoxicity of phthalates with the generation of reactive oxygen species (ROS) during the metabolization process (ASGHARI; SAEIDNIA; ABDOLLAHI, 2015; ERKEKOĞLU et al., 2010; ERKEKOGLU; KOCER-GUMUSEL, 2014). However, we did not observe an increase in DNA damage in the two versions of the comet assay, alkaline and oxidative. For this reason, we infer that DiPeP, in the doses administered, its metabolites or possible ROS from exposure, could not cause DNA damage. As mentioned earlier, we believe that the system for combating oxidative stress in the liver was efficient, and thus, sufficient to protect the cell from both membrane damage and its genetic material.

The kidney was the organ whose biomarkers were significantly affected by exposure to DiPeP. Divided into two portions, anterior and posterior, that perform different functions such as hematopoiesis and excretion (SOLDATOV, 2005; UDROIU, 2006; ZWOLLO et al., 2005), respectively, suffered different impacts concerning DNA damage caused by exposure to DiPeP. In the posterior kidney, phthalate did not promote a significant increase in DNA damage in any treatment dose. Besides that, in the same portion of kidney, the LPO was reduced in all treatments with DiPeP. According to Qu et al. (2014), the reduction of lipid peroxidation may indicate that antioxidants' production was sufficient to remove free radicals and thus prevent oxidative damage to cells.

Even in the posterior kidney, although there were no changes to GST and CAT between exposed and control groups, we observed a reduction in the SOD and GPx activities of in all treatments with DiPeP. The SOD catalyzes the dismutation of superoxide anions forming H_2O_2, which are removed by CAT and GPx (VAN DER OOST; BEYER; VERMEULEN, 2003). Our results for SOD and GPx corroborate the data published by Qu et al. (2014), in which fish were exposed intraperitoneally to nine different phthalates, after four days showed a reduction in the activity of these enzymes. The reduction in GPx activity in our study may be related to the reduction in GSH observed in all groups exposed to DiPeP. Since GSH is a GPx cofactor, its activity becomes limited. Thus, we can infer that DiPeP causes an imbalance in the posterior kidney antioxidant system, which can promote the increase and bioavailability of reactive oxygen species, such as superoxide anion, and other varieties of peroxides not metabolized by CAT.

Studies suggest that phthalate metabolites, monoalkyl phthalate esters (MPE), are eliminated by the gills and kidneys (HU et al., 2016). In the kidneys, DBP showed different intensity of the effect, between portions, concerning genetic material (MARTINS et al., 2016). In the present study, opposite to the posterior kidney, we found in the anterior kidney significant increases in the scores of the groups treated with phthalate compared to the NC group. Our results corroborate the responses obtained in our study with the same experimental design with exposure to DBP. Like the present study, DBP promoted increased damage to the genetic material of only the anterior kidney. However, this increase was only evident at the dose of 125ng.g^{-1} (Oya-Silva et al., In preparation).

The neurotoxic potential of phthalates in fish has already been reported in the literature, indicating the brain as one of the target tissues of DBP and DEP (JEE et al., 2009; POOPAL et al., 2017; XU et al., 2013b). In our study, the activity of muscle AChE was reduced in all treatments with DiPeP, corroborating the results presented by Jee et al. (2009) in which *Pseudobagrus fulvidraco* exposed to doses of 100 to 1000 mg.Kg^{-1} of DBP for eight weeks. In the brain tissue, DiPeP promoted an increase in AChE activity at doses of 5 and 25ng.g^{-1}, however, the treatment with 125ng.g^{-1} caused a drastic reduction in the activity of the enzyme. Jee et al. (2009) and Poopal et al. (2017) observed significant reductions in the brain AChE activity after subchronic exposures to DBP by the trophic and water, respectively. The increase in AChE activity is related to apoptotic events, being considered a promising biomarker of cell death in vitro and in vivo assays (ZHANG; GREENBERG, 2012). Considering this bias, the non-induction of damage to brain tissue DNA and the AChE response, we believe that, in the brain, exposure to DiPeP could induced apoptosis. However, at lower doses the speed of this process occurred more mildly and continuously, and at 125ng.g^{-1} the process already shows the effect of excessive cell death and thus, the production of AChE would be reduced in the brain. Thus, the result of the comet assay may have been masked by the excess of pulverized cells, with extreme damage and not meeting the minimum classification criteria. But to confirm this hypothesis, are necessary studies using biomarkers of death cell.

DiPeP has more toxic potential than DBP. The most affected tissue in both studies was the kidney, evidenced by the increase in DNA damage, reduced activity of antioxidant enzymes GSH and GPx. The responses generated by exposure to DiPeP were more intense than DBP, since all exposed groups showed the same response, while exposure to DBP induced responses only in treatments with the highest doses. Besides, DiPeP showed more significant neurotoxicity than DBP, since all doses had the potential to reduce AChE activity in muscle tissue, which was not observed with DBP (Oya-Silva et al., In preparation).

The mechanisms of excretion and the metabolic pathways of phthalates are still not fully understood. Toxicity studies of DiPeP are scarce in the literature. In fish, this is the first report of effects caused by trophic exposure to DiPeP. Thus, new proposals for *in vivo* tests are needed, with different exposure routes to a phthalate, mainly with species native to South America, where DiPeP production is abundant. Studies on exposure to its metabolites are also interesting, as in the environmental scenario both

will be present, and there is evidence that MPEs are as toxic as their respective phthalates (JIAO et al., 2020; PARK et al., 2020). Besides that, in vitro tests with exposure to DiPeP and its metabolites to liver, kidney and brain cells, for example, are essential to be performed to elucidate the mechanisms of metabolism and direct effects on these tissues.

5.5 Conclusion

The constant trophic exposure to DiPeP revealed nephrotoxic and neurotoxic potential. The kidney's antioxidant system has been compromised and may have allowed increased damage to the genetic material of kidney cells. Neurotoxicity evidenced mainly in muscle tissue can cause long-term problems in the locomotion and survival of exposed organisms. The genetic and biochemical biomarkers set proved to be efficient for assessing the risks of exposure to phthalates. However, in the specific case of DiPeP, further studies are still needed, adding to the biomarkers used in this study, other complementary ones such as histological, cell death, for example, and the quantification of phthalate and its active metabolite in tissues and water.

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6. CONSIDERAÇÕES FINAIS

No presente estudo, foram apresentadas evidências de efeitos tóxicos em exposições ao DBP e DiPeP no rim, fígado, cérebro e músculo, mesmo em concentrações menores que as mensuradas no ambiente aquático para o DBP. Pela primeira vez, foi demostrado que a ingestão prolongada de DBP e DiPeP em baixas doses, pode causar alterações na atividade de enzimas e moléculas não enzimáticas do sistema antioxidante de *Rhamda quelen*. No fígado, o principal efeito foi o aumento da atividade de GST, que pode nos indicar que a etapa de biotransformação do DBP e DiPeP em MPEs ocorra nesse órgão. O desequilíbrio observado no sistema antioxidante, no rim posterior, pode ser devido aos monos ésters de ftalato e espécies reativas de oxigênio produzidos durante a metabolização do ftalato.

Além do sistema antioxidante, as baixas doses DiPeP foram genotóxicas para o rim anterior, enquanto que somente a maior dose de DBP apresentou esse efeito no mesmo tecido. A genotoxicidade renal pode ser decorrente do comprometimento do órgão, promovido pela exposição ao DBP e DiPeP. No cérebro, ambos ftalatos demonstraram aumento de AChE cerebral, entretanto o DiPeP reduziu significativamente a atividade da enzima na maior dose. Além disso, o DiPeP também mostrou maior potencial tóxico ao sistema nervoso, pois reduziu a atividade da AChE muscular em todas as doses.

Pelos resultados obtidos neste estudo, o DiPeP demostrou maior toxicidade, uma vez que apresentou efeitos em biomarcadores que o DBP não causou alteração, como redução da GPx. E, em biomarcadores nos quais o DBP alterou em apenas alguns tratamentos, o DiPeP mostrou efeito em todas as doses de tratamento.

São necessários mais estudos com modelos de exposição à baixas doses e/ou concentrações e por longos períodos para elucidar possíveis vias metabólicas, de acordo com a via de administração. É interessante também a execução de estudos com os metabólitos dos ftalatos, pois podem ter maior potencial tóxico e também estão biodisponíveis no ambiente. Especificamente, em relação ao DiPeP, os dados na literatura são escassos, desta forma faz-se necessário tanto estudos com exposições controladas em bioensaios, utilizando biomarcadores precoces, e agregando outros não empregados nesse estudo, como biomarcadores histológicos e de morte celular, por exemplo.

A construção de banco de dados, sobre efeitos causados pela exposição à concentrações e/ou doses ambientalmente relevantes, é essencial para a determinação de limites de exposição seguros para o ecossistema aquático, bem como para o desenvolvimento de regulamentações a respeito do uso e descartes industriais adequados.

REFERÊNCIAS

ABDEL DAIEM, M. M.; RIVERA-UTRILLA, J.; OCAAMPO-PÉREZ, R.; MÉNDEZ-DÍAS, J. D.; SÁNCHEZ-POLO, M. Environmental impact of phthalic acid esters and their removal from water and sediments by different technologies - A review. **Journal of Environmental Management**, v. 109, p. 164–178, 2012.

ABIPLAST. **Perfil 2019**. Disponível em: <http://www.abiplast.org.br/wp-content/uploads/2020/09/Perfil_2019_web_abiplast.pdf>.

ADAMS, W. J.; BIDDINGER, G. R.; ROBILLARD, K. A. A summary of the acute toxicity of 14 phthalate esters to representative aquatic organisms. **Environmental Toxicology and Chemistry**, v. 14, n. 9, p. 1569–1574, 1995.

ADENIYI, A. A.; OKEDEYI, O. O.; YUSUF, K. A. Flame ionization gas chromatographic determination of phthalate esters in water, surface sediments and fish species in the Ogun river catchments, Ketu, Lagos, Nigeria. **Environmental Monitoring and Assessment**, v. 172, n. 1–4, p. 561–569, 2011.

AEBI, H. [13] Catalase in vitro. **Methods in Enzymology**, v. 105, p. 121–126, 1984.
AKAISHI, F. M. **Aplicação de biomarcadores de contaminação ambiental em estudos de laborátório e monitoramento em campo**. [s.l.] Universidade Federal do Paraná, 2003.

AL-SABTI, K.; METCALFE, C. D. Fish micronuclei for assessing genotoxicity in water. **Genetic Toxicology**, v. 343, p. 121–135, 1995.

AL-SALEH, I.; AL-RAJUDI, T.; AL-QUDAIHI, G.; MANOGARAN, P. Evaluating the potential genotoxicity of phthalates esters (PAEs) in perfumes using in vitro assays. **Environmental Science and Pollution Research**, v. 24, n. 30, p. 23903–23914, 2017..

ANDERSON, D.; YU, T.-W.; HINÇAL, F. Effect of Some Phthalate Esters in Human Cells in the Comet Assay. **Teratogenesis, Carcinogenesis, and Mutagenesis**, v. 19, p. 275–280, 1999.

AOKI, K. A. A.; HARRIS, C. A.; KATSIADAKI, I.; SUMPTER, J. P. Evidence suggesting that di-n-butyl phthalate has antiandrogenic effects in fish. **Environmental Toxicology and Chemistry**, v. 30, n. 6, p. 1338–1345, 2011.

ASGHARI, M. H.; SAEIDNIA, S.; ABDOLLAHI, M. A review on the biochemical and molecular mechanisms of phthalate-induced toxicity in various organs with a focus on the reproductive system. **International Journal of Pharmacology**, v. 11, n. 2, p. 95–105, 2015.

ATLI, G.; ALPTEKIN, Ö.; TÜKEL, S.; CANLI, M. Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish Oreochromis niloticus. **Comparative Biochemistry and Physiology - C Toxicology and Pharmacology**, 2006.

AYLLON, F.; GARCIA-VAZQUEZ, E. Induction of micronuclei and other nuclear abnormalities in European minnow Phoxinus phoxinus and mollie Poecilia latipinna : an assessment of the fish micronucleus test. **Mutation Research**, v. 467, p. 177–186, 2000.

AZQUETA, A.; MEIER, S.; PRIESTLEY, C.; GUTZKOW, K. B.; BRUNBORG, G.; SALLETTE, J.; SOUSSALINE, F.; COLLINS, A. The influence of scoring method on variability in results obtained with the comet assay. **Mutagenesis**, v. 26, n. 3, p. 393–399, 2011.

BARCELLOS, L. J. G. KREUTZ, L. C.; QUEVEDO, R. M.; FIOREZE, I.; CERICATO, L.; SOSO, A. B.; FAGUNDES, M.; CONRAD, J.; BALDISSERA, R. K.; BRUSCHI, A.; RITTER, F. Nursery rearing of jundia (Quoy & Gaimard) in cages : cage type , stocking density and stress response to confinement. **Aquaculture**, v. 232, p. 383–394, 2004.

BENLI, A. C. K.; ERKMEN, B.; ERKOC, F. Genotoxicity of sub-lethal di-n-butyl phthalate (DBP) in Nile tilapia (Oreochromis niloticus). **Arhiv za Higijenu Rada i Toksikologiju**, v. 67, n. 1, p. 25–30, 2016.

BERTONCELLO SOUZA, M.; PASSONI, M. T.; PÄLMKE, C.; MEYER, K. B.; VENTURELLI, A. C.; ARAÚJO, G.; CASTILHOS, B. S.; MORAIS, N. M.; DALSENTER, P. R.; SWAN, S. H.; KOCH, H. M.; MARTINO-ANDRADE, A. J. Unexpected, ubiquitous exposure of pregnant Brazilian women to diisopentyl phthalate, one of the most potent antiandrogenic phthalates. **Environment International**, v. 119, n. April, p. 447–454, 2018.

BHATIA, H.; KUMAR, A.; DU, J.; CHAPMAN, J.; MCLAUGHLIN, M. Di-n-butyl phthalate causes antiestrogenic effects in female murray rainbowfish (Melanotaenia fluviatilis). **Environmental Toxicology and Chemistry**, v. 32, n. 10, p. 2335–2344, 2013.

BHATIA, H.; KUMAR, A.; OGINO, Y.; GREGG, A.; CHAPMAN, J.; MCLAUGHLIN, M. J.; IGUCHI, T. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (Melanotaenia fluviatilis). **Aquatic Toxicology**, v. 149, p. 103–115, 2014.

BOMBAIL, V.; AW, D.; GORDON, E.; BATTY, J. Application of the comet and micronucleus assays to butter ® sh (Pholis gunnellus) erythrocytes from the Firth of Forth , Scotland. **Chemosphere**, v. 44, p. 383–392, 2001.

BOMBARDELLI, R. A.; MÖRSCHBÄCHER, E. F.; CAMPAGNOLO, R.; SANCHES, E. A.; SYPERRECK, M. A.. Dose inseminante para fertilização artificial de ovócitos de jundiá cinza, Rhamdia quelen (Quoy & Gaimardm, 1824). **Revista Brasileira de Zootecnia**, v. 35, n. 4, p. 1251–1257, 2006.

BORAN, H.; TERZI, S. Bis(2-ethylhexyl) phthalate induces DNA strand breaks and gene expression alterations in larval zebrafish Danio rerio. **Toxicology and Industrial Health**, v. 35, n. 8, p. 520–529, 2019.

BOTELHO, G. G. K.; GOLIN, M.; BUFALO, A. C.; MORAIS, R. N.; DALSENTER, P.

R.; MARTINO-ANDRADE, A. J. Reproductive Effects of Di (2-ethylhexyl) phthalate in Immature Male Rats and Its Relation to Cholesterol , Testosterone , and Thyroxin Levels. **Archives of Environmental Contamination and Toxicology**, v. 57, p. 777–784, 2009.

BRADFORD, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizinf the Principle of Protein-Dye Binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BROMAGE, E. S.; KAATTARI, I. M.; ZWOLLO, P.; KAATTARI, S. L. Plasmablast and Plasma Cell Production and Distribution in Trout Immune Tissues. **The Journal of Immunology**, v. 173, n. 12, p. 7317–7323, 2004.

BUCHELI, T. D.; FENT, K. Induction of Cytochrome P450 as a Biomarker for Environmental Contamination in Aquatic Ecosystems. **Environmental Science and Pollution Research**, v. 25, n. 3, p. 201–268, 1995.

CARRASCO, K. R.; TILBURY, K. L.; MYERS, M. S. Assessment of the Piscine Micronuc eus Test as an in situ. **Canadian Journal of Fisheries and Aquatic Sciences**, v. 47, n. 3, p. 2123–2136, 1990.

ÇAVAŞ, T.; ERGENE-GÖZÜKARA, S. Genotoxicity evaluation of metronidazole using the piscine micronucleus test by acridine orange fluorescent staining. **Environmental Toxicology and Pharmacology**, v. 19, n. 1, p. 107–111, 2005.

CESTARI, M. M.; LEMOS, P. M. M.; RIBEIRO, C. A. O.; COSTA, J. R. M. A.; PELLETIER, E.; FERRARO, M. V. M.; MANTOVANI, M. S.; FENOCCHIO, A. S. 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**, v. 27, n. 2, 2004.

CHEN, X.; XU, S.; TAN, T.; LEE, T. S.; CHENG, S. H.; LEE, F. W. F.; XU, S. J. L.; HO, K. C. Toxicity and Estrogenic Endocrine Disrupting Activity of Phthalates and Their Mixtures. **International Journal of Environmental Research and Public Health**, v. 11, p. 3156–3168, 2014.

CHENG, Z.; NIE, X.; WANG, H.; WONG, M. Risk assessments of human exposure to bioaccessible phthalate esters through market fish consumption. **Environment International**, v. 57–58, p. 75–80, 2013.

CHEON, Y.-P. Di-(2-ethylhexyl) Phthalate (DEHP) and Uterine Histological Characteristics. **Development & Reproduction**, v. 24, n. 1, p. 1–17, 2020.

CHO, Y. S.; CHOI, B. N.; KIM, K. H.; KIM, S. K.; KIM, D. S.; BANG, I. C.; NAM, Y. K. Differential expression of Cu / Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (Oplegnathus fasciatus). **Aquaculture**, v. 253, p. 667–679, 2006.

COLLINS, A. R.; DOBSON, V. L.; DUSINKÁ, M.; KENNEDY, G.; STETINA, R. The comet assay: what can it really tell us? **Mutation Research**, v. 375, p. 183–193,

1997.

COLLINS, A. R.; DUTHIE, S. J.; DOBSON, V. L. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte dna. **Carcinogenesis**, v. 14, n. 9, p. 1733–1735, 1993.

CURI, T. Z.; SILVA, G. N.; PASSONI, M. T.; TOLOUEI, S. E. L.; MELDOLA, H.; ROMANO, R. M.; GRECHI, N.; DALSENTER, P. R.; MARTINO-ANDRADE, A. J. In Utero and Lactational Exposure to Diisopentyl Phthalate Induces Fetal Toxicity and Antiandrogenic Effects in Rats. **Toxicological Sciences**, v. 171, n. 2, p. 347–358, 2019.

DAVID, R. M.; MOORE, M. R.; CIFONE, M. A.; FINNEY, D. C.; GUEST, D. Chronic Peroxisome Proliferation and Hepatomegaly Associated with the Hepatocellular Tumorigenesis of Di (2-Ethylhexyl) Phthalate and the Effects of Recovery. **Toxicological Sciences**, v. 50, p. 195–205, 1999.

DO NASCIMENTO FILHO, I.; MÜHLEN, C. V.; SCHOSSLER, P.; CARAMÃO, E. B. Identification of some plasticizers compounds in landfill leachate. **Chemosphere**, v. 50, p. 657–663, 2003.

DOMÍNGUEZ-MORUECO, N.; GONZÁLEZ-ALONSO, S.; VALCÁRCEL, Y. Phthalate occurrence in rivers and tap water from central Spain. **Science of the Total Environment**, v. 500–501, p. 139–146, 2014.

DU, L. LI, G.; LIU, M.; LI, Y.; YIN, S.; ZHAO, J.; ZHANG, X. Evaluation of DNA damage and antioxidant system induced by di-n-butyl phthalates exposure in earthworms (Eisenia fetida). **Ecotoxicology and Environmental Safety**, v. 115, p. 75–82, 2015.

ELLMAN, G. L. COURTNEY, K. D.; ANDRES JR., V.; FEATHERSTONE, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. **Biochemical Pharmacology**, 1961.

RKEKOGLU, P.; RACHIDI, W.; DE ROSA, V.; GIRAY, B.; FAVIER, A.; HINCAL, F. Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells. **Free Radical Biology and Medicine**, v. 49, n. 4, p. 559–566, 2010.

ERKEKOĞLU, P.; KOCER-GUMUSEL, B. Genotoxicity of phthalates. **Toxicology Mechanisms and Methods**, v. 24, n. 9, p. 616–626, 2014.

ERKMEN, B.; BENLI, A. Ç. K.; ANGUS, H. H.; YILDIRIM, Z.; MERTO, R.; ERKOÇ, F. Impact of sublethal di-n-butyl phthalate on the aquaculture fish species Nile tilapia (Oreochromis niloticus): histopathology and oxidative stress assessment. **Aquaculture Research**, v. 48, n. 2, p. 675–685, 2015.

FERRARO, M. V. M.; FENOCCHIO, A. S.; MANTOVANI, M. S.; RIBEIRO, C. O.; CESTARI, M. M. Mutagenic effects of tributyltin and inorganic 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**, v. 27, n. 1, p. 103–107, 2004.

FERREIRA, D.; MOTTA, A. C.; KREUTZ, L. C.; TONI, C.; LORO, V. L.; BARCELLOS, L. J. G. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. **Chemosphere**, v. 79, n. 9, p. 914–921, 2010.

FERREIRA, I. D.; MORITA, D. M. EX-SITU BIOREMEDIATION OF BRAZILIAN SOIL CONTAMINATED WITH PLASTICIZERS PROCESS WASTES. **Brazilian Journal of Chemical Engineering**, v. 29, n. 01, p. 77–86, 2012.

FRENZILLI, G.; NIGRO, M.; LYONS, B. P. The Comet assay for the evaluation of genotoxic impact in aquatic environments. **Mutation Research - Reviews in Mutation Research**, v. 681, n. 1, p. 80–92, 2009.

GAO, D. W.; WEN, Z. D. Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. **Science of the Total Environment**, v. 541, p. 986–1001, 2016.

GAO, R.; YUAN, Z.; ZHAO, Z.; GAO, X. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. **Bioelectrochemistry and Bioenergetics**, v. 45, n. 1, p. 41–45, 1998.

GAO, X.; LI, J.; WANG, X.; ZHOU, J.; FAN, B.; LI, W.; LIU, Z. Exposure and ecological risk of phthalate esters in the Taihu Lake basin, China. **Ecotoxicology and Environmental Safety**, v. 171, n. December 2018, p. 564–570, 2019.

GHELFI, A.; RIBAS, J. L. C.; GUILOSKI, I. C.; BETTIM, F. L.; PIANCINI, L. D. S.; CESTARI, M. M.; PEREIRA, A. J.; SASSAKI, G. L.; ASSIS, H. C. S. Evaluation of Biochemical , Genetic and Hematological Biomarkers in a Commercial Catfish Rhamdia quelen Exposed to Diclofenac. **Bulletin of Environmental Contamination and Toxicology**, v. 96, p. 49–54, 2016.

GHISI, N. D. C.; RAMSDORF, W. A.; FERRARO, M. V. M.; ALMEIDA, M. I. M.; RIBEIRO, C. A. O.; CESTARI, M. M. Evaluation of genotoxicity in Rhamdia quelen (Pisces, Siluriformes) after sub-chronic contamination with Fipronil. **Environmental Monitoring and Assessment**, v. 180, n. 1–4, p. 589–599, 2011.

GHORPADE, N.; MEHTA, V.; KHARE, M.; SINKAR, P.; KRISHNAN, S.; RAO, C. V. Toxicity Study of Diethyl Phthalate on Freshwater Fish Cirrhina mrigala. **Ecotoxicology and Environmental Safety**, v. 53, p. 255–258, 2002.

GOBAS, F. A. P. C.; IKONOMOU, M. Bioaccumulation of Phthalate Esters in Aquatic Food-Webs. v. 3, p. 201–225, 2003.

GOKSØYR, A.; ANDERSSON, T.; BUHLER, D. R.; STEGEMAN, J. J.; WILLIAMS, D. E.; FÖRLIN, L. Immunochemical cross-reactivity of β-naphthoflavone-inducible cytochrome P450 (P450IA) in liver microsomes from different fish species and rat. **Fish Physiology and Biochemistry**, v. 9, n. 1, p. 1–13, 1991.

GOMES, L. DE C.; GOLOMBIESKI, J. I.; GOMES, A. R. C.; BALDISSEROTTO, B. Biologia do Jundiá Rhamdia quelen (TELEOSTEI, PIMELODIDAE). **Ciência Rural**, v. 30, n. 1, p. 179–185, 2000.

GONTIJO, Á. M. D. M. C.; BARRETO, R. E.; SPEIT, G.; REYES, V. A. V.; VOLPATO, G. L.; SALVADORI, D. M. F. Anesthesia of fish with benzocaine does not interfere with comet assay results. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 534, n. 1–2, p. 165–172, 2003.

GOUTTE, A.; ALLIOT, F.; BUDZINSKI, H.; SIMONNET-LAPRADE, C.; SANTOS, R.; LACHAUX, V.; MACIEJEWSKI, K.; MENACH, K. L.; LABADIE, P. Trophic Transfer of Micropollutants and Their Metabolites in an Urban Riverine Food Web. **Environmental Science and Technology**, v. 54, n. 13, p. 8043–8050, 2020.

GRASSI MILANO, E.; BASARI, F.; CHIMENTI, C. Adrenocortical and adrenomedullary homologs in eight species of adult and developing teleosts: Morphology, histology, and immunohistochemistry. **General and Comparative Endocrinology**, v. 108, n. 3, p. 483–496, 1997.

GUILOSKI, I. C.; PIANCINI, L. D. S.; DAGOSTIM, A. C.; CALADO, S.; L.; M.; FÁVARO, L. F.; BOSCHEN, S. L.; CESTARI, M. M.; CUNHA, C.; ASSIS, H. C. S. Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish Rhamdia quelen. **Ecotoxicology and Environmental Safety**, v. 139, n. December 2016, p. 291–300, 2017a.

GUILOSKI, I. C.; RIBAS, J. L. C.; PIANCINI, L. D. S.; DAGOSTIM, A. C.; CIRIO, S. M.; FÁVARO, L. F.; BOSCHEN, S. L.; CESTARI, M. M., CUNHA, C.; ASSIS, H. C. S. Paracetamol causes endocrine disruption and hepatotoxicity in male fish Rhamdia quelen after subchronic exposure. **Environmental Toxicology and Pharmacology**, v. 53, p. 111–120, 2017b.

GUSTAVINO, B.; SCORNAJENGHI, K. A.; MINISSI, S.; CICCOTTI, E. Micronuclei induced in erythrocytes of Cyprinus carpio (teleostei , pisces) by X-rays and colchicine. **Genetic Toxicology and Environmental Mutagenesis**, v. 494, p. 151–159, 2001.

GUTTERIDGE, J. M. C.; HALLIWELL, B. Free Radicals and Antioxidants in the Year 2000 A Historical Look to the Future. **Annals of the New York Academy of Sciences**, v. 899, p. 136–147, 2000.

HAI, D. Q.; VARGA, I. S.; MATKOVICS, B. Effects of an organophosphate on the antioxidant systems of fish tissues. **Acta biologica Hungarica**, 1995.

HEDDLE, J. A. A rapid in vivo test for chromosomal damage. **Mutation Research**, v. 18, p. 187–190, 1973.

HEUDORF, U.; MERSCH-SUNDERMANN, V.; ANGERER, J. Phthalates : Toxicology and exposure. **International Journal of Hygiene and Environmental Health**, v. 210, p. 623–634, 2007.

HOOFTMAN, R. N.; DE RAAT, W. K. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethyl methanesulphonate. **Mutation Research Letters**, 1982.

HU, T.; FU, Q.; LIU, X.; ZHANG, H.; DONG, M. Increased acetylcholinesterase and capase-3 expression in the brain and peripheral immune system of focal cerebral ischemic rats. **Journal of Neuroimmunology**, v. 211, n. 1–2, p. 84–91, 2009.

HU, X.; GU, Y.; HUANG, W.; YIN, D. Phthalate monoesters as markers of phthalate contamination in wild marine organisms. **Environmental Pollution**, v. 218, p. 410–418, 2016.

JARMOŁOWICZ, S.; DEMSKA-ZAKĘŚ, K.; ZAKĘŚ, Z. Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (Sander lucioperca) Phthalates , which are classified as endocrine disruptor compounds (EDCs), were first created in the 1920s for commercial applications , and th. **Acta Veterinaria Brno**, v. 82, p. 197–201, 2013.

JEE, J. H.; KOO, J. G.; KEUM, Y. H.; PARK, K. H.; CHOI, S. H.; KANG, J. C. Effects of dibutyl phthalate and di-ethylhexyl phthalate on acetylcholinesterase activity in bagrid catfish, Pseudobagrus fulvidraco (Richardson). **Journal of Applied Ichthyology**, v. 25, n. 6, p. 771–775, 2009.

JIAN GE; LI, M.; LIN, F.; ZHAO, J.; HAN, B. Study on metabolism of N-Butyl Benzyl Phthalate (BBP) and Dibutyl Phthalate (DBP) in Ctenopharyngodon idellus by GC and LC-MS/MS. **African Journal of Agricultural Research**, v. 7, n. 12, p. 1855–1862, 2012.

JIANG, Z.; HUNT, J. V; WOLFF, S. P. Ferrous Ion Oxidation in the Presence of Xylenol Orange for Detection of Lipid Hydroperoxide in Low Density Lipoprotein. **Analytical Biochemistry**, v. 202, n. 2, p. 384–389, 1992.

JIAO, Y.; TAO, Y.; YANG, Y.; DIOGENE, T.; YU, H.; HE, Z.; HAN, W.; CHEN, Z.; WU, P.; ZHANG, Y. Monobutyl phthalate (MBP) can dysregulate the antioxidant system and induce apoptosis of zebrafish liver. **Environmental Pollution**, v. 257, p. 113517, 2020.

KANG, J.; JEE, J. H.; KOO, J.; KEUM, Y.; JO, S.; PARK, K. H. Anti-oxidative status and hepatic enzymes following acute administration of diethyl phthalate in olive flounder Paralichthys olivaceus , a marine culture fish. **Ecotoxicology and Environmental Safety**, v. 73, n. 6, p. 1449–1455, 2010.

KAVITHA, P.; RAO, J. V. Oxidative stress and locomotor behaviour response as biomarkers for assessing recovery status of mosquito fish, Gambusia affinis after lethal effect of an organophosphate pesticide , monocrotophos. **Pesticide Biochemistry and Physiology**, v. 87, p. 182–188, 2007.

KEEN, J. H.; HABIG, W. H.; JAKOBY, W. B. Mechanism for the Several Activities of the Glutathione. **The Journal of Biological Chemistry**, v. 251, n. 20, p. 6183–6188, 1976.

KHALIL, S. R.; ELHAKIM, Y. A.; EL-MURR, A. E. Sublethal concentrations of di-n-butyl phthalate promote biochemical changes and DNA damage in juvenile Nile tilapia (Oreochromis niloticus). **Japanese Journal of Veterinary Research**, v. 64, n. 1, p. 67–80, 2016.

KIMBER, I.; DEARMAN, R. J. An assessment of the ability of phthalates to influence immune and allergic responses. **Toxicology**, v. 271, n. 3, p. 73–82, 2010.

KLEINSASSER, N. H.; KASTENBAUER, E. R.; WEISSACHER, H.; MUENZENRIEDER, R. K.; HARRÉUS, U. A. Phthalates Demonstrate Genotoxicity on Human Mucosa of the Upper Aerodigestive Tract. **Environmental and Molecular Mutagenesis**, v. 35, p. 9–12, 2000a.

KLEINSASSER, N. H.; WALLNER, B. C.; KASTENBAUER, E. R.; MUENZENRIEDER, R. K.; HARRÉUS, U. A. Comparing the genotoxic sensitivities of human peripheral blood lymphocytes and mucosa cells of the upper aerodigestive tract using the Comet assay. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 467, n. 1, p. 21–30, 2000b.

KLEINSASSER, N. H.; WALLNER, B. C.; KASTENBAUER, E. R.; WEISSACHER, H.; HARRÉUS, U. A. Genotoxicity of Di-Butyl-Phthalate and Di-Iso-Butyl-Phthalate in Human Lymphocytes and Mucosal Cells. **Teratogenesis, Carcinogenesis, and Mutagenesis**, v. 21, p. 189–196, 2001.

KLEMZ, C.; SILVA DE ASSIS, H. C. EFEITOS DO ENDOSULFANO NA ATIVIDADE DA ACETILCOLINESTERASE DE CASCUDO (Ancistrus multispinnis, FISH, TELEOSTEI). **Revista Acadêmica: Ciência Animal**, v. 3, n. 4, p. 51, 2017.

KLINGELFUS, T.; COSTA, P. M.; SCHERER, M.; CESTARI, M. M. DNA damage in the kidney tissue cells of the fish rhamdia quelen after trophic contamination with aluminum sulfate. **Genetics and Molecular Biology**, v. 38, n. 4, p. 499–506, 2015.

KLINGELFUS, T.; LIROLA, J. R.; OYA-SILVA, L. F.; DISNER, G. R.; VICENTINI, M.; NADALINE, M. J. B.; ROBLES, J. C. Z.; TREIN, L. M.; VOIGT, C. L.; SILVA DE ASSIS, H. C.; MELA, M.; LEME, D. D.; CESTARI, M. M. Acute and long-term effects of trophic exposure to silver nanospheres in the central nervous system of a neotropical fish Hoplias intermedius. **NeuroToxicology**, 2017.

KOO, H. J.; LEE, B. M. Estimated exposure to phthalates in cosmetics and risk assessment. **Journal of Toxicology and Environmental Health, Part A**, 2010.

KOPPEN, G.; AZQUETA, A.; POURRUT, B.; BRUNBORG, G.; COLLINS, A. R.; LANGIE, S. A. S. The next three decades of the comet assay: A report of the 11th International Comet Assay Workshop. **Mutagenesis**, v. 32, n. 3, p. 397–408, 2017.

KWOK, K. W. H.; LEUNG, K. M. Y.; LUI, G. S. G.; CHU, V. K. H.; LAM, P. K. S.; MORRITT, D.; MALTBY, L.; BROCK, T. C. M.; BRINK, P. J. V.; WARNE, M. ST.; CRANE, M. Comparison of Tropical and Temperate Freshwater Animal Species' Acute Sensitivities to Chemicals: Implications for Deriving Safe Extrapolation Factors.

Integrated Envoronmental Assessment and Management, v. 3, n. 1, p. 49–67, 2007.

LATIF, M.; FAHEEM, M.; ASMATULLAH. Study of Oxidative Stress and Histo-Biochemical Biomarkers of Diethyl Phthalate Induced Toxicity in a Cultureable Fish, Labeo rohita. **Pakistan Veterinary Journal**, v. 40, n. 02, p. 202–208, 2020.

LIU, H.; LIANG, H.; LIANG, Y.; ZHANG, D.; WANG, C.; CAI, H.; SHVARTSEV, S. L. Distribution of phthalate esters in alluvial sediment: A case study at JiangHan Plain, Central China. **Chemosphere**, v. 78, n. 4, p. 382–388, 2010.

LUZIA, M. R. F.; SARROUH, B.; LOFRANO, R. C. Z. Evaluation of the biodegradation of phthalic contaminants originated from polyvinyl chloride commercial films. **Acta Scientiarum - Technology**, v. 42, n. 1, p. 1–10, 2020.

MANKIDY, R.; WISEMAN, S.; MA, H.; GIESY, J. P. Biological impact of phthalates. **Toxicology Letters**, v. 217, n. 1, p. 50–58, 2013.

MARCHIORO, M. I.; BALDISSEROTTO, B. Sobrevivência de alevinos de Jundiá (Rhamdia quelen Quoy & Gaimard, 1824) à variação de salinidade da água. **Ciência Rural**, v. 29, n. 2, p. 315–318, 1999.

MARIONNET, D.; DESCHAUX, P.; REYNAUD, S. Possible implication of macrophages in the regulation of cytochrome P450 activities in carp (Cyprinus carpio). **Fish and Shellfish Immunology**, v. 21, p. 80–91, 2006.

MARTINO-ANDRADE, A. J.; MORIS, R. N.; BOTELHO, G. G. K.; MULLER, G.; GRANDE, S. W.; CARPENTIERI, G. B.; LEÃO, G. M. C.; DALSENTER, P. R. Coadministration of active phthalates results in disruption of foetal testicular function in rats. **International Journal of Andrology**, v. 32, p. 704–712, 2009.

MARTINO-ANDRADE, A. J.; CHAHOUD, I. Reproductive toxicity of phthalate esters. **Molecular Nutrition and Food Research**, v. 54, p. 148–157, 2010.

MARTINS, K.; APPLEGATE, B.; HAGEDORN, B.; KENNISH, J.; ZWOLLO, P. Di(2-ethylhexyl) phthalate inhibits B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues of the rainbow trout. **Fish and Shellfish Immunology**, v. 44, n. 1, p. 332–341, 2015.

MARTINS, K.; HAGEDORN, B.; ALI, S.; KENNISH, J.; APPLEGATE, B.; LEU, M.; EPP, L.; PALLISTER, C.; ZWOLLO, P. Tissue Phthalate Levels Correlate With Changes in Immune Gene Expression in a Population of Juvenile Wild Salmon. **Archives of Environmental Contamination and Toxicology**, v. 71, n. 1, p. 35–47, 2016.

MATHIAS, F. T.; FOCKINK, D. H.; DISNER, G. R.; PRODOCIMO, V.; RIBAS, J. L. C.; RAMOS, L. P.; CESTARI, M. M.; SILVA DE ASSIS, H. C. Effects of low concentrations of ibuprofen on freshwater fish Rhamdia quelen. **Environmental Toxicology and Pharmacology**, v. 59, n. 2010, p. 105–113, 2018.

MEAGHER, E. A.; FITZGERALD, G. A. INDICES OF LIPID PEROXIDATION IN VIVO: STRENGTHS AND LIMITATIONS. **Free Radical Biology and Medicine**, v. 28, n. 12, p. 1745–1750, 2000.

MINISSI, S.; CICCOTTI, E.; RIZZONI, M. Micronucleus test in erythrocytes of Barbus plebejus (Teleostei , Pisces) from two natural environments : a bioassay for the in situ detection of mutagens in freshwater. **Genetic Toxicology**, v. 367, p. 245–251, 1996.

MONTAGNER, C. C.; SODRÉ, F. F.; ACAYABA, R. D.; VIDAL, C.; CAMPESTRINI, I.; LOCATELLI, M. A.; PESCARA, I. C.; ALBUQUERQUE, A. F.; UMBUZEIRO, G. A.; JARDIM, W. F. Ten Years-Snapshot of the Occurrence of Emerging Contaminants in Drinking, Surface and Ground Waters and Wastewaters from São Paulo State, Brazil. **Journal of the Brazilian Chemical Society**, v. 30, n. 3, p. 614–632, 2019.

MONTAGNER, C. C.; JARDIM, W. F. Spatial and seasonal variations of pharmaceuticals and endocrine disruptors in the Atibaia River, São Paulo State (Brazil). **Journal of the Brazilian Chemical Society**, v. 22, n. 8, p. 1452–1462, 2011.

MURSALIN; HARIYADI, P.; PURNOMO, E. H.; ANDARWULAN, N.; FARDIZ, D. Crystallization kinetics of coconut oil based on Avrami model. **International Food Research Journal**, v. 23, n. 4, p. 1355–1360, 2016.

NAGORKA, R.; KOSCHORRECK, J. Trends for plasticizers in German freshwater environments – Evidence for the substitution of DEHP with emerging phthalate and non-phthalate alternatives. **Environmental Pollution**, v. 262, p. 114237, 2020.

NELSON, J. S.; GRANDE, T. C.; WILSON, M. V. H. **Fishes of the World: Fifth Edition**. [s.l: s.n.].

NET, S.; SEMPÉRÉ, R.; DELMONT, A.; PALUSELLI, A.; OUDDANE, B. Occurrence, Fate, Behavior and Ecotoxicological State of Phthalates in Di ff erent Environmental Matrices. **Environmental Science & Technology**, v. 49, p. 4019–4035, 2015.

NEUBERT DA SILVA, G.; CURI, T. Z.; TOLOUEI, S. R. L.; PASSONI, M. T.; HEY, G. B. S.; ROMANO, R. M.; MARTINO-ANDRADE, A. J.; DALSENTER, P. R. Effects of diisopentyl phthalate exposure during gestation and lactation on hormone-dependent behaviours and hormone receptor expression in rats. **Journal of Neuroendocrinology**, v. 31, n. 12, p. 1–12, 2019.

NIKINMAA, M. Environmental regulation of the function of circulating erythrocytes via changes in age distribution in teleost fish: Possible mechanisms and significance. **Marine Genomics**, v. 49, n. July, p. 100717, 2020.

O'BRIEN, M. L.; SPEAR, B. T.; GLAUERT, H. P. Role of Oxidative Stress in Peroxisome Carcinogenesis Proliferator-mediated. **Critical Reviews in Toxicology**, v. 35, p. 61–88, 2005.

OLIVE, P. L.; BANÁTH, J. P.; DURAND, R. E. DNA Damage and Repair in Tumor

Heterogeneity in Radiation-Induced and Normal Cells Measured Using the “Comet” Assay. **Radiation Research**, v. 122, p. 86–94, 1990.

OLIVEIRA-JÚNIOR, A. A.; TAVARES-DIAS, M.; MARCON, J. L. Biochemical and hematological reference ranges for Amazon freshwater turtle, Podocnemis expansa (Reptilia: Pelomedusidae), with morphologic assessment of blood cells. **Research in Veterinary Science**, v. 86, n. 1, p. 146–151, 2009.

PAGLIA, D. E.; VALENTINE, W. N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. **The Journal of Laboratory and Clinical Medicine**, v. 70, p. 158–169, 1967.

PANDRANGI, R.; PETRAS, M.; RALPH, S.; VRZOC, M. Alkaline Single Cell Gel (Comet) Assay and Genotoxicity Monitoring Using Bullheads and Carp. **Environmental and Molecular Mutagenesis**, v. 26, p. 345–356, 1995.

PARK, C. G.; SUNG, B.; RYU, C. S.; KIM, Y. J. Mono-(2-ethylhexyl) phthalate induces oxidative stress and lipid accumulation in zebrafish liver cells. **Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology**, v. 230, 2020.

PAYNE, J. F.; FANCEY, L. L.; RAHIMTULA, A. D.; PORTER, E.L. **Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoringComparative Biochemistry and Physiology. Part C, Comparative**, v. 86, n 2, p. 233-245, 1987.

PEIJNENBURG, W. J. G. M.; STRUIJS, J. Occurrence of phthalate esters in the environment of the Netherlands. **Ecotoxicology and Environmental Safety**, v. 63, n. 2, p. 204–215, 2006.

PEREIRA, L. S.; RIBAS, J. L. C.; VICARI, T.; SILVA, S. B.; STIVAL, J.; BALDAN, A. P.; VALDEZ DOMINGOS, F. X.; GRASSI, M. T.; CESTARI, M. M.; SILCA DE ASSIS, H. C. Effects of ecologically relevant concentrations of cadmium in a freshwater fish. **Ecotoxicology and Environmental Safety**, v. 130, p. 29–36, 2016.

PÉREZ, E. R.; CARDOSO, D. R.; FRANCO, D. W. Analysis of the alcohols, esters and carbonyl compounds in fusel oil samples. **Quimica Nova**, v. 24, n. 1, p. 10–12, 2001.

PERUSSOLO, M. C.; GUILOSKI, I. C.; LIROLA, J. R.; FOCKINK, D. .; CORSO, C. R.; BOZZA, D. C.; PRODOCIMO, V.; MELA, M.; RAMOS, L. P.; CESTARI, M. M.; ACCO, A.; SILVA DE ASSIS, H. C. Integrated biomarker response index to assess toxic effects of environmentally relevant concentrations of paracetamol in a neotropical catfish (Rhamdia quelen). **Ecotoxicology and Environmental Safety**, v, 182, 2019.

PIANCINI, L. D. S.; SANTOS, G. S.; TINCANI, F. H.; CESTARI, M. M. Piscine micronucleus test and the comet assay reveal genotoxic effects of Atrazine herbicide in the neotropical fish Rhamdia quelen. **Ecotoxicology and Environmental Contamination**, 2015a.

PIANCINI, L. D. S.; GUILOSKI, I. C.; SILVA DE ASSIS, H. C.; CESTARI, M. M. Mesotrione herbicide promotes biochemical changes and DNA damage in two fish species. **Toxicology Reports**, v. 2, p. 1157–1163, 2015b.

POOPAL, R. K.; RAMESH, M.; MARUTHAPPAN, V.; RAJENDRAN, R. B. Potential effects of low molecular weight phthalate esters (C16H22O4 and C12H14O4) on the freshwater fish: Cyprinus carpio. **Toxicology Research**, v. 6, n. 4, p. 505–520, 2017.

QU, R.; FENG, M.; SUN, P.; WANG, Z. A Comparative Study on Antioxidant Status Combined with Integrated Biomarker Response in Carassius auratus Fish Exposed to Nine Phthalates. **Environmental Toxicology**, v. 30, n. 10, p. 1125–1134, 2014.

RAMSDORF, W. A.; FERRARO, M. V. M.; OLIVEIRA-RIBEIRO, C.A.; COSTA, J. R. M.; CESTARI, M. M. Genotoxic evaluation of different doses of inorganic lead (PbII) in Hoplias malabaricus. **Environmental Monitoring and Assessment**, 2009.

RAMZI, A.; GIREESHKUMAR, T. R.; RAHMAN, K. H.; BALACHANDRAN, K. K.; SHAMEEM, K.; CHACKO, J.; CHANDRAMOHANAKUMAR, N. Phthalic acid esters – A grave ecological hazard in Cochin estuary, India. **Marine Pollution Bulletin**, v. 152, 2020.

REEVES, J. F.; DAVIES, S. J.; DOOD, N. J. F.; JHA, A. N. Hydroxyl radicals (OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. **Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis**, v. 640, n. 1–2, p. 113–122, 2008.

RHODES, J. E.; ADAMS, W. J.; BIDDINGER, G. R.; ROBILLARD, K. A.; GORSUCH, J. W. Chronic toxicity of 14 phthalate esters to Daphnia magna and rainbow trout (Oncorhynchus mykiss). **Environmental Toxicology and Chemistry**, v. 14, n. 11, p. 1967–1976, 1995.

ROCHA, B. A.; ASIMAKOPOULOS, A. G.; BARBORA JR, F.; KANNAN, K. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. **Science of the Total Environment**, v. 586, p. 152–162, 2017.
ROJAS, E.; LOPEZ, M. C.; VALVERDE, M. Single cell gel electrophoresis assay : methodology and applications. **Journal of Chromatography b**, v. 722, p. 225–254, 1999.

ROMERO-FRANCO, M.; HERNÁNDEZ-RAMÍREZ, R. U.; CALAFAT, A. M.; CEBRIÁN, M. E.; NEEDHAM, L. L.; TEITELBAUM, S.; WOLFF, M. S.; LÓPEZ-CARRILLO, L. Personal care product use and urinary levels of phthalate metabolites in Mexican women. **Environment International**, v. 37, p. 867–871, 2011.

RUSYN, I.; PETERS, J. M.; CUNNINGHAM, M. L. Modes of Action and Species-Specific Effects of Di- (2-ethylhexyl) Phthalate in the Liver. **Critical Reviews in Toxicology**, v. 36, p. 459–479, 2006.

SARAH, J. M.; SHANE, J. W.; LANGLOIS, V. S. Influence of Lipophilicity on the

Toxicity of Bisphenol A and Phthalates to Aquatic Organisms. **Bulletin of Environmental Contamination and Toxicology**, v. 97, n. 1, p. 4–10, 2016.

SCHETTLER, T. Human exposure to phthalates via consumer products. **International Journal of Andrology**, v. 29, p. 134–139, 2006.

SCHMID, W. The micronucleus test. **Mutation Research**, v. 31, p. 9–15, 1975.

SEDLAK, J.; LINDSAY, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. **Analytical Biochemistry**, v. 25, p. 192–205, 1968.

S SELVARAJ, K. K.; SUNDARAMOORTHY, G.; RAVICHANDRAN, P. K.; GIRIJAN, G. K.; SAMPATH, S.; RAMASWAMY, B. R. Phthalate esters in water and sediments of the Kaveri River, India: environmental levels and ecotoxicological evaluations. **Environmental Geochemistry and Health**, v. 37, n. 1, p. 83–96, 2014.

SEPPERUMAL, U.; SAMINATHAN, S. Effect of diethylphthalate on the haematological parameters of the freshwater fish Oreochromismossambicus (Tilapia). **European Journal of Zoological Research**, v. 2, n. 4, p. 55–59, 2013.

SHEN, C.; WEI, J.; WANG, T.; WANG, Y. Acute toxicity and responses of antioxidant systems to dibutyl phthalate in neonate and adult Daphnia magna. **PeerJ**, v. 2019, n. 3, 2019.

SINGH, N. P.; MACCOY, M. T.; TICE, R. R.; SCHNEIDER, W. L. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells'. **Experimental Cell Research**, v. 175, p. 184–191, 1988.

SOLDATOV, A. A. Peculiarities of organization and functioning of the fish red blood system. **Journal of Evolutionary Biochemistry and Physiology**, v. 41, n. 3, p. 272–281, 2005.

SPEIT, G.; HARTMANN, A. The Comet Assay (Single-Cell Gel Test) A Sensitive Genotoxicity Test for the Detection of DNA Damage and Repair. **Methods in Molecular Biology**, v. 113, n. DNA Repair Protocols: Eukaryotic Systems, p. 203–212, 1999.

STAPLES, C. A.; PETERSON, D. R.; PARKERTON, T. F.; ADAMS, W. J. The environmental fate of phthalate esters: a literature review. **Chemosphere**, v. 35, n. 4, p. 667–749, 1997a.

STAPLES, C. A.; ADAMS, W. J.; PARKERTON, T. F.; GORSUCH, J. W.; BIDDINGER, G. R.; REINERT, K. H. Aquatic of toxicity of eighteen phthalates esters. **Environmental Toxicology and Chemistry**, v. 16, n. 5, p. 875–891, 1997b.

STAPLES, C. A.; PARKERTON, T. F.; PETERSON, D. R. A risk assessment of selected phthalate esters in North American and Western European surface waters. **Chemosphere**, v. 40, n. 8, p. 885–891, 2000.

STEGEMAN, J. J.; MARIUS, B.; GIULIO, R. T.; LARS, F.; FOWLER, B. A.; SANDERS, B. M.; VELD, P. A. V. Molecular responses to environmental contamination: Enzyme and protein systems as indicators of chemical exposure and effect. In: **Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress**. 1. ed. CRC Press, 2018.

SUN, J.; HUANG, J.; ZHANG, A.; LIU, W.; CHENG, W. Occurrence of phthalate esters in sediments in Qiantang River, China and inference with urbanization and river flow regime. **Journal of Hazardous Materials**, v. 248–249, n. 1, p. 142–149, 2013.

SWAN, S. H. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. **Environmental Research**, v. 108, n. 2, p. 177–184, 2008.

TAVARES-DIAS, M.; MORAES, F. R. DE. Hematological parameters for the Brycon orbignyanus Valenciennes, 1850 (Osteichthyes: Characidae) intensively bred. **Hidrobiológica**, v. 16, n. 3, p. 271–274, 2006.

TEBAS, S. DE O. G.; BARAÑANO, A. G.; PINHEIRO, P. F.; LACERDA, V. Mild conditions in the transesterification reaction of babassu oil and purified fusel oil. **Chemical Engineering Communications**, 2020.

TICE, R. R.; AGURELL, E.; ANDERSON, D.; BURLINSON, B.; HARTMANN, A.; KOBAYASHI, H.; MIYAMAE, Y.; ROJAS, E.; RYU, J. C.; SASAKI, Y. F. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. **Environmental and Molecular Mutagenesis**, v. 35, n. 3, p. 206–221, 2000.

TIWARI, M.; SAHU, S. K.; PANDIT, G. G. Environmental distribution and ecotoxicological concerns of phthalic acid esters in creek ecosystem. **Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering**, v. 54, n. 4, p. 328–336, 2019.

U.S.EPA. Preliminary Materials for the Integrated Risk Information System (IRIS) Toxicological Review of Butyl Benzyl Phthalate (BBP) (CASRN 85-68-7). **U.S. Environmental Protection Agency, Washington, DC, EPA/635/R-13/302**, p. 1–215, 2015.

UDROIU, I. The micronucleus test in piscine erythrocytes. **Aquatic Toxicology**, v. 79, p. 201–204, 2006.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environmental Toxicology and Pharmacology**, v. 13, p. 57–149, 2003.

ABDEL DAIEM, M. M. et al. Environmental impact of phthalic acid esters and their removal from water and sediments by different technologies - A review. **Journal of Environmental Management**, v. 109, p. 164–178, 2012.

ABIPLAST. **Perfil 2019**. Disponível em: <http://www.abiplast.org.br/wp-

content/uploads/2020/09/Perfil_2019_web_abiplast.pdf>. Acesso em: 27 jul. 2020.

ADAMS, W. J.; BIDDINGER, G. R.; ROBILLARD, K. A. A summary of the acute toxicity of 14 phthalate esters to representative aquatic organisms. **Environmental Toxicology and Chemistry**, v. 14, n. 9, p. 1569–1574, 1995.

ADENIYI, A. A.; OKEDEYI, O. O.; YUSUF, K. A. Flame ionization gas chromatographic determination of phthalate esters in water, surface sediments and fish species in the Ogun river catchments, Ketu, Lagos, Nigeria. **Environmental Monitoring and Assessment**, v. 172, n. 1–4, p. 561–569, 2011.

AEBI, H. [13] Catalase in vitro. **Methods in Enzymology**, v. 105, p. 121–126, 1984.
AKAISHI, F. M. **Aplicação de biomarcadores de contaminação ambiental em estudos de laborátório e monitoramento em campo**. [s.l.] Universidade Federal do Paraná, 2003.

AL-SABTI, K.; METCALFE, C. D. Fish micronuclei for assessing genotoxicity in water. **Genetic Toxicology**, v. 343, p. 121–135, 1995.

AL-SALEH, I.; AL-RAJUDI, T.; AL-QUDAIHI, G.; MANOGARAN, P. Evaluating the potential genotoxicity of phthalates esters (PAEs) in perfumes using in vitro assays. **Environmental Science and Pollution Research**, v. 24, n. 30, p. 23903–23914, 2017.

ANDERSON, D.; YU, T.-W.; HINÇAL, F. Effect of Some Phthalate Esters in Human Cells in the Comet Assay. **Teratogenesis, Carcinogenesis, and Mutagenesis**, v. 19, p. 275–280, 1999.

AOKI, K. A. A.; HARRIS, C. A.; KATSIADAKI, I.; SUMPTER, J. P. Evidence suggesting that di-n-butyl phthalate has antiandrogenic effects in fish. **Environmental Toxicology and Chemistry**, v. 30, n. 6, p. 1338–1345, 2011.

ASGHARI, M. H.; SAEIDNIA, S.; ABDOLLAHI, M. A review on the biochemical and molecular mechanisms of phthalate-induced toxicity in various organs with a focus on the reproductive system. **International Journal of Pharmacology**, v. 11, n. 2, p. 95–105, 2015.

ATLI, G.; ALPTEKIN, Ö.; TÜKEL, S.; CANLI, M. Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish Oreochromis niloticus. **Comparative Biochemistry and Physiology - C Toxicology and Pharmacology**, 2006.

AYLLON, F.; GARCIA-VAZQUEZ, E. Induction of micronuclei and other nuclear abnormalities in European minnow Phoxinus phoxinus and mollie Poecilia latipinna : an assessment of the fish micronucleus test. **Mutation Research**, v. 467, p. 177–186, 2000.

AZQUETA, A.; MEIER, S.; PRIESTLEY, C.; GUTZKOW, K. B.; BRUNBORG, G.; SALLETTE, J.; SOUSSALINE, F.; COLLINS, A. The influence of scoring method on variability in results obtained with the comet assay. **Mutagenesis**, v. 26, n. 3, p. 393–

399, 2011.

BARCELLOS, L. J. G. KREUTZ, L. C.; QUEVEDO, R. M.; FIOREZE, I.; CERICATO, L.; SOSO, A. B.; FAGUNDES, M.; CONRAD, J.; BALDISSERA, R. K.; BRUSCHI, A.; RITTER, F. Nursery rearing of jundia (Quoy & Gaimard) in cages : cage type , stocking density and stress response to confinement. **Aquaculture**, v. 232, p. 383–394, 2004.

BENLI, A. C. K.; ERKMEN, B.; ERKOC, F. Genotoxicity of sub-lethal di-n-butyl phthalate (DBP) in Nile tilapia (Oreochromis niloticus). **Arhiv za Higijenu Rada i Toksikologiju**, v. 67, n. 1, p. 25–30, 2016.

BERTONCELLO SOUZA, M.; PASSONI, M. T.; PÄLMKE, C.; MEYER, K. B.; VENTURELLI, A. C.; ARAÚJO, G.; CASTILHOS, B. S.; MORAIS, N. M.; DALSENTER, P. R.; SWAN, S. H.; KOCH, H. M.; MARTINO-ANDRADE, A. J. Unexpected, ubiquitous exposure of pregnant Brazilian women to diisopentyl phthalate, one of the most potent antiandrogenic phthalates. **Environment International**, v. 119, n. April, p. 447–454, 2018.

BHATIA, H.; KUMAR, A.; DU, J.; CHAPMAN, J.; MCLAUGHLIN, M. Di-n-butyl phthalate causes antiestrogenic effects in female murray rainbowfish (Melanotaenia fluviatilis). **Environmental Toxicology and Chemistry**, v. 32, n. 10, p. 2335–2344, 2013.

BHATIA, H.; KUMAR, A.; OGINO, Y.; GREGG, A.; CHAPMAN, J.; MCLAUGHLIN, M. J.; IGUCHI, T. Di-n-butyl phthalate causes estrogenic effects in adult male Murray rainbowfish (Melanotaenia fluviatilis). **Aquatic Toxicology**, v. 149, p. 103–115, 2014.

BOMBAIL, V.; AW, D.; GORDON, E.; BATTY, J. Application of the comet and micronucleus assays to butter ® sh (Pholis gunnellus) erythrocytes from the Firth of Forth , Scotland. **Chemosphere**, v. 44, p. 383–392, 2001.

BOMBARDELLI, R. A.; MÖRSCHBÄCHER, E. F.; CAMPAGNOLO, R.; SANCHES, E. A.; SYPERRECK, M. A.. Dose inseminante para fertilização artificial de ovócitos de jundiá cinza, Rhamdia quelen (Quoy & Gaimardm, 1824). **Revista Brasileira de Zootecnia**, v. 35, n. 4, p. 1251–1257, 2006.

BORAN, H.; TERZI, S. Bis(2-ethylhexyl) phthalate induces DNA strand breaks and gene expression alterations in larval zebrafish Danio rerio. **Toxicology and Industrial Health**, v. 35, n. 8, p. 520–529, 2019.

BOTELHO, G. G. K.; GOLIN, M.; BUFALO, A. C.; MORAIS, R. N.; DALSENTER, P. R.; MARTINO-ANDRADE, A. J. Reproductive Effects of Di (2-ethylhexyl) phthalate in Immature Male Rats and Its Relation to Cholesterol , Testosterone , and Thyroxin Levels. **Archives of Environmental Contamination and Toxicology**, v. 57, p. 777–784, 2009.

BRADFORD, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizinf the Principle of Protein-Dye Binding. **Analytical Biochemistry**, v. 72, p. 248–254, 1976.

BROMAGE, E. S.; KAATTARI, I. M.; ZWOLLO, P.; KAATTARI, S. L. Plasmablast and Plasma Cell Production and Distribution in Trout Immune Tissues. **The Journal of Immunology**, v. 173, n. 12, p. 7317–7323, 2004.

BUCHELI, T. D.; FENT, K. Induction of Cytochrome P450 as a Biomarker for Environmental Contamination in Aquatic Ecosystems. **Environmental Science and Pollution Research**, v. 25, n. 3, p. 201–268, 1995.

CARRASCO, K. R.; TILBURY, K. L.; MYERS, M. S. Assessment of the Piscine Micronuc eus Test as an in situ. **Canadian Journal of Fisheries and Aquatic Sciences**, v. 47, n. 3, p. 2123–2136, 1990.

ÇAVAŞ, T.; ERGENE-GÖZÜKARA, S. Genotoxicity evaluation of metronidazole using the piscine micronucleus test by acridine orange fluorescent staining. **Environmental Toxicology and Pharmacology**, v. 19, n. 1, p. 107–111, 2005.

CESTARI, M. M.; LEMOS, P. M. M.; RIBEIRO, C. A. O.; COSTA, J. R. M. A.; PELLETIER, E.; FERRARO, M. V. M.; MANTOVANI, M. S.; FENOCCHIO, A. S. 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**, v. 27, n. 2, 2004.

CHEN, X.; XU, S.; TAN, T.; LEE, T. S.; CHENG, S. H.; LEE, F. W. F.; XU, S. J. L.; HO, K. C. Toxicity and Estrogenic Endocrine Disrupting Activity of Phthalates and Their Mixtures. **International Journal of Environmental Research and Public Health**, v. 11, p. 3156–3168, 2014.

CHENG, Z.; NIE, X.; WANG, H.; WONG, M. Risk assessments of human exposure to bioaccessible phthalate esters through market fish consumption. **Environment International**, v. 57–58, p. 75–80, 2013.

CHEON, Y. Di-(2-ethylhexyl) Phthalate (DEHP) and Uterine Histological Characteristics. **Development & Reproduction**, v. 24, n. 1, p. 1–17, 2020.

CHO, Y. S.; CHOI, B. N.; KIM, K. H.; KIM, S. K.; KIM, D. S.; BANG, I. C.; NAM, Y. K. Differential expression of Cu / Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (Oplegnathus fasciatus). **Aquaculture**, v. 253, p. 667–679, 2006.

COLLINS, A. R.; DOBSON, V. L.; DUSINKÁ, M.; KENNEDY, G.; STETINA, R. The comet assay: what can it really tell us? **Mutation Research**, v. 375, p. 183–193, 1997.

COLLINS, A. R.; DUTHIE, S. J.; DOBSON, V. L. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte dna. **Carcinogenesis**, v. 14, n. 9, p. 1733–1735, 1993.

CURI, T. Z.; SILVA, G. N.; PASSONI, M. T.; TOLOUEI, S. E. L.; MELDOLA, H.; ROMANO, R. M.; GRECHI, N.; DALSENTER, P. R.; MARTINO-ANDRADE, A. J. In

Utero and Lactational Exposure to Diisopentyl Phthalate Induces Fetal Toxicity and Antiandrogenic Effects in Rats. **Toxicological Sciences**, v. 171, n. 2, p. 347–358, 2019.

DAVID, R. M.; MOORE, M. R.; CIFONE, M. A.; FINNEY, D. C.; GUEST, D. Chronic Peroxisome Proliferation and Hepatomegaly Associated with the Hepatocellular Tumorigenesis of Di (2-Ethylhexyl) Phthalate and the Effects of Recovery. **Toxicological Sciences**, v. 50, p. 195–205, 1999.

DO NASCIMENTO FILHO, I.; MÜHLEN, C. V.; SCHOSSLER, P.; CARAMÃO, E. B. Identification of some plasticizers compounds in landfill leachate. **Chemosphere**, v. 50, p. 657–663, 2003.

DOMÍNGUEZ-MORUECO, N.; GONZÁLEZ-ALONSO, S.; VALCÁRCEL, Y. Phthalate occurrence in rivers and tap water from central Spain. **Science of the Total Environment**, v. 500–501, p. 139–146, 2014.

DU, L.; LI, G.; LIU, M.; LI, Y.; YIN, S.; ZHAO, J.; ZHANG, X. Evaluation of DNA damage and antioxidant system induced by di-n-butyl phthalates exposure in earthworms (Eisenia fetida). **Ecotoxicology and Environmental Safety**, v. 115, p. 75–82, 2015.

ELLMAN, G. L.; COURTNEY, K. D.; ANDRES JR, V.; FEATHERSTONE, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. **Biochemical Pharmacology**, 1961.

ERKEKOGLU, P.; RACHIDI, W.; DE ROSA, V.; GIRAY, B.; FAVIER, A.; HINCAL, F. Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells. **Free Radical Biology and Medicine**, v. 49, n. 4, p. 559–566, 2010.

ERKEKOGLU, P.; KOCER-GUMUSEL, B. Genotoxicity of phthalates. **Toxicology Mechanisms and Methods**, v. 24, n. 9, p. 616–626, 2014.

ERKMEN, B.; BENLI, A. Ç. K.; ANGUS, H. H.; YILDIRIM, Z.; MERTO, R.; ERKOÇ, F. Impact of sublethal di-n-butyl phthalate on the aquaculture fish species Nile tilapia (Oreochromis niloticus): histopathology and oxidative stress assessment. **Aquaculture Research**, v. 48, n. 2, p. 675–685, 2015.

FERRARO, M. V. M.; FENOCCHIO, A. S.; MANTOVANI, M. S.; RIBEIRO, C. O.; CESTARI, M. M. Mutagenic effects of tributyltin and inorganic 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**, v. 27, n. 1, p. 103–107, 2004.

FERREIRA, D.; MOTTA, A. C.; KREUTZ, L. C.; TONI, C.; LORO, V. L.; BARCELLOS, L. J. G. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. **Chemosphere**, v. 79, n. 9, p. 914–921, 2010.

FERREIRA, I. D.; MORITA, D. M. EX-SITU BIOREMEDIATION OF BRAZILIAN SOIL

CONTAMINATED WITH PLASTICIZERS PROCESS WASTES. **Brazilian Journal of Chemical Engineering**, v. 29, n. 01, p. 77–86, 2012.

FRENZILLI, G.; NIGRO, M.; LYONS, B. P. The Comet assay for the evaluation of genotoxic impact in aquatic environments. **Mutation Research - Reviews in Mutation Research**, v. 681, n. 1, p. 80–92, 2009.

GAO, D. W.; WEN, Z. D. Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. **Science of the Total Environment**, v. 541, p. 986–1001, 2016.

GAO, R.; YUAN, Z.; ZHAO, Z.; GAO, X. Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. **Bioelectrochemistry and Bioenergetics**, v. 45, n. 1, p. 41–45, 1998.

GAO, X.; LI, J.; WANG, X.; ZHOU, J.; FAN, B.; LI, W.; LIU, Z. Exposure and ecological risk of phthalate esters in the Taihu Lake basin, China. **Ecotoxicology and Environmental Safety**, v. 171, n. December 2018, p. 564–570, 2019.

GHELFI, A.; RIBAS, J. L. C.; GUILOSKI, I. C.; BETTIM, F. L.; PIANCINI, L. D. S.; CESTARI, M. M.; PEREIRA, A. J.; SASSAKI, G. L.; ASSIS, H. C. S. Evaluation of Biochemical , Genetic and Hematological Biomarkers in a Commercial Catfish Rhamdia quelen Exposed to Diclofenac. **Bulletin of Environmental Contamination and Toxicology**, v. 96, p. 49–54, 2016.

GHISI, N. D. C.; RAMSDORF, W. A.; FERRARO, M. V. M.; ALMEIDA, M. I. M.; RIBEIRO, C. A. O.; CESTARI, M. M. Evaluation of genotoxicity in Rhamdia quelen (Pisces, Siluriformes) after sub-chronic contamination with Fipronil. **Environmental Monitoring and Assessment**, v. 180, n. 1–4, p. 589–599, 2011.

GHORPADE, N.; MEHTA, V.; KHARE, M.; SINKAR, P.; KRISHNAN, S.; RAO, C. V. Toxicity Study of Diethyl Phthalate on Freshwater Fish Cirrhina mrigala. **Ecotoxicology and Environmental Safety**, v. 53, p. 255–258, 2002.

GOBAS, F. A. P. C.; IKONOMOU, M. Bioaccumulation of Phthalate Esters in Aquatic Food-Webs. v. 3, p. 201–225, 2003.

GOKSØYR, A.; ANDERSSON, T.; BUHLER, D. R.; STEGEMAN, J. J.; WILLIAMS, D. E.; FÖRLIN, L. Immunochemical cross-reactivity of β-naphthoflavone-inducible cytochrome P450 (P450IA) in liver microsomes from different fish species and rat. **Fish Physiology and Biochemistry**, v. 9, n. 1, p. 1–13, 1991.

GOMES, L. DE C.; GOLOMBIESKI, J. I.; GOMES, A. R. C.; BALDISSEROTTO, B. Biologia do Jundiá Rhamdia quelen (TELEOSTEI, PIMELODIDAE). **Ciência Rural**, v. 30, n. 1, p. 179–185, 2000.

GONTIJO, Á. M. D. M. C.; BARRETO, R. E.; SPEIT, G.; REYES, V. A. V.; VOLPATO, G. L.; SALVADORI, D. M. F. Anesthesia of fish with benzocaine does not interfere with comet assay results. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 534, n. 1–2, p. 165–172, 2003.

GOUTTE, A.; ALLIOT, F.; BUDZINSKI, H.; SIMONNET-LAPRADE, C.; SANTOS, R.; LACHAUX, V.; MACIEJEWSKI, K.; MENACH, K. L.; LABADIE, P. Trophic Transfer of Micropollutants and Their Metabolites in an Urban Riverine Food Web. **Environmental Science and Technology**, v. 54, n. 13, p. 8043–8050, 2020.

GRASSI MILANO, E.; BASARI, F.; CHIMENTI, C. Adrenocortical and adrenomedullary homologs in eight species of adult and developing teleosts: Morphology, histology, and immunohistochemistry. **General and Comparative Endocrinology**, v. 108, n. 3, p. 483–496, 1997.

GUILOSKI, I. C.; PIANCINI, L. D. S.; DAGOSTIM, A. C.; CALADO, S.; L.; M.; FÁVARO, L. F.; BOSCHEN, S. L.; CESTARI, M. M.; CUNHA, C.; ASSIS, H. C. S. Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish Rhamdia quelen. **Ecotoxicology and Environmental Safety**, v. 139, n. December 2016, p. 291–300, 2017a.

GUILOSKI, I. C.; RIBAS, J. L. C.; PIANCINI, L. D. S.; DAGOSTIM, A. C.; CIRIO, S. M.; FÁVARO, L. F.; BOSCHEN, S. L.; CESTARI, M. M., CUNHA, C.; ASSIS, H. C. S. Paracetamol causes endocrine disruption and hepatotoxicity in male fish Rhamdia quelen after subchronic exposure. **Environmental Toxicology and Pharmacology**, v. 53, p. 111–120, 2017b.

GUSTAVINO, B.; SCORNAJENGHI, K. A.; MINISSI, S.; CICCOTTI, E. Micronuclei induced in erythrocytes of Cyprinus carpio (teleostei , pisces) by X-rays and colchicine. **Genetic Toxicology and Environmental Mutagenesis**, v. 494, p. 151–159, 2001.

GUTTERIDGE, J. M. C.; HALLIWELL, B. Free Radicals and Antioxidants in the Year 2000 A Historical Look to the Future. **Annals of the New York Academy of Sciences**, v. 899, p. 136–147, 2000.

HAI, D. Q.; VARGA, I. S.; MATKOVICS, B. Effects of an organophosphate on the antioxidant systems of fish tissues. **Acta biologica Hungarica**, 1995.
HEDDLE, J. A. A rapid in vivo test for chromosomal damage. **Mutation Research**, v. 18, p. 187–190, 1973.

HEUDORF, U.; MERSCH-SUNDERMANN, V.; ANGERER, J. Phthalates : Toxicology and exposure. **International Journal of Hygiene and Environmental Health**, v. 210, p. 623–634, 2007.

HOOFTMAN, R. N.; DE RAAT, W. K. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow Umbra pygmaea by ethyl methanesulphonate. **Mutation Research Letters**, 1982.

HU, T.; FU, Q.; LIU, X.; ZHANG, H.; DONG, M. Increased acetylcholinesterase and capase-3 expression in the brain and peripheral immune system of focal cerebral ischemic rats. **Journal of Neuroimmunology**, v. 211, n. 1–2, p. 84–91, 2009.

HU, X.; GU, Y.; HUANG, W.; YIN, D. Phthalate monoesters as markers of phthalate

contamination in wild marine organisms. **Environmental Pollution**, v. 218, p. 410–418, 2016.

JARMOŁOWICZ, S.; DEMSKA-ZAKĘŚ, K.; ZAKĘŚ, Z. Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (Sander lucioperca) Phthalates , which are classified as endocrine disruptor compounds (EDCs), were first created in the 1920s for commercial applications , and th. **Acta Veterinaria Brno**, v. 82, p. 197–201, 2013.

JEE, J. H.; KOO, J. G.; KEUM, Y. H.; PARK, K. H.; CHOI, S. H.; KANG, J. C. Effects of dibutyl phthalate and di-ethylhexyl phthalate on acetylcholinesterase activity in bagrid catfish, Pseudobagrus fulvidraco (Richardson). **Journal of Applied Ichthyology**, v. 25, n. 6, p. 771–775, 2009.

JIAN GE; LI, M.; LIN, F.; ZHAO, J.; HAN, B. Study on metabolism of N-Butyl Benzyl Phthalate (BBP) and Dibutyl Phthalate (DBP) in Ctenopharyngodon idellus by GC and LC-MS/MS. **African Journal of Agricultural Research**, v. 7, n. 12, p. 1855–1862, 2012.

JIANG, Z.; HUNT, J. V; WOLFF, S. P. Ferrous Ion Oxidation in the Presence of Xylenol Orange for Detection of Lipid Hydroperoxide in Low Density Lipoprotein. **Analytical Biochemistry**, v. 202, n. 2, p. 384–389, 1992.

JIAO, Y.; TAO, Y.; YANG, Y.; DIOGENE, T.; YU, H.; HE, Z.; HAN, W.; CHEN, Z.; WU, P.; ZHANG, Y. Monobutyl phthalate (MBP) can dysregulate the antioxidant system and induce apoptosis of zebrafish liver. **Environmental Pollution**, v. 257, p. 113517, 2020.

KANG, J.; JEE, J. H.; KOO, J.; KEUM, Y.; JO, S.; PARK, K. H. Anti-oxidative status and hepatic enzymes following acute administration of diethyl phthalate in olive flounder Paralichthys olivaceus , a marine culture fish. **Ecotoxicology and Environmental Safety**, v. 73, n. 6, p. 1449–1455, 2010.

KAVITHA, P.; RAO, J. V. Oxidative stress and locomotor behaviour response as biomarkers for assessing recovery status of mosquito fish, Gambusia affinis after lethal effect of an organophosphate pesticide , monocrotophos. **Pesticide Biochemistry and Physiology**, v. 87, p. 182–188, 2007.

KEEN, J. H.; HABIG, W. H.; JAKOBY, W. B. Mechanism for the Several Activities of the Glutathione. **The Journal of Biological Chemistry**, v. 251, n. 20, p. 6183–6188, 1976.

KHALIL, S. R.; ELHAKIM, Y. A.; EL-MURR, A. E. Sublethal concentrations of di-n-butyl phthalate promote biochemical changes and DNA damage in juvenile Nile tilapia (Oreochromis niloticus). **Japanese Journal of Veterinary Research**, v. 64, n. 1, p. 67–80, 2016.

KIMBER, I.; DEARMAN, R. J. An assessment of the ability of phthalates to influence immune and allergic responses. **Toxicology**, v. 271, n. 3, p. 73–82, 2010.

KLEINSASSER, N. H.; KASTENBAUER, E. R.; WEISSACHER, H.; MUENZENRIEDER, R. K.; HARRÉUS, U. A. Phthalates Demonstrate Genotoxicity on Human Mucosa of the Upper Aerodigestive Tract. **Environmental and Molecular Mutagenesis**, v. 35, p. 9–12, 2000a.

KLEINSASSER, N. H.; WALLNER, B. C.; KASTENBAUER, E. R.; MUENZENRIEDER, R. K.; HARRÉUS, U. A. Comparing the genotoxic sensitivities of human peripheral blood lymphocytes and mucosa cells of the upper aerodigestive tract using the Comet assay. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 467, n. 1, p. 21–30, 2000b.

KLEINSASSER, N. H.; WALLNER, B. C.; KASTENBAUER, E. R.; WEISSACHER, H.; HARRÉUS, U. A. Genotoxicity of Di-Butyl-Phthalate and Di-Iso-Butyl-Phthalate in Human Lymphocytes and Mucosal Cells. **Teratogenesis, Carcinogenesis, and Mutagenesis**, v. 21, p. 189–196, 2001.

KLEMZ, C.; SILVA DE ASSIS, H. C. EFEITOS DO ENDOSULFANO NA ATIVIDADE DA ACETILCOLINESTERASE DE CASCUDO (Ancistrus multispinnis, FISH, TELEOSTEI). **Revista Acadêmica: Ciência Animal**, v. 3, n. 4, p. 51, 2017.

KLINGELFUS, T.; COSTA, P. M.; SCHERER, M.; CESTARI, M. M. DNA damage in the kidney tissue cells of the fish rhamdia quelen after trophic contamination with aluminum sulfate. **Genetics and Molecular Biology**, v. 38, n. 4, p. 499–506, 2015.

KLINGELFUS, T.; LIROLA, J. R.; OYA-SILVA, L. F.; DISNER, G. R.; VICENTINI, M.; NADALINE, M. J. B.; ROBLES, J. C. Z.; TREIN, L. M.; VOIGT, C. L.; SILVA DE ASSIS, H. C.; MELA, M.; LEME, D. D.; CESTARI, M. M. Acute and long-term effects of trophic exposure to silver nanospheres in the central nervous system of a neotropical fish Hoplias intermedius. **NeuroToxicology**, 2017.

KOO, H. J.; LEE, B. M. Estimated exposure to phthalates in cosmetics and risk assessment. **Journal of Toxicology and Environmental Health, Part A**, 2010.

KOPPEN, G.; AZQUETA, A.; POURRUT, B.; BRUNBORG, G.; COLLINS, A. R.; LANGIE, S. A. S. The next three decades of the comet assay: A report of the 11th International Comet Assay Workshop. **Mutagenesis**, v. 32, n. 3, p. 397–408, 2017.

KWOK, K. W. H.; LEUNG, K. M. Y.; LUI, G. S. G.; CHU, V. K. H.; LAM, P. K. S.; MORRITT, D.; MALTBY, L.; BROCK, T. C. M.; BRINK, P. J. V.; WARNE, M. ST.; CRANE, M. Comparison of Tropical and Temperate Freshwater Animal Species' Acute Sensitivities to Chemicals: Implications for Deriving Safe Extrapolation Factors. **Integrated Envoronmental Assessment and Management**, v. 3, n. 1, p. 49–67, 2007.

LATIF, M.; FAHEEM, M.; ASMATULLAH. Study of Oxidative Stress and Histo-Biochemical Biomarkers of Diethyl Phthalate Induced Toxicity in a Cultureable Fish, Labeo rohita. **Pakistan Veterinary Journal**, v. 40, n. 02, p. 202–208, 2020.

LIU, H.; LIANG, H.; LIANG, Y.; ZHANG, D.; WANG, C.; CAI, H.; SHVARTSEV, S. L. Distribution of phthalate esters in alluvial sediment: A case study at JiangHan Plain,

Central China. **Chemosphere**, v. 78, n. 4, p. 382–388, 2010.

LUZIA, M. R. F.; SARROUH, B.; LOFRANO, R. C. Z. Evaluation of the biodegradation of phthalic contaminants originated from polyvinyl chloride commercial films. **Acta Scientiarum - Technology**, v. 42, n. 1, p. 1–10, 2020.

MANKIDY, R.; WISEMAN, S.; MA, H.; GIESY, J. P. Biological impact of phthalates. **Toxicology Letters**, v. 217, n. 1, p. 50–58, 2013.

MARCHIORO, M. I.; BALDISSEROTTO, B. Sobrevivência de alevinos de Jundiá (Rhamdia quelen Quoy & Gaimard, 1824) à variação de salinidade da água. **Ciência Rural**, v. 29, n. 2, p. 315–318, 1999.

MARIONNET, D.; DESCHAUX, P.; REYNAUD, S. Possible implication of macrophages in the regulation of cytochrome P450 activities in carp (Cyprinus carpio). **Fish and Shellfish Immunology**, v. 21, p. 80–91, 2006.

MARTINO-ANDRADE, A. J.; MORIS, R. N.; BOTELHO, G. G. K.; MULLER, G.; GRANDE, S. W.; CARPENTIERI, G. B.; LEÃO, G. M. C.; DALSENTER, P. R. Coadministration of active phthalates results in disruption of foetal testicular function in rats. **International Journal of Andrology**, v. 32, p. 704–712, 2009.

MARTINO-ANDRADE, A. J.; CHAHOUD, I. Reproductive toxicity of phthalate esters. **Molecular Nutrition and Food Research**, v. 54, p. 148–157, 2010.

MARTINS, K.; APPLEGATE, B.; HAGEDORN, B.; KENNISH, J.; ZWOLLO, P. Di(2-ethylhexyl) phthalate inhibits B cell proliferation and reduces the abundance of IgM-secreting cells in cultured immune tissues of the rainbow trout. **Fish and Shellfish Immunology**, v. 44, n. 1, p. 332–341, 2015.

MARTINS, K.; HAGEDORN, B.; ALI, S.; KENNISH, J.; APPLEGATE, B.; LEU, M.; EPP, L.; PALLISTER, C.; ZWOLLO, P. Tissue Phthalate Levels Correlate With Changes in Immune Gene Expression in a Population of Juvenile Wild Salmon. **Archives of Environmental Contamination and Toxicology**, v. 71, n. 1, p. 35–47, 2016.

MATHIAS, F. T.; FOCKINK, D. H.; DISNER, G. R.; PRODOCIMO, V.; RIBAS, J. L. C.; RAMOS, L. P.; CESTARI, M. M.; SILVA DE ASSIS, H. C. Effects of low concentrations of ibuprofen on freshwater fish Rhamdia quelen. **Environmental Toxicology and Pharmacology**, v. 59, n. 2010, p. 105–113, 2018.

MEAGHER, E. A.; FITZGERALD, G. A. INDICES OF LIPID PEROXIDATION IN VIVO: STRENGTHS AND LIMITATIONS. **Free Radical Biology and Medicine**, v. 28, n. 12, p. 1745–1750, 2000.

MINISSI, S.; CICCOTTI, E.; RIZZONI, M. Micronucleus test in erythrocytes of Barbus plebejus (Teleostei , Pisces) from two natural environments : a bioassay for the in situ detection of mutagens in freshwater. **Genetic Toxicology**, v. 367, p. 245–251, 1996.

MONTAGNER, C. C.; SODRÉ, F. F.; ACAYABA, R. D.; VIDAL, C.; CAMPESTRINI, I.; LOCATELLI, M. A.; PESCARA, I. C.; ALBUQUERQUE, A. F.; UMBUZEIRO, G. A.; JARDIM, W. F. Ten Years-Snapshot of the Occurrence of Emerging Contaminants in Drinking, Surface and Ground Waters and Wastewaters from São Paulo State, Brazil. **Journal of the Brazilian Chemical Society**, v. 30, n. 3, p. 614–632, 2019.

MONTAGNER, C. C.; JARDIM, W. F. Spatial and seasonal variations of pharmaceuticals and endocrine disruptors in the Atibaia River, São Paulo State (Brazil). **Journal of the Brazilian Chemical Society**, v. 22, n. 8, p. 1452–1462, 2011.

MURSALIN; HARIYADI, P.; PURNOMO, E. H.; ANDARWULAN, N.; FARDIZ, D. Crystallization kinetics of coconut oil based on Avrami model. **International Food Research Journal**, v. 23, n. 4, p. 1355–1360, 2016.

NAGORKA, R.; KOSCHORRECK, J. Trends for plasticizers in German freshwater environments – Evidence for the substitution of DEHP with emerging phthalate and non-phthalate alternatives. **Environmental Pollution**, v. 262, 2020.

NELSON, J. S.; GRANDE, T. C.; WILSON, M. V. H. **Fishes of the World: Fifth Edition**, v 71, p 35-47, 2016.

NET, S.; SEMPÉRÉ, R.; DELMONT, A.; PALUSELLI, A.; OUDDANE, B. Occurrence, Fate, Behavior and Ecotoxicological State of Phthalates in Di ff erent Environmental Matrices. **Environmental Science & Technology**, v. 49, p. 4019–4035, 2015.

NEUBERT DA SILVA, G.; CURI, T. Z.; TOLOUEI, S. R. L.; PASSONI, M. T.; HEY, G. B. S.; ROMANO, R. M.; MARTINO-ANDRADE, A. J.; DALSENTER, P. R. Effects of diisopentyl phthalate exposure during gestation and lactation on hormone-dependent behaviours and hormone receptor expression in rats. **Journal of Neuroendocrinology**, v. 31, n. 12, p. 1–12, 2019.

NIKINMAA, M. Environmental regulation of the function of circulating erythrocytes via changes in age distribution in teleost fish: Possible mechanisms and significance. **Marine Genomics**, v. 49, n. July, p. 100717, 2020.

O'BRIEN, M. L.; SPEAR, B. T.; GLAUERT, H. P. Role of Oxidative Stress in Peroxisome Carcinogenesis Proliferator-mediated. **Critical Reviews in Toxicology**, v. 35, p. 61–88, 2005.

OLIVE, P. L.; BANÁTH, J. P.; DURAND, R. E. DNA Damage and Repair in Tumor Heterogeneity in Radiation-Induced and Normal Cells Measured Using the "Comet" Assay. **Radiation Research**, v. 122, p. 86–94, 1990.

OLIVEIRA-JÚNIOR, A. A.; TAVARES-DIAS, M.; MARCON, J. L. Biochemical and hematological reference ranges for Amazon freshwater turtle, Podocnemis expansa (Reptilia: Pelomedusidae), with morphologic assessment of blood cells. **Research in Veterinary Science**, v. 86, n. 1, p. 146–151, 2009.

PAGLIA, D. E.; VALENTINE, W. N. Studies on the quantitative and qualitative

characterization of erythrocyte glutathione peroxidase. **The Journal of Laboratory and Clinical Medicine**, v. 70, p. 158–169, 1967a.

PAGLIA, D.; VALENTINE, W. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. **Journal Laboratory Clinical Medicine**, v. 70, p. 158–169, 1967b.

PANDRANGI, R.; PETRAS, M.; RALPH, S.; VRZOC, M. Alkaline Single Cell Gel (Comet) Assay and Genotoxicity Monitoring Using Bullheads and Carp. **Environmental and Molecular Mutagenesis**, v. 26, p. 345–356, 1995.

PARK, C. G.; SUNG, B.; RYU, C. S.; KIM, Y. J. Mono-(2-ethylhexyl) phthalate induces oxidative stress and lipid accumulation in zebrafish liver cells. **Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology**, v. 230, 2020.

PAYNE, J. F.; FANCEY, L. L.; RAHIMTULA, A. D.; PORTER, E.L. **Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoringComparative Biochemistry and Physiology. Part C, Comparative**, v. 86, n 2, p. 233-245, 1987.

PEIJNENBURG, W. J. G. M.; STRUIJS, J. Occurrence of phthalate esters in the environment of the Netherlands. **Ecotoxicology and Environmental Safety**, v. 63, n. 2, p. 204–215, 2006.

PEREIRA, L. S.; RIBAS, J. L. C.; VICARI, T.; SILVA, S. B.; STIVAL, J.; BALDAN, A. P.; VALDEZ DOMINGOS, F. X.; GRASSI, M. T.; CESTARI, M. M.; SILCA DE ASSIS, H. C. Effects of ecologically relevant concentrations of cadmium in a freshwater fish. **Ecotoxicology and Environmental Safety**, v. 130, p. 29–36, 2016.

PÉREZ, E. R.; CARDOSO, D. R.; FRANCO, D. W. Analysis of the alcohols, esters and carbonyl compounds in fusel oil samples. **Quimica Nova**, v. 24, n. 1, p. 10–12, 2001.

PERUSSOLO, M. C.; GUILOSKI, I. C.; LIROLA, J. R.; FOCKINK, D. .; CORSO, C. R.; BOZZA, D. C.; PRODOCIMO, V.; MELA, M.; RAMOS, L. P.; CESTARI, M. M.; ACCO, A.; SILVA DE ASSIS, H. C. Integrated biomarker response index to assess toxic effects of environmentally relevant concentrations of paracetamol in a neotropical catfish (Rhamdia quelen). **Ecotoxicology and Environmental Safety**, v, 182, 2019.

PIANCINI, L. D. S.; SANTOS, G. S.; TINCANI, F. H.; CESTARI, M. M. Piscine micronucleus test and the comet assay reveal genotoxic effects of Atrazine herbicide in the neotropical fish Rhamdia quelen. **Ecotoxicology and Environmental Contamination**, 2015a.

PIANCINI, L. D. S.; GUILOSKI, I. C.; SILVA DE ASSIS, H. C.; CESTARI, M. M. Mesotrione herbicide promotes biochemical changes and DNA damage in two fish species. **Toxicology Reports**, v. 2, p. 1157–1163, 2015b.

POOPAL, R. K.; RAMESH, M.; MARUTHAPPAN, V.; RAJENDRAN, R. B. Potential effects of low molecular weight phthalate esters (C16H22O4 and C12H14O4) on the freshwater fish: Cyprinus carpio. **Toxicology Research**, v. 6, n. 4, p. 505–520, 2017.

QU, R.; FENG, M.; SUN, P.; WANG, Z. A Comparative Study on Antioxidant Status Combined with Integrated Biomarker Response in Carassius auratus Fish Exposed to Nine Phthalates. **Environmental Toxicology**, v. 30, n. 10, p. 1125–1134, 2014.

RAMSDORF, W. A.; FERRARO, M. V. M.; OLIVEIRA-RIBEIRO, C.A.; COSTA, J. R. M.; CESTARI, M. M. Genotoxic evaluation of different doses of inorganic lead (PbII) in Hoplias malabaricus. **Environmental Monitoring and Assessment**, 2009.

RAMZI, A.; GIREESHKUMAR, T. R.; RAHMAN, K. H.; BALACHANDRAN, K. K.; SHAMEEM, K.; CHACKO, J.; CHANDRAMOHANAKUMAR, N. Phthalic acid esters – A grave ecological hazard in Cochin estuary, India. **Marine Pollution Bulletin**, v. 152, 2020.

REEVES, J. F.; DAVIES, S. J.; DOOD, N. J. F.; JHA, A. N. Hydroxyl radicals (OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. **Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis**, v. 640, n. 1–2, p. 113–122, 2008.

RHODES, J. E.; ADAMS, W. J.; BIDDINGER, G. R.; ROBILLARD, K. A.; GORSUCH, J. W. Chronic toxicity of 14 phthalate esters to Daphnia magna and rainbow trout (Oncorhynchus mykiss). **Environmental Toxicology and Chemistry**, v. 14, n. 11, p. 1967–1976, 1995.

ROCHA, B. A.; ASIMAKOPOULOS, A. G.; BARBORA JR, F.; KANNAN, K. Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage. **Science of the Total Environment**, v. 586, p. 152–162, 2017.

ROJAS, E.; LOPEZ, M. C.; VALVERDE, M. Single cell gel electrophoresis assay : methodology and applications. **Journal of Chromatography b**, v. 722, p. 225–254, 1999.

ROMERO-FRANCO, M.; HERNÁNDEZ-RAMÍREZ, R. U.; CALAFAT, A. M.; CEBRIÁN, M. E.; NEEDHAM, L. L.; TEITELBAUM, S.; WOLFF, M. S.; LÓPEZ-CARRILLO, L. Personal care product use and urinary levels of phthalate metabolites in Mexican women. **Environment International**, v. 37, p. 867–871, 2011.

RUSYN, I.; PETERS, J. M.; CUNNINGHAM, M. L. Modes of Action and Species-Specific Effects of Di- (2-ethylhexyl) Phthalate in the Liver. **Critical Reviews in Toxicology**, v. 36, p. 459–479, 2006.

SARAH, J. M.; SHANE, J. W.; LANGLOIS, V. S. Influence of Lipophilicity on the Toxicity of Bisphenol A and Phthalates to Aquatic Organisms. **Bulletin of Environmental Contamination and Toxicology**, v. 97, n. 1, p. 4–10, 2016.

SCHETTLER, T. Human exposure to phthalates via consumer products.

International Journal of Andrology, v. 29, p. 134–139, 2006.

SCHMID, W. The micronucleus test. **Mutation Research**, v. 31, p. 9–15, 1975.
SEDLAK, J.; LINDSAY, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. **Analytical Biochemistry**, v. 25, p. 192–205, 1968.

SELVARAJ, K. K.; SUNDARAMOORTHY, G.; RAVICHANDRAN, P. K.; GIRIJAN, G. K.; SAMPATH, S.; RAMASWAMY, B. R. Phthalate esters in water and sediments of the Kaveri River, India: environmental levels and ecotoxicological evaluations. **Environmental Geochemistry and Health**, v. 37, n. 1, p. 83–96, 2014.

SEPPERUMAL, U.; SAMINATHAN, S. Effect of diethylphthalate on the haematological parameters of the freshwater fish Oreochromismossambicus (Tilapia). **European Journal of Zoological Research**, v. 2, n. 4, p. 55–59, 2013.

SHEN, C.; WEI, J.; WANG, T.; WANG, Y. Acute toxicity and responses of antioxidant systems to dibutyl phthalate in neonate and adult Daphnia magna. **PeerJ**, v. 2019, n. 3, 2019.

SINGH, N. P.; MACCOY, M. T.; TICE, R. R.; SCHNEIDER, W. L. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells'. **Experimental Cell Research**, v. 175, p. 184–191, 1988.

SOLDATOV, A. A. Peculiarities of organization and functioning of the fish red blood system. **Journal of Evolutionary Biochemistry and Physiology**, v. 41, n. 3, p. 272–281, 2005.

SPEIT, G.; HARTMANN, A. The Comet Assay (Single-Cell Gel Test) A Sensitive Genotoxicity Test for the Detection of DNA Damage and Repair. **Methods in Molecular Biology**, v. 113, n. DNA Repair Protocols: Eukaryotic Systems, p. 203–212, 1999.

STAPLES, C. A.; PETERSON, D. R.; PARKERTON, T. F.; ADAMS, W. J. The environmental fate of phthalate esters: a literature review. **Chemosphere**, v. 35, n. 4, p. 667–749, 1997a.

STAPLES, C. A.; ADAMS, W. J.; PARKERTON, T. F.; GORSUCH, J. W.; BIDDINGER, G. R.; REINERT, K. H. Aquatic of toxicity of eighteen phthalates esters. **Environmental Toxicology and Chemistry**, v. 16, n. 5, p. 875–891, 1997b.

STAPLES, C. A.; PARKERTON, T. F.; PETERSON, D. R. A risk assessment of selected phthalate esters in North American and Western European surface waters. **Chemosphere**, v. 40, n. 8, p. 885–891, 2000.

STEGEMAN, J. J.; MARIUS, B.; GIULIO, R. T.; LARS, F.; FOWLER, B. A.; SANDERS, B. M.; VELD, P. A. V. Molecular responses to environmental contamination: Enzyme and protein systems as indicators of chemical exposure and effect. In: **Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress**. 1. ed. CRC Press, 2018.

SUN, J.; HUANG, J.; ZHANG, A.; LIU, W.; CHENG, W. Occurrence of phthalate esters in sediments in Qiantang River, China and inference with urbanization and river flow regime. **Journal of Hazardous Materials**, v. 248–249, n. 1, p. 142–149, 2013.

SWAN, S. H. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. **Environmental Research**, v. 108, n. 2, p. 177–184, 2008.

TAVARES-DIAS, M.; MORAES, F. R. DE. Hematological parameters for the Brycon orbignyanus Valenciennes, 1850 (Osteichthyes: Characidae) intensively bred. **Hidrobiológica**, v. 16, n. 3, p. 271–274, 2006.

TEBAS, S. DE O. G.; BARAÑANO, A. G.; PINHEIRO, P. F.; LACERDA, V. Mild conditions in the transesterification reaction of babassu oil and purified fusel oil. **Chemical Engineering Communications**, 2020.

TICE, R. R.; AGURELL, E.; ANDERSON, D.; BURLINSON, B.; HARTMANN, A.; KOBAYASHI, H.; MIYAMAE, Y.; ROJAS, E.; RYU, J. C.; SASAKI, Y. F. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing. **Environmental and Molecular Mutagenesis**, v. 35, n. 3, p. 206–221, 2000.

TIWARI, M.; SAHU, S. K.; PANDIT, G. G. Environmental distribution and ecotoxicological concerns of phthalic acid esters in creek ecosystem. **Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering**, v. 54, n. 4, p. 328–336, 2019.

U.S.EPA. Preliminary Materials for the Integrated Risk Information System (IRIS) Toxicological Review of Butyl Benzyl Phthalate (BBP) (CASRN 85-68-7). **U.S. Environmental Protection Agency, Washington, DC, EPA/635/R-13/302**, p. 1–215, 2015.

UDROIU, I. The micronucleus test in piscine erythrocytes. **Aquatic Toxicology**, v. 79, p. 201–204, 2006.

VAN DER OOST, R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environmental Toxicology and Pharmacology**, v. 13, p. 57–149, 2003.

VANDENBERG, L. N.; COLBORN, T.; HAYES, T. B.; HEINDEL, J. J.; JACOBS, D. R.; LEE, D.; SHIODA, T.; SOTO, A. M.; VOM SAAL, F. S.; WELSHONS, W. V.; ZOELLER, R. T.; MUERS, J. P. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. **Endocrine Revews**, v. 33, n. 3, p. 378–455, 2012.

VETHAAK, A. D.; LAHR, J.; SCHRAP, S. M.; BELFROID, A. C.; RIJS, G. B. J.; GERRITSEN, A.; BOER, J.; BULDER, A. S.; GRINWIS, G. C. M.; KUIPER, R. V.; LEGLER, J.; MURK, T. A. J.; PEIJNENBURG, W.; VERHAAR, H. J. M.; VOOGT, P. An integrated assessment of estrogenic contamination and biological effects in the

aquatic environment of The Netherlands. **Chemosphere**, v. 59, p. 511–524, 2005.

WANG, J.; BO, L.; LI, L.; WANG, D.; CHEN, G.; CHRISTIE, P.; TENG, Y. Occurrence of phthalate esters in river sediments in areas with different land use patterns. **Science of the Total Environment**, v. 500–501, p. 113–119, 2014.

WANG, T.; WEI, J.; WANG, L.; LU, W.; ZHANG, Q.; WANG, Y. Effect of Dibutyl phthalate on antioxidant parameters and related gene expression in Daphnia magna. **IOP Conference Series: Earth and Environmental Science**, v. 227, n. 5, 2019.

WANG, Y.; CHEN, H.; LONG, C.; TSAI, C.; HSIEH, T.; HSU, C.; TSAI, E. Possible mechanism of phthalates-induced tumorigenesis. **Kaohsiung Journal of Medical Sciences**, v. 28, p. S22–S27, 2012.

WANG, Y.; WANG, T.; BAN., Y.; SHEN, C.; SHEN, Q.; CHAI, X.; ZHAO, W.; WEI, J. Di-(2-ethylhexyl) Phthalate Exposure Modulates Antioxidant Enzyme Activity and Gene Expression in Juvenile and Adult Daphnia magna. **Archives of Environmental Contamination and Toxicology**, v. 75, n. 1, p. 145–156, 2018.

WITORSCH, R. J.; THOMAS, J. A. Personal care products and endocrine disruption : A critical review of the literature. **Critical Reviews in Toxicology**, v. 40, n. s3, p. 1–30, 2010.

WITTASSEK, M.; KOCH, H. M.; ANGERER, J.; BRÜNING, T. Assessing exposure to phthalates – The human biomonitoring approach. **Molecular Nutrition and Food Research**, v. 55, p. 7–31, 2011.

XU, H.; SHAO, X.; ZHANG, Z.; ZOU, Y.; WU, X.; YANG, L. Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in zebrafish embryos. **Ecotoxicology and Environmental Safety**, v. 93, p. 39–44, 2013a.

XU, H.; SHAO, X.; ZHANG, Z.; ZOU, Y.; CHEN, Y.; HAN, S.; WANG, S.; WU, X.; YANG, L.; CHEN, Z. Effects of di-n-butyl phthalate and diethyl phthalate on acetylcholinesterase activity and neurotoxicity related gene expression in embryonic zebrafish. **Bulletin of Environmental Contamination and Toxicology**, v. 91, n. 6, p. 635–639, 2013b.

XU, H.; DONG, X.; ZHANG, Z.; YANG, M.; WU, X.; LIU, H.; LAO, Q.; LI, C. Assessment of immunotoxicity of dibutyl phthalate using live zebrafish embryos. **Fish and Shellfish Immunology**, v. 45, n. 2, p. 286–292, 2015.

XU, S.; ZHANG, H.; PAO, P.; LEE, A.; WANG, J.; CHAN, Y. S.; MANOO III, F. A. M.; CHAN, S. W.; CHENG, S. H.; CHEN, X. Exposure to phthalates impaired neurodevelopment through estrogenic effects and induced DNA damage in neurons. **Aquatic Toxicology**, v. 222, p. 105469, 2020.

YANG, W. K.; CHIANG, L.; TAI, S.; CHEN, P. Environmentally relevant concentrations of di(2-ethylhexyl)phthalate exposure alter larval growth and locomotion in medaka fish via multiple pathways. **Science of the Total**

Environment, v. 640–641, p. 512–522, 2018.

ZAPATA, A. Ultrastructural study of the teleost fish kidney. **Developmental and Comparative Immunology**, v. 3, n. C, p. 55–65, 1979.

ZHANG, X. J.; YANG, L.; ZHAO, Q.; CAEN, J. P.; HE, H. Y.; JIN, Q. H.; GUO, L. H.; ALEMANY, M.; ZHANG, L. Y.; SHI, Y. F. Induction of acetylcholinesterase expression during apoptosis in various cell types. **Cell Death and Differentiation**, v. 9, n. 8, p. 790–800, 2002.

ZHANG, X. J.; GREENBERG, D. S. Acetylcholinesterase involvement in apoptosis. **Frontiers in Molecular Neuroscience**, v. 5, n. MARCH, p. 1–15, 2012.

ZHENG, Q.; FENG, M.; DAI, Y. Comparative antioxidant responses in liver of Carassius auratus exposed to phthalates: An integrated biomarker approach. **Environmental Toxicology and Pharmacology**, v. 36, p. 741–749, 2013.

ZWOLLO, P.; COLE, S.; BROMAGE, E.; KAATTARI, S. B Cell Heterogeneity in the Teleost Kidney: Evidence for a Maturation Gradient from Anterior to Posterior Kidney. **The Journal of Immunology**, v. 174, n. 11, p. 6608–6616, 2005.

ANEXO

Supplementary Material

Supplementary Table 1 - Hematological parameters of the control groups of *Rhamdia quelen*.

Treatments	Parameters		
	Erythrocytes10^6 /mL	Leukocytes /mL	Thrombocytes /mL
CFC	2.45±0.83	22.73±10.16	57.68±39.33
NC	2.64±0.64	33.36±16.59	60.43±28.07

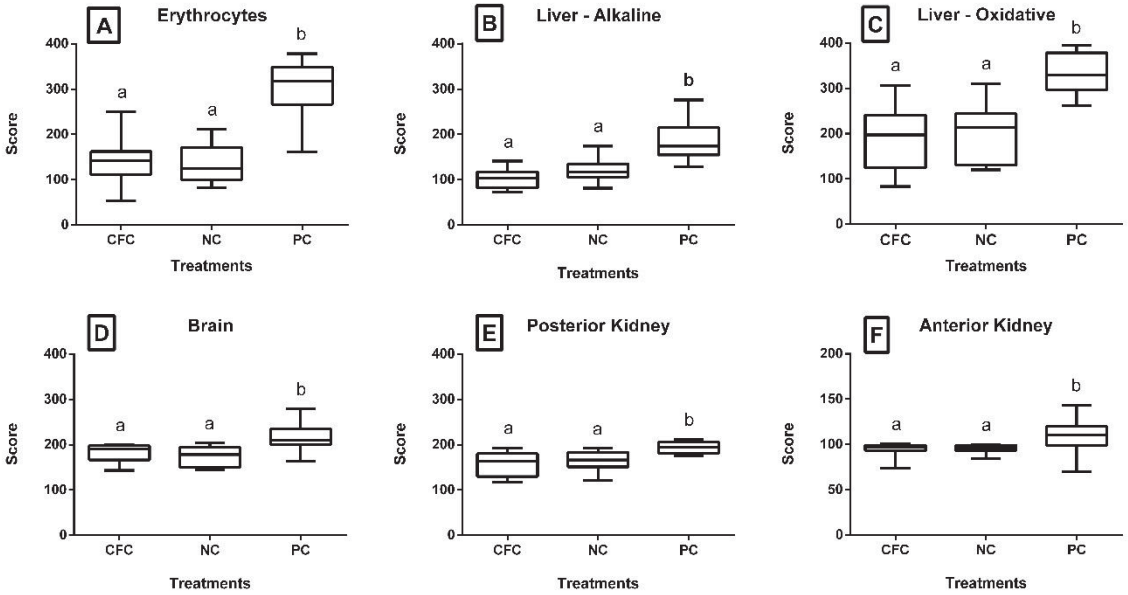
Values are presented as mean ± standard deviation.
CFC – commercial food control
NC - negative control.
There was no statistical difference between CFC and NC($p > 0.05$)

Supplementary Table 2 - Frequency of micronuclei (MN) and nuclear erythrocyte alterations (NEA) in *Rhamdia quelen*, discriminate in blebbed, lobed, notched, vacuolated and Total NEA (MN + NEA)

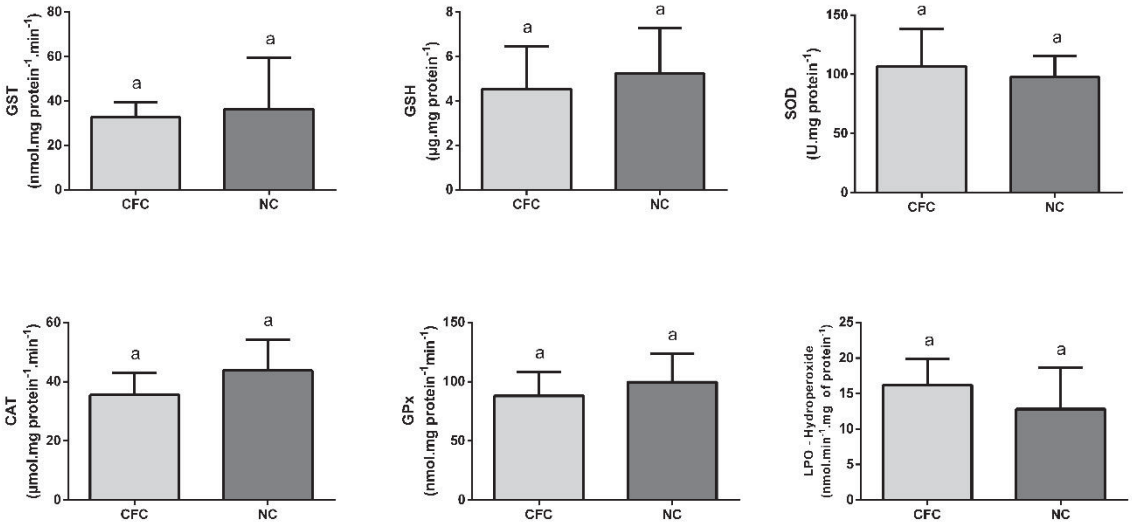
Treatments	MN	*Blebbed*	*Notched*	*Vacuolated*	Total NEA
CFC	0 (0 / 0)	0 (0 /1)	0 (0 / 0)	3.5 (1.25/4.75)	4.5 (3/6)
NC	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)	2 (1 / 6)	3 (2 / 8)

Values are expressed as median and interquartile (1st Q / 3rd Q).
CFC – commercial food control
NC - negative control.
There was no statistical difference between CFC and NC ($p > 0.05$)

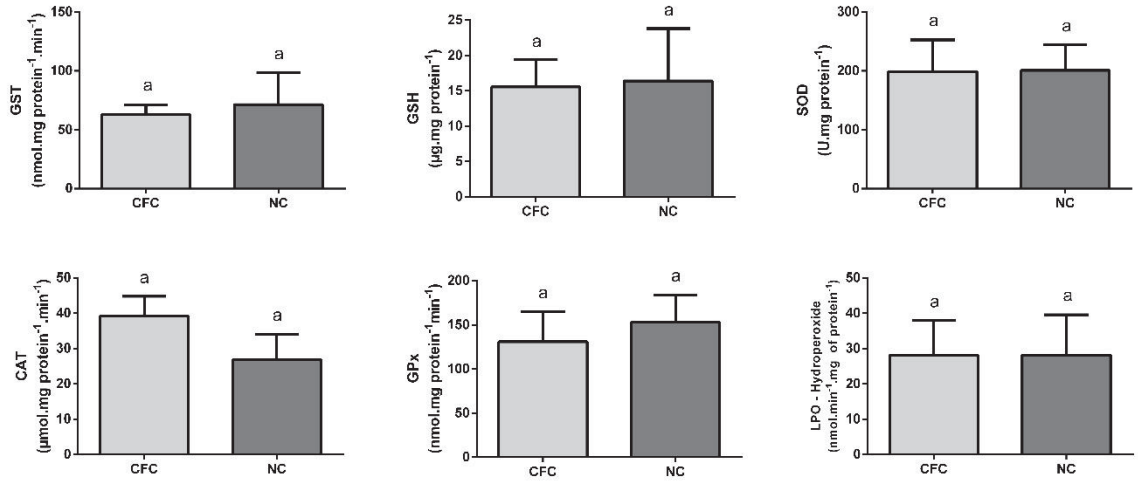
Supplementary Figure 1 - DNA damage of *Rhamdia quelen* detected by the comet assay in the control groups. CFC: commercial food control; NC: negative control; PC: positive control. The data are represented by medians and quartiles. Different lowercase letters indicate statistica statistical differences between groups ($p < 0.05$).



Supplementary Figure 2 - Biochemical biomarkers evaluated in control groups for the liver of the *Rhamdia quelen* of the control groups. CFC: commercial food control; NC: negative control. The data are presented by means and standard deviation. Different lowercase letters indicate statistical differences between groups ($p < 0.05$).



Supplementary Figure 3 - Biochemical biomarkers evaluated in control groups for the posterior kidney of the *Rhamdia quelen* of the control groups. CFC: commercial food control; NC: negative control. The data are presented by means and standard deviation. Different lowercase letters indicate statistical differences between groups ($p < 0.05$).



Supplementary Figure 4 - Cerebral (A) and muscular (B) acetylcholinesterase (AChE) activities quantified in control groups of *Rhamdia quelen*. CFC: commercial food control; NC: negative control. The data are represented by mean and standard deviation. Different lowercase letters indicate statistical differences between groups ($p < 0.05$).