UNIVERSIDADE FEDERAL DO PARANÁ

JULIANA RORATTO LIROLA

AVALIAÇÃO DA TOXICIDADE DO DI-N-BUTIL FTALATO (DBP) E DI-ISO-PENTIL FTALATO (DiPeP) ISOLADOS E EM MISTURA UTILIZANDO EMBRIÕES E LARVAS DE *Danio rerio* E *Rhamdia quelen*

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 título de Doutora em Genética.

Orientadora: Prof ª. Dr ª. Marta Margarete Cestari.
Coorientadora: Prof ª. Dr ª. Daniela Morais Leme.

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"Dizem que nosso destino está ligado à nossa terra, que ela é parte de nós assim como nós somos dela. Outros dizem que o destino é costurado como um tecido, onde a vida de um determina a de muitos outros. É a única coisa que buscamos, ou que lutamos para mudar, alguns nunca encontram o destino, mas outros são levados a ele."

- Merida (Valente, Walt Disney)

RESUMO

Os ftalatos são contaminantes emergentes de alto volume de produção, devido a sua ampla utilização no ramo industrial, usados principalmente como plastificantes. Esses compostos não estão covalentemente ligados a estrutura do plástico, sendo facilmente liberados no ambiente. O di-n-butil ftalato (DBP) está entre os ftalatos mais utilizados no mundo, sendo classificado pela *European Chemicals Agency* (ECHA) como uma substância com alto potencial tóxico, com capacidade de interferir na formação dos órgãos sexuais de mamíferos e no desenvolvimento embrionário de anfíbios e peixes. Já o di-iso-pentil ftalato (DiPeP) é muito utilizado no Brasil, por ser um produto secundário da produção de etanol e é considerado como um ftalato com forte potencial antiandrogênico. São poucos os estudos que avaliam a toxicidade de misturas entre ftalatos, entretanto essa avaliação é mais ambientalmente realística, pois os organismos são expostos simultaneamente a vários compostos. O *Fish Embryo Extend Toxicity* (FEET) teste é uma adaptação no tempo de exposição ao teste de toxicidade em embriões de peixes (FET), padronizado pela *Organisation for Economic Co-operation and Development* (OECD), o qual utiliza o peixe *Danio rerio*. A transposição desse teste para outras espécies de peixes tem se mostrado viável, e por este motivo, o objetivo desse estudo foi transpor o FEET teste para a espécie nativa da América do Sul *Rhamdia quelen* e comparar com a espécie modelo *Danio rerio,* (*zebrafish*) por meio de parâmetros de letalidade e subletalidade e pela avaliação de biomarcadores bioquímicos e genético. A metodologia seguiu o protocolo 236/2013 da OECD e também com uma adaptação no período de exposição de 96 para 168 horas, onde os ovos recém fertilizados foram expostos ao DBP e ao DiPeP em concentrações que variam de 0,001 a 0,125 $mg.L^{-1}$ e a mistura entre DBP + DiPeP (MIX) na faixa de 0,001 a 0,031 $mg.L^{-1}$, além dos controles negativo, de solvente (metanol 0,1%) e positivo (3,4-dicloroalanina). Os biomarcadores foram avaliados em 96 horas e em 168 horas de exposição. Os ftalatos induziram mortalidade, malformações no desenvolvimento embrionário, alterações comportamentais, neurotoxicidade, estresse oxidativo, peroxidação lipídica e genotoxicidade nas larvas de peixe. O DBP foi mais tóxico quando analisamos os resultados obtidos pelo teste de toxicidade, como mortalidade e malformações em embriões e larvas. Já o DiPeP causou maiores danos a macromoléculas, como lipídios. A MIX intensificou a toxicidade do DBP e do DiPeP, causando danos morfológicos em baixas concentrações e alterando a atividade da enzima glutationa S-transferase. A genotoxicidade foi observada somente em 168 horas de exposição aos ftalatos isolados e as MIX, indicando que a extensão do tempo de exposição é válida para este biomarcador. A espécie *R. quelen* demonstrou ser adequada para o FET e FEET teste, apresentando maior sensibilidade do que *D. rerio* aos ftalatos isolados e em mistura. Assim, concluímos que a transposição do FEET teste para *R. quelen* foi viável, demonstrando que essa espécie nativa pode ser uma alternativa para testes de toxicidade com embriões e larvas.

Palavras-chave: Biomarcadores. Ensaio cometa. Jundiá. *Zebrafish*.

ABSTRACT

Phthalates are emerging contaminants of high production volume, due to widespread use the industrial sector, used mainly as plasticizers. These compounds are not covalently linked to plastic structure, easily reaching the environment. Di-n-butyl phthalate (DBP) is among the most widely used phthalates in the world, classified by European Chemicals Agency (ECHA) as substance with high toxic potential in formation of the mammals sexual organs, and in the embryonic development of amphibians and fish. Di-iso-pentyl phthalate (DiPeP) is widely used in Brazil, because it is a secondary product of ethanol production, considered a phthalate with strong antiandrogenic potential. There are few studies that evaluate the phthalates mixture's toxicity, however the evaluation of mixtures (MIX) are more environmentally realistic, because the organisms are simultaneously exposed to several compounds. The Fish Embryo Extend Toxicity (FEET) test is an adaptation in time of exposure to fish embryo toxicity test (FET), standardized by Organization for Economic Co-operation and Development (OECD), using the model fish species *Danio rerio*. The transpose of FEET test to other fish species has been shown to be viable, for this reason, the aim of study was to transpose the FEET test on native to South America species *Rhamdia quelen* and to compare with *D. rerio* (zebrafish), through lethality and subletality parameters and by the evaluation of biochemical and genetic biomarkers. The methodology followed the OECD test guideline 236/2013, and also an adaptation to exposure time from 96 to 168 hours, in which newly fertilized eggs were exposed to DBP and DiPeP in concentrations ranging from 0.001 to 0.125 $mg.L^{-1}$ and the mixture between DBP + DiPeP (MIX), in the ranges of 0.001 to 0.031 $mg.L^{-1}$, in addition to negative, solvent (0.1% methanol) and positive (3.4 dicloroalanina) controls. Biomarkers were evaluated at 96 hours and at 168 hours exposure. Phthalates induced mortality, embryonic development malformations, behavioral changes, neurotoxicity, oxidative stress, lipid peroxidation and genotoxicity in fish larvae. DBP was more toxic when we analyzed the results obtained by toxicity test, such as mortality and embryos and larvae malformations. DiPeP caused greater macromolecules damage, such as lipids. MIX intensified the DBP and DiPeP toxicity, causing morphological damage at low concentrations and changing the glutathione S-transferase (GST) enzyme activity. Genotoxicity was observed only at 168 hours of exposure to isolated phthalates and MIX, this indicates that exposure time extension is valid for this biomarker. The *R. quelen* species proved to be adequate for the FET and FEET test than *D. rerio*, presenting greater sensitivity to isolated and MIX between phthalates. Thus, we concluded that the transpose of FEET test to *R. quelen* was feasible, demonstrating that this native species can be an alternative for embryos and larvae toxicity tests.

Key-words: Biomarkers. Comet assay. Jundiá. Zebrafish.

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AChE	Acetilcolinesterase
CAS	*Chemical Abstract Service Registry*
CL_{50} / LC_{50}	Concentração letal média
CH	Deformações estruturais no córion
CEUA	Comissão de Ética no Uso de Animais
CI	Controle interno da placa
CPE	Extrato de hipófise da carpa
DBP	Di-n-butil ftalato
DEP	Di-etil ftalato
DEHP	Bis (2-etilhexil) ftalato
DHpP	Di-heptilo ftalato
DIDP	Di-iso-decil ftalato
DMP	Di-metil ftalato
DiPeP	Di-iso-pentil ftalato
DNA	Ácido desoxiribonucleico
DTNB	5,5-ditio-bis-2nitro-benzoato
DW	Nanismo
EChA	*European Chemicals Agency*
ED	Desreguladores endócrinos
EDC	Compostos desreguladores endócrinos
FET	*Fish Embryo Toxicity* teste
FEET	*Fish Embryo Extend Toxicity* teste
FOX	Ferrous oxidation / Xylenol orange method
HG	Hora-grau
HM	Hemorragia
HPF	Horas após a fertilização
H_2O_2	Peróxidos de hidrogênio
GR	Crescimento corporal reduzido
GST	Glutationa S-transferase
LMP	Agarose de baixo ponto de fusão
LPO	Peroxidação lipídica
MC	Microcefalia

MIX	Mistura entre o DBP e DiPeP
NC	Controle negativo
NS	Diferença significativa com o controle negativo
OECD	*Organisation for Economic Co-operation and Development*
PC	Controle positivo
PCC	Controle positivo do ensaio cometa
PE	Edema pericárdico
PI	Pigmentação alterada
PVC	Cloreto de polivinila
RB	Barbilhões reduzidos
ROS	Espécies reativas de Oxigênio
SB	Bexiga natatória não inflada
SC	Controle de solvente
SD	Deformidades no esqueleto
UE	Olhos não desenvolvidos
UV-C	Radiação Ultravioleta do tipo C
YA	Má absorção do saco vitelino
YE	Edema no saco vitelino

SUMÁRIO

1 INTRODUÇÃO

Os ftalatos são compostos presentes na formulação de uma grande quantidade de produtos comercializados, principalmente em plásticos, com o intuito de aumentar a flexibilidade e resistência desses produtos (MARTINO-ANDRADE et al., 2010; HANNAS et al., 2011; XU et al., 2015; BENJAMIN et al., 2017; ROCHA et al., 2017; SOUZA et al., 2018; SEYOUM e PRADHAN, 2019). Os ftalatos não são quimicamente ligados à matriz do plástico, e dessa forma chegam facilmente ao ambiente, o que tem causado preocupação quanto a toxicidade dessas substâncias (STAPLES et al., 1997; CHRISTEN et al., 2012; ROCHA et al., 2017; MOLINO et al., 2019).

O di-n-butil ftalato (DBP) está entre os ftalatos mundialmente mais utilizados na indústria, e já é bem descrito como tóxico ao sistema reprodutivo de mamíferos, devido ao seu alto potencial antiandrogênico (CHRISTEN et al., 2012; MACHTINGER et al., 2018; SOUZA et al., 2018), bem como seus efeitos negativos ao desenvolvimento embrionário de larvas de peixes e de anfíbios (STAPLES et al., 1997; ORTIZ-ZARRAGOITIA, TRANT e CAJARAVILLE, 2006; XU e GYE, 2018; PU et al., 2019).

Outro ftalato de importância neste contexto, principalmente no Brasil, é o di-iso-pentil ftalato (DiPeP), resultante da fermentação do açúcar durante a produção de etanol, tendo portanto sua produção favorecida no Brasil (SOUZA et al., 2018), sendo muito utilizado como plastificante em produtos de cloreto de polivinila (PVC) (ROCHA et al., 2017). Dessa forma, a presença desse composto tem sido relatada em amostras ambientais e biológicas em nosso país (DO NASCIMENTO FILHO et al., 2003; FERREIRA e MORITA, 2012; ROCHA et al., 2017; SOUZA et al., 2018). O DiPeP, tal como o DBP é relatado como um ftalato com forte capacidade de desregulação endócrina (SOUZA et al., 2018).

O potencial tóxico dos ftalatos no sistema reprodutor de mamíferos já é bem conhecido, no entanto, poucos estudos avaliam os efeitos ecotoxicológicos desses compostos (YANG et al., 2018). Já é descrito que alguns ftalatos podem alterar o desenvolvimento embrionário dos peixes e causar malformações, afetando a sobrevivência dos embriões e larvas (ORTIZ-ZARRAGOITIA, TRANT e CAJARAVILLE, 2006; YANG et al., 2018; PU et al., 2019).

Considerando um cenário ambiental realista, os seres vivos são expostos a uma mistura entre ftalatos, entretanto, pouco se sabe sobre a toxicidade causada pela co-exposição a esses compostos (CHRISTEN et al., 2012). A avaliação das misturas é válida quando os ftalatos apresentam diferentes potenciais de toxicidade e mecanismos de ação semelhantes (MANKIDY et al., 2013), como é o caso do DBP e do DiPeP. Ademais, no Brasil ocorre a exposição simultânea ao DBP e ao DiPeP (ROCHA et al., 2017), no entanto, não existe nenhum estudo que avalie a toxicidade da mistura destes ftalatos.

O *Fish Embryo Acute Toxicity Test* (FET teste) é um teste de toxicidade em peixes padronizado internacionalmente pela OECD (*Organisation for Economic Co-operation and Development*) sob o protocolo 236/2013 com embriões de *Danio rerio* (*zebrafish*, peixe zebra ou paulistinha) (OECD, 2013). *D. rerio* é uma espécie modelo que apresenta muitas características que são vantajosas para testes toxicológicos, como genoma sequenciado, pequeno tamanho, desova abundante, rápido desenvolvimento embrionário, embriões transparentes, além de permitir a exposição a misturas entre compostos químicos (LAMMER et al., 2009; GAMSE e GORELICK, 2016; ANDRADE et al., 2018; OLIVEIRA DE FARIAS et al., 2019).

Essas características que permitem a aplicação do teste de toxicidade em embriões não são exclusivas de *D. rerio*. Como exemplo, a espécie *Rhamdia quelen* é uma espécie nativa da América do Sul, com abundante desova por indução hormonal, córion transparente, rápido desenvolvimento embriológico, e ainda são sensíveis à compostos tóxicos, podendo assim, cumprir os critérios de espécie a ser utilizada no FET teste (RODRIGUES-GALDINO et al., 2009; BRITO et al., 2017; SCHREIBER et al., 2017).

A avaliação de parâmetros de subletalidade no FET teste é uma adaptação que pode torna-lo mais sensível a ação de compostos químicos (SEHONOVA et al., 2016; NORBERG-KING et al. 2018; KRZYKWA, SAEID e JEFFRIES, 2019). Uma outra adaptação ao FET teste é a extensão do período de exposição de 96 (FET teste convencional) para 168 horas de exposição, o chamado *Fish Embryo Extend Toxicity* (FEET) teste. O FEET teste tem se mostrado adequado para avaliação de alguns produtos químicos (OLIVEIRA DE FARIAS et al., 2019), pois as larvas têm se mostrado mais sensíveis a exposição aos xenobióticos do que os embriões (DANG, VEN e KIENHUIS, 2017; STELZER et al., 2018).

A comparação da sensibilidade entre embriões de *D. rerio* e de *Rhamdia quelen* em um teste padronizado pelas agências regulatórias, com critérios rígidos de validação, poderia acelerar o processo de inclusão de novas espécies em protocolos de toxicidade aguda e subcrônica, como o FET teste (DANG, VEN e KIENHUIS, 2017; SCHREIBER et al., 2017). Assim, esse estudo teve como objetivo avaliar a toxicidade do DBP e do DiPeP isolados e em mistura em *Danio rerio* e *Rhamdia quelen* por meio de ensaios com embriões e larvas (FET e FEET teste) e de biomarcadores bioquímicos e genético.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Investigar a toxicidade dos ftalatos DBP e DiPeP isolados e em mistura em embriões e larvas da espécie modelo *Danio rerio* e na espécie nativa da América do Sul *Rhamdia quelen* a fim de avaliar se essa espécie nativa poderia apresentar uma alternativa mais viável para estudos futuros que utilizem o FET teste no contexto brasileiro.

1.2.2 Objetivos específicos

- Analisar os *endpoints* de letalidade e de subletalidade em embriões e larvas de *D. rerio* e *R. quelen* expostas ao DBP, DiPeP e MIX isolados e em mistura;
- Verificar a viabilidade do FET teste com extensão do tempo de exposição (FEET teste) nas espécies *Danio rerio* e *Rhamdia quelen*;
- Transpor o FEET teste para *R. quelen* e verificar sua viabilidade, comparando a sensibilidade das espécies;
- Avaliar os efeitos em biomarcadores genético (ensaio cometa) e bioquimicos (AChE, GST e LPO) em larvas de *D. rerio* e *R. quelen* expostas ao DBP e ao DiPeP isolados e em mistura.

2 REVISÃO BIBLIOGRÁFICA

2.1 FTALATOS

Os ftalatos ou ésteres do ácido ftálico são produtos químicos orgânicos e sintéticos, amplamente utilizados como aditivos e plastificantes para aumentarem a durabilidade, a flexibilidade e a resistência de produtos industriais (BENJAMIN et al, 2017; ROCHA et al., 2017; SOUZA et al., 2018; SEYOUM e PRADHAN, 2019). Dessa forma, tem um amplo espectro de aplicações (CHRISTEN et al., 2012) como em tintas, adesivos, selantes, cosméticos, produtos de higiene pessoal, embalagens de alimentos, roupas, brinquedos e produtos hospitalares (MARTINO-ANDRADE et al., 2010; HANNAS et al., 2011; XU et al., 2015).

Os ftalatos são considerados compostos de alto volume de produção (CHRISTEN et al., 2012), já que a estimativa do uso global de plastificantes foi em torno de $10,3 \times 10^6$ toneladas no ano de 2019 (ZHANG et al., 2019a). O aumento do uso de ftalatos resultou na contaminação do ecossistema aquático, onde esses compostos atingiram a concentração aproximada acima de 0,05 $mg.L^{-1}$ na água (SEYOUM e PRADHAN, 2019a), ameaçando diretamente a vida (VENTRICE et al., 2013; KATSIKANTAMI et al., 2016; BENJAMIN et al., 2017; POOPAL et al., 2020). Ademais, esses compostos não estão quimicamente ligados à matriz polimérica, sendo facilmente liberados no meio ambiente (STAPLES et al., 1997; CHRISTEN et al., 2012; ROCHA et al., 2017; MOLINO et al., 2019).

Esta classe de compostos tem atraído muita atenção dos pesquisadores e das agências reguladoras por conta da associação já relatada destes com distúrbios reprodutivos (MARTINO-ANDRADE et al., 2010; SOUZA et al., 2018). A União Européia, a Suíça e os Estados Unidos da América têm legislações que restringem a presença dos ftalatos bis (2-etilhexil) ftalato (DEHP), benzil butil ftalato (BBP), di-isobutil ftalato (DIBP) e o di-n-butil ftalato (DBP) em cosméticos e em brinquedos infantis desde 2003 (CHRISTEN et al., 2012). No Brasil, ftalatos como o DEHP, o DBP e o di-iso-pentil ftalato (DiPeP) foram proibidos em 2016, devido à sua toxicidade (ANVISA, 2016). Apesar das regulamentações, não ocorre monitoramento para estabelecer os níveis de referência e avaliar o cumprimento das regulamentações (SOUZA et al., 2018).

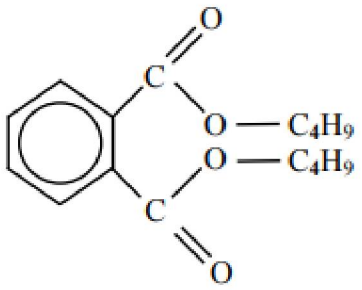
Quando expostos aos ftalatos, os seres humanos e os organismos não aquáticos podem absorvê-los via inalatória, oral ou dérmica, chegando ao sangue e sendo posteriormente transformados em metabolitos primários e secundários no fígado, a fim de se tornarem mais solúveis para que ocorra sua excreção (KOCH et al., 2005; BENJAMIN et al, 2017; WANG, ZHU e KURUNTHACHALAM, 2019). Assim, esses compostos podem interagir com diferentes tecidos e órgãos e causar efeitos a longo prazo (VENTRICE et al., 2013; POOPAL et al., 2020).

Sabe-se que os ftalatos são potentes desreguladores endócrinos, podendo se ligar a receptores hormonais e imitar ou antagonizar a ação do ligante hormonal natural (BRADLEE e THOMAS, 2003), levando a graves efeitos antiandrogênicos já bem descritos em ratos (BENJAMIN et al., 2017; MACHTINGER et al., 2018). Estudos em mamíferos mostraram que os ftalatos têm efeitos adversos no desenvolvimento de órgãos reprodutivos (CHRISTEN et al., 2012; KATSIKANTAMI et al., 2016).

A toxicidade dos ftalatos está relacionada ao comprimento da sua cadeia lateral, a qual pode variar de C1 a C13 (BENJAMIN et al, 2017). De modo geral, os ftalatos com baixo peso molecular (C1 a C4) são mais tóxicos, enquanto que ftalatos com cadeia superior a 6 carbonos não apresentam toxicidade intrínseca para organismos aquáticos, devido a sua insolubilidade na água (STAPLES et al., 1997; BRADLEE e THOMAS, 2003; BENJAMIN et al., 2017).

Dentre a classe dos ftalatos, o di-n-butil ftalato (DBP) (FIGURA 1) apresenta quatro carbonos em sua cadeia lateral (C4), é um dos compostos mais utilizados na indústria (XU e GYE, 2018), sendo classificado como uma substância de elevada preocupação pela *European Chemicals Agency* (ECHA, 2004). O DBP já foi associado ao estresse oxidativo, genotoxicidade (DU et al., 2014, LI, YIN e ZHAO, 2017), peroxidação lipídica, apoptose (JENG, 2014), malformações em embriões de peixes e de anfíbios (ORTIZ-ZARRAGOITIA, TRANT e CAJARAVILLE, 2006; XU e GYE, 2018; PU et al., 2019), levando a redução na expectativa de vida (SEYOUM e PRADHAN, 2019) e a morte desses animais (PU et al., 2019).

FIGURA 1. Estrutura química do Di-n-butil ftalato (DBP).

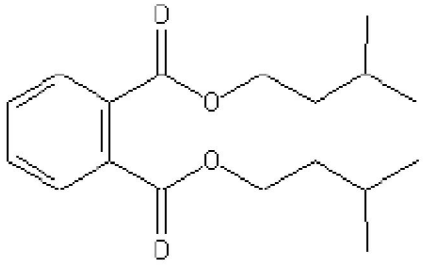


FONTE: ECHA, 2004.

O di-iso-pentil ftalato (DiPeP) (FIGURA 2) ou di-isoamil ftalato tem cadeia composta por 5 carbonos - comprimento intermediário (SOUZA et al., 2018), sendo usado como plastificante em produtos de PVC e na fabricação de propulsores e explosivos de nitrocelulose (ECHA, 2016; ROCHA et al., 2017). O DiPeP tem sido relatado como um ftalato com forte potencial antiandrogênico em mamíferos, exibindo maior capacidade de desregulação endócrina do que outros ftalatos, isso indica que sua estrutura atua com potencial tóxico similar aos demais (ROCHA et al., 2017; SOUZA et al., 2018).

O DiPeP é sintetizado pela esterificação do álcool isoamílico com o anidrido ftálico (SOUZA et al., 2018), sendo que o álcool isoamílico é um produto resultante da fermentação do açúcar na produção de etanol. Por sua vez, o etanol e seus derivados são produzidos em abundância no Brasil, favorecendo a produção do DiPeP, assim esse é o único país onde sua presença foi relatada em amostras ambientais, como no solo e em aterros sanitários (DO NASCIMENTO FILHO et al., 2003; FERREIRA e MORITA, 2012; SOUZA et al., 2018). Além disso, seus metabólitos foram quantificados na urina de crianças e de mulheres grávidas brasileiras (ROCHA et al., 2017; SOUZA et al., 2018).

FIGURA 2. Estrutura química do Di-iso-pentil ftalato.



FONTE: ECHA, 2016.

Como apresentado anteriormente, é necessário conhecer o potencial tóxico de cada composto isolado, porém, no ambiente os organismos são diariamente expostos por meio de diferentes rotas, a vários ftalatos simultaneamente, mas os riscos desta exposição não são bem compreendidos (MANKIDY et al., 2013; JOHANSSON et al., 2016; ZHOU, GAO e FLAWS, 2017; SOUZA et al., 2018; HSIEH et al., 2019; NEIER et al., 2019). Assim, estudos que se assemelham a uma exposição cotidiana são necessários na avaliação de risco dos ftalatos, a fim de evitar que seus efeitos sejam subestimados (CHRISTEN et al., 2012; ROCHA et al., 2017; ZHOU, GAO e FLAWS, 2017).

Estudos têm demonstrado que as misturas entre os ftalatos podem ter diferentes tipos de efeitos de interação, como: adição de dose ou efeito, sinergismo ou antagonismo e assim alterar vários parâmetros biológicos (CHRISTEN et al., 2012; LI, YIN e ZHAO, 2017; NEIER et al., 2019). Cada mistura terá um efeito combinatório diferente, mas de maneira geral a toxicidade das misturas parece ser maior do que a dos ftalatos isolados (CHRISTEN et al., 2012).

Sabe-se que diferentes ftalatos podem atuar na mesma via biológica, assim, quando os ftalatos têm diferentes potenciais de toxicidade mas mecanismos de ação semelhantes, a avaliação das misturas é válida, pois a interação entre eles pode potencializar seus efeitos tóxicos (MANKIDY et al., 2013). O DBP e o DiPeP apresentam alto potencial de desregulação endócrina, principalmente na inibição da produção de testosterona em fetos de ratos (SOUZA et al., 2018; CURI et al., 2019).

Os ftalatos de baixo peso molecular são transformados em metabólitos primários e secundários, estes tem a capacidade de interagir erroneamente, com fatores de transcrição presentes no núcleo (BENJAMIN et al., 2017) e dessa forma interferir no sistema endócrino. Sendo principalmente produtos químicos lipofílicos, os ftalatos são prontamente absorvidos pelo sangue ou pelos fluidos humanos e

rapidamente transformados nos respectivos metabolitos primários e secundários; antes da excreção, alguns desses metabólitos interagiriam erroneamente (xenosensores) com o sistema de sinalização endócrino molecular como ligantes (agonista ou antagonista ou co-ativador) de residentes - resultando em efeitos adversos à saúde.

A maioria dos estudos sobre a toxicidade dos ftalatos investigam as consequências no sistema reprodutivo de mamíferos e poucos estudos avaliam os efeitos ecotoxicológicos nos estágios iniciais de vida dos organismos aquáticos (YANG et al., 2018). A exposição a ftalatos pode afetar o desenvolvimento embrionário de anfíbios (XU e GYE, 2018) e de peixes (ORTIZ-ZARRAGOITIA, TRANT e CAJARAVILLE, 2006; PU et al., 2019). Tal efeito está relacionado ao estresse oxidativo causado principalmente pelo di-etil ftalato (DEP) e pelo DEHP (MANKIDY et al., 2013; YANG et al., 2018). Já o DBP, apesar de causar menor estresse oxidativo (MANKIDY et al., 2013), pode causar malformações na coluna, edemas no saco vitelino, defeitos cardíacos e bexiga natatória não inflada em embriões e larvas de peixes (ORTIZ-ZARRAGOITIA, TRANT e CAJARAILLE, 2006; PU et al., 2019).

2.2 DESENVOLVIMENTO EMBRIONÁRIO DE PEIXES

A descrição dos estágios do desenvolvimento embrionário é de grande importância, pois permite conhecer a biologia e a sistemática da espécie, permitindo sua identificação nos ambientes naturais, bem como no estudo de malformações decorrentes da contaminação da água (PEREIRA et al., 2006; RODRIGUES-GALDINO et al., 2009). Além disso, a maioria das espécies de peixe são mais sensíveis a contaminação aquática, a variações no pH, salinidade, alcalinidade, turbidez e a concentração de oxigênio dissolvido nas fases iniciais da vida (PEREIRA et al., 2006).

As condições ideiais para o desenvolvimento embrionário podem variar de acordo com a espécie, sendo a temperatura um fator crítico para o crescimento dos peixes (RODRIGUES-GALDINO et al., 2009). O desenvolvimento embrionário ocorre de maneira similar entre as espécies de peixes teleósteos, no qual consiste dos seguintes estágios: zigoto, clivagem, blástula, gástrula, segmentação, pharyngula e larval, e essas fases variam no tempo em que ocorre em cada espécie (KIMMEL et

al., 1995; GOMES et al., 2000; PEREIRA et al., 2006; RODRIGUES-GALDINO et al., 2009; ALVES et al., 2016).

O estágio de zigoto ocorre logo após a fertilização (0 horas) e é caracterizado pela movimentação do citoplasma e o surgimento do pólo animal (RODRIGUES-GALDINO et al., 2009; ALVES et al., 2016). Em exemplares de *Danio rerio* a 28.5 °C e de *Rhamdia quelen* a 27 °C, esse estágio tem duração de 40 min (KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009).

O estágio de clivagem consiste em diversas divisões mitóticas no polo animal, as clivagens em teleósteos são do tipo meroblástica, e após cada divisão, os blastômeros diminuem de tamanho, chegando a 64 ao fim desse estágio (RODRIGUES-GALDINO et al., 2009; ALVES et al., 2016). Em *D. rerio* a clivagem finaliza em 2 horas após a fertilização (hpf) (KIMMEL et al., 1995), enquanto que em *R. quelen* tem duração de 1h e 45 min hpf (RODRIGUES-GALDINO et al., 2009).

Na blástula surge a blastoderme, e após uma intensa divisão celular apresenta o formato achatado, levando ao movimento morfogênico, iniciando a epibolia (KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009; ALVES et al., 2016). Esse estágio dura até 3h e 15 min hpf em embriões de *D. rerio* a 28.5 °C (KIMMEL et al., 1995) e 4h e 45 min hpf em embriões de *Rhamdia quelen* a 27 °C (RODRIGUES-GALDINO et al., 2009).

O estágio da gástrula é caracterizado por movimentos de involução, convergência e extensão da epibolia, e levam a migração celular a fim de promover a diferenciação dos tecidos e formar as camadas germinativas primárias e o eixo embrionário (KIMMEL et al., 1995; ALVES et al., 2016). A gástrula finaliza em 5h e 15 min hph tanto em *D. rerio* quanto em *R. quelen* (KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009).

Na segmentação ocorre a diferenciação das regiões cefálica e caudal (ALVES et al., 2016), surgem os somitos, a notocorda e inicia-se a organogênese. Ocorre a compartimentação do cérebro, surge o intestino primitivo, os batimentos cardíacos e ocorre o alongamento do corpo (GOMES et al., 2000; KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009; ALVES et al., 2016). O estágio de segmentação finaliza após 35 hpf em embriões de *D. rerio* a 28.5 °C (KIMMEL et al., 1995) e em 11h e 30 min hpf em embriões de *R. quelen* a 27 °C (RODRIGUES-GALDINO et al., 2009).

No estágio pharyngula os embriões tem o corpo alongado e bilateral, apresentam intensa mobilidade, circulação sanguínea, movimentos operculares e arcos branquiais (KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009). Esse é considerado o melhor estágio para comparar a morfologia de embriões de vertebrados de diferentes espécies (KIMMEL et al., 1995). A pharyngula finaliza em 42 hpf em *D. rerio* (KIMMEL et al., 1995) e em 19 hpf em *R. quelen* (RODRIGUES-GALDINO et al., 2009).

O estágio larval é caracterizado pela larva recém eclodida e finaliza com a completa absorção do vitelo. As larvas apresentam os olhos e o corpo pigmentados, ocorre o inflar da bexiga natatória e inicia-se a alimentação exógena (KIMMEL et al., 1995; RODRIGUES-GALDINO et al., 2009). Larvas de *D. rerio* a 28.5 °C iniciam o estágio larval por volta de 48 hpf (KIMMEL et al., 1995) enquanto as larvas de *R. quelen* a 27 °C eclodem em torno das 20 hpf (RODRIGUES-GALDINO et al., 2009).

2.3 USO DE EMBRIÕES DE PEIXE EM TESTES ECOTOXICOLÓGICOS

O uso de embriões em testes de toxicidade surgiu como uma substituição ao teste de toxicidade aguda realizado com peixes adultos, sendo considerado um método alternativo ao uso de animais pela legislação europeia (DANG, VEN e KIENHUIS, 2017). Segundo a Diretiva 86/609/EEC da União Europeia o termo "animal" é definido como “qualquer animal vertebrado não-humano vivo, incluindo formas larvares autónomas, mas excluindo formas fetais ou embrionárias", sendo estes protegidos pela diretiva.

O momento em que a proteção dos animais começa a valer para cada espécie pela diretiva é determinado por características como início da alimentação exógena da larva e o desprendimento do intestino (HALDER et al., 2010). Para exemplares de *Danio rerio* a proteção é válida até 96 horas pós-fertilização (KIMMEL et al., 1995), enquanto que para *Rhamdia quelen* a proteção é válida por até 90 horas pós-fertilização (RODRIGUES-GALDINO et al., 2009). Assim, testes com embriões são considerados um refinamento dentro do Princípio dos 3Rs proposto por Russell e Birch em 1959, onde busca-se reduzir o desconforto causado aos animais por meio de técnicas menos invasivas (BRAUNBECK et al., 2014).

A utilização de embriões de peixes apresentam várias vantagens, como períodos mais curtos de exposição, obtenção de resultados mais rápidos, menor

custo, o uso de pequenas quantidades das substâncias a serem testadas e a necessidade de apenas adultos reprodutores, além disso, é possível implementar facilmente *endpoints* de subletalidade (LAMMER et al., 2009; STELZER et al., 2018).

A avaliação desses *endpoints* de subletalidade aumenta a sensibilidade do FET teste (KRZYKWA, SAEID e JEFFRIES, 2019), permitindo avaliar compostos que possam alterar ou interromper o desenvolvimento de tecidos e órgãos e causar alterações comportamentais, comprometendo a sobrevivência de embriões e larvas de peixes (RIVERO-WENDT et al., 2016; SEHONOVA et al., 2016; ANDRADE et al., 2018).

Neste contexto, o teste de toxicidade em embriões de peixes aprimorou a tal ponto que foi internacionalmente padronizado, visto como uma das metodologias mais rigorosamente validadas (LAMMER et al., 2009; BRAUNBECK et al., 2014). O *Fish Embryo Acute Toxicity Test* (FET teste) foi desenvolvido pela *Organisation for Economic Co-operation and Development* (OECD) através do protocolo 236 (OECD, 2013) que utiliza embriões de *Danio rerio* como organismo modelo para avaliar a toxicidade aguda de substâncias químicas nos estágios embrionários.

O princípio do teste é expor os ovos recém fertilizados ao xenobiótico e avaliar os parâmetros de letalidade, como a coagulação dos ovos, a ausência de formação de somitos e de deslocamento da cauda do saco vitelínico e de batimentos cardíacos a cada 24 horas. Ao final das 96 horas de exposição é possível determinar a toxicidade aguda e calcular a Concentração Letal média (CL_{50}) (OECD, 2013).

2.4 *FISH EMBRYO EXTEND TOXICITY* (FEET) TESTE

O FET teste avalia a toxicidade a curto prazo nas fases iniciais do desenvolvimento em embriões e larvas recém-eclodidas de peixes. No entanto, há uma crescente demanda por testes com maior período de exposição a fim de avaliar a sobrevivência, o crescimento e o comportamento das larvas (NORBERG-KING et al., 2018). A extensão no tempo de exposição de 96 para 168 horas é uma adaptação ao teste que permite a avaliação dos parâmetros de letalidade e de subletalidade (OLIVEIRA DE FARIAS et al., 2019), sendo então chamado de *Fish Embryo Extend Toxicity* (FEET) teste.

Estudos têm demonstrado que com o passar do tempo os valores da concentração letal tendem a diminuir, indicando que o aumento da toxicidade está

relacionado aos estágios do desenvolvimento embrionário. Dessa maneira, embriões têm demonstrado ser mais resistentes à exposição a compostos químicos do que as larvas recém eclodidas (DANG, VEN e KIENHUIS, 2017; STELZER et al., 2018). Ademais, na avaliação de compostos com modo de ação específica, um maior tempo de exposição pode ser adequado para identificar neurotoxicantes que podem alterar o comportamento das larvas (NORBERG-KING et al., 2018; OLIVEIRA DE FARIAS et al., 2019).

Algumas características presentes nos embriões, podem torná-los menos sensíveis do que as larvas a ação de xenobióticos. Tais características são a presença do córion, o qual funciona como uma barreira que protege o embrião dos compostos químicos com alto peso molecular; a ausência de brânquias completamente formadas, sendo menor a absorção dos xenobióticos presentes na água e por fim a capacide de metabólica limitada dos embriões (BRAUNBECK et al., 2014; DANG, VEN e KIENHUIS, 2017; LOURENÇO et al., 2017; STELZER et al., 2018; OLIVEIRA DE FARIAS et al., 2019).

O estágio larval tem se mostrado ser o período de vida mais sensível (STELZER et al., 2018), pois tem uma fisiologia e ecologia únicas, sendo mais sensíveis do que embriões e do que adultos, pois sofrem maior pressão seletiva (BAGATTO, PELSTER e BURGGREN, 2001). Dessa forma, as malformações observadas nessa fase podem ter efeitos prolongados e mais frequentemente levar à morte, constituindo assim um bom modelo de estudo para se investigar o risco de exposição a xenobióticos (AZEVEDO-LINHARES et al., 2018).

2.5 TRANSPOSIÇÃO DO FEET TESTE PARA *Rhamdia quelen*

Danio rerio é considerado um modelo confiável para a avaliação dos efeitos de vários xenobióticos e tem sido utilizado há mais de 60 anos devido a características que são vantajosas em estudos toxicológicos (GAMSE e GORELICK, 2016). A utilização de *D. rerio* permite a exposição a misturas, pois ele pode absorver todos os compostos administrados na água; sua organogênese é externa; os ovos são permeáveis e transparentes, permitindo a visualização do desenvolvimento embrionário (BRAUNBECK et al., 2014; GAMSE e GORELICK, 2016; ANDRADE et al., 2018).

As características mencionadas como vantajosas na toxicologia não são exclusivas dessa espécie. O atual protocolo 236 (OECD, 2013) é aplicável apenas ao *D. rerio*, entretanto, tem se sugerido a realização de pesquisas a fim de fornecer a base técnica para desenvolver o FET teste com outras espécies (LAMMER et al., 2009; DANG, VEN e KIENHUIS, 2017; NORBERG-KING et al., 2018), visto que espécies modelo como *Danio rerio* tem sua relevância ecológica restrita ao seu local de origem (SCHREIBER et al., 2017).

A transposição do protocolo do FET teste tem se mostrado viável para outras espécies de peixe, como *Misgurnus fossilis* a qual é nativa da Europa (SCHREIBER et al., 2017), *Oryzias latipes* nativa da Ásia (LAMMER et al., 2009; SCHILLER et al., 2014), *Pimephales promelas* nativa da América do Norte e da América Central (LAMMER et al., 2009) e *Clarias gariepinus* que é nativa da África (DANG, VEN e KIENHUIS, 2017). Além disso, essas espécies têm provado serem mais sensíveis do que *D. rerio*, e com maior relevância ecológica na região a ser investigada.

Dessa forma, espécies nativas tem um alto potencial para atender os critérios de validação e serem utilizadas como espécie padrão no teste de toxidade em uma região particular (SCHREIBER et al., 2017). Espécies de peixe que apresentam transparência na membrana embrionária, rápido desenvolvimento e desova abundante, podem cumprir os critérios do FET teste (SCHREIBER et al., 2017), tal como *Rhamdia quelen*.

A espécie *R. quelen* pertence à família Heptapteridae, é um peixe neotropical de água doce, que vive perto da vegetação em rios e lagos de água calma (GUILOSKI et al., 2017). *R. quelen* é onívora, nativa da América do Sul e por ser uma espécie muito utilizada na psicultura, há uma série de estudos sobre a reprodução da espécie, facilitando a obtenção dos ovos (BRITO et al., 2017; AZEVEDO-LINHARES et al., 2018).

Além disso, *R. quelen* tem alta taxa de fertilização, rápido desenvolvimento embrionário, no qual a eclosão se dá em torno das 20 horas após a fertilização (hpf), e características importantes como o início da alimentação exógena, o inflar da bexiga natatória e o surgimento dos três pares de barbilhões, que ocorrem em torno das 72 hpf (GOMES et al., 2000; PEREIRA et al., 2006; DE AMORIM et al., 2009, RODRIGUES-GALDINO et al., 2009; GOES et al., 2016; BRITO et al., 2017).

Ademais, é uma espécie sensível a ação de xenobióticos, sendo comulmente utilizada na fase adulta em testes ecotoxicológicos (DE AMORIM et al., 2009,

PIANCINI et al., 2015; GOES et al., 2016; PEREIRA et al., 2016; BRITO et al., 2017; GUILOSKI et al., 2017; PERUSSOLO et al., 2019), no entanto, poucos estudos ecotoxicológicos utilizam as fases iniciais da vida de *R. quelen* (BRITO et al., 2017).

2.6 BIOMARCADORES BIOQUÍMICOS

Os biomarcadores podem ser definidos como alterações moleculares, genéticas, bioquímicas ou alterações fisiológicas em órgãos ou tecidos que indicam a exposição a determinado composto químico (LAM e GRAY, 2003), podendo ser considerados uma medida preventiva contra danos mais severos (VAN DER OOST, BEYER e VERMEULEN, 2003). A avaliação de biomarcadores em tecidos de peixe fornece bons parâmetros na investigação da toxicidade de xenobióticos (POOPAL et al., 2020).

A utilização dos biomarcadores bioquímicos junto ao FET teste é adequado, pois fornece resultados a nível celular e molecular, afim de definir o modo de ação do composto estudado (RIVERO-WENDT et al., 2016). Além do que, a adição desses indicadores pode auxiliar no entendimento dos mecanismos responsáveis por respostas comportamentais e morfológicas observados nas larvas (OLIVEIRA DE FARIAS et al., 2019).

2.6.1 Acetilcolinesterase (AChE)

As colinesterases são enzimas que degradam o neurotransmissor acetilcolina nas sinapses colinérgicas, e são importantes na regulação da homeostase do corpo, sendo essenciais para a neurotransmissão (RIVERO-WENDT et al., 2016; YANG et al., 2018). A acetilcolinesterase (AChE) é um biomarcador adequado no estudo de compostos de ação neurotóxica (KAVIRAJ e GUPTA, 2014) e alterações na ativação ou inibição da atividade dessa enzima ocorrem em função da exposição a compostos químicos em diferentes espécies de teleósteos (YANG et al., 2018).

Os biomarcadores enzimáticos, como a AChE são mais sensíveis do que os efeitos de subletalidade observados pelo FET teste. Assim, associar os efeitos do FEET teste com a avaliação da atividade da AChE, permite a correlação dos efeitos como mudanças no padrão de locomoção e alterações comportamentais nas larvas

com as alterações no sistema nervoso central do peixe (YANG et al., 2018; KRZYKWA, SAEID e JEFFRIES, 2019).

Os ftalatos tem a capacidade de modular a atividade da AChE, comumente diminuindo a atividade da enzima causada pelo acúmulo de acetilcolina (BASHA e RADHA, 2020), pela diminuição na capacidade antioxidante do organismo (AMARA et al., 2018) ou pelo dano tecidual (POOPAL et al., 2020). O DEHP pode reduzir a atividade da AChE em tecidos cardíacos de ratos (AMARA et al., 2018), já o di-heptilo ftalato (DHpP) e o di-iso-decil ftalato (DIDP) causaram uma diminuição na atividade da AChE no intestino, brânquias, cérebro e músculo de *D. rerio* (POOPAL et al., 2020) e o DBP reduziu a atividade dessa enzima no hipocampo de ratos (BASHA e RADHA, 2020).

2.6.2 Glutationa-S-transferase (GST)

A biotransformação é o processo de conversão de um xenobiótico em um composto hidrossolúvel para facilitar a excreção e esse processo ocorre em duas fases (VAN DER OOST, BEYER e VERMEULEN, 2003). As enzimas da fase I, adicionam um grupamento funcional polar, afim de tornar o composto mais hidrofílico. Já as enzimas da fase II realizam a conjugação de um composto endógeno, como tripeptídeo glutationa, ao metabólito resultante da fase I (SEVERO et al., 2020).

A glutationa S-transferase (GST) é uma enzima multifuncional que participa dos processos de detoxificação da fase II (BHAGAT, INGOLE e SINGH, 2016), sendo uma das enzimas mais importantes na metabolização de compostos químicos (ALLOCATI, et al., 2018). As enzimas antioxidantes, como a GST protegem a célula contra danos oxidativos e da peroxidação, os quais podem danificar macromoléculas (DU et al., 2014; BHAGAT, INGOLE e SINGH, 2016).

Quando há um desequilíbrio entre a produção de espécies reativas de Oxigênio (ROS) e a capacidade do organismo de eliminá-las, ocorre o estresse oxidativo (SEHONOVA et al., 2016; ROCHA et al., 2017; XIANG et al., 2017; ZHANG et al.; 2019b). As ROS são moléculas tóxicas para a célula, as quais podem causar danos a proteínas, membranas celulares e DNA (XIANG et al., 2017; ZHANG et al.; 2019b).

A exposição aos ftalatos pode aumentar os níveis de ROS nas células, alterando a atividade de enzimas antioxidantes como a GST (MANKIDY et al., 2013;

JENG, 2014; ROCHA et al., 2017; AMARA et al., 2018; YANG et al., 2018; MOLINO et al., 2019; SEYOUM e PRADHAN, 2019). O aumento na atividade da GST foi relatado na exposição a ftalatos como DEHP em ostras (XIANG et al., 2017) e em *Daphnia magna* (WANG et al., 2018) e em minhocas (*Eisenia fetida*) expostas ao DBP (DU et al., 2014) e ao di-metil ftalato (DMP) (SONG et al., 2019).

A exposição ao DEHP, ao DMP e ao DBP levou a um aumento na atividade da GST, seguido de uma diminuição significativa na atividade dessa enzima após alguns dias de exposição. Esse padrão sugere que o aumento na atividade da GST ocorre para compensar os danos induzidos pelos ftalatos, e que após um período de exposição a célula excede sua capacidade antioxidante, levando a uma diminuição na atividade dessa enzima (DU et al., 2014; XIANG et al., 2017; WANG et al., 2018; SONG et al., 2019).

2.6.3 Peroxidação lipídica (LPO)

A peroxidação lipídica (LPO) é um processo molecular de origem natural, no entanto, o aumento de ROS pode atacar os ácidos graxos poliinsaturados e se as defesas antioxidantes se esgotam, ocorrerá um aumento nos níveis de LPO (JENG, 2014; BHAGAT, INGOLE e SINGH, 2016; AMARA et al., 2018; WANG et al., 2018; SONG et al., 2019; ZHANG et al., 2019b).

A LPO acontece pela degradação oxidativa nas membranas celulares, o que pode alterar a fluidez da membrana, aumentando assim a sua permeabilidade, e consequentemente, causar apoptose ou necrose (BHAGAT, INGOLE e SINGH, 2016; SONG et al., 2019). A LPO é um dos danos mais importantes causados pelo estresse oxidativo (AMARA et al., 2018).

Como já mencionado, ftalatos podem causar estresse oxidativo e assim, aumentar os níveis de LPO (MANKIDY et al., 2013; DU et al., 2014; JENG, 2014; SONG et al., 2019). A exposição ao DEP e ao DEHP levaram a um aumento nos níveis de LPO em embriões do peixe *Pimephales promelis* (MANKIDY et al., 2013). O DBP também pode causar aumento de LPO, como observado em espermatócitos (JENG, 2014) e em minhocas (DU et al., 2014).

2.7 BIOMARCADOR GENÉTICO

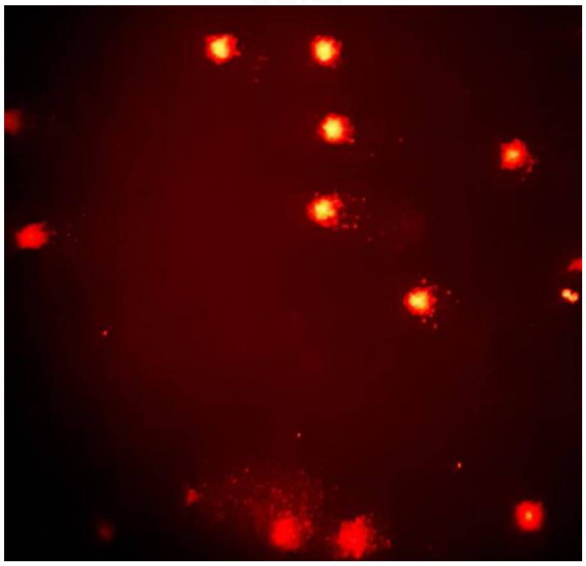
O ensaio cometa ou *Single cell gel electrophoresis* é um biomarcador genético que detecta lesões genômicas (GONTIJO *et al.*, 2003). O ensaio cometa detecta danos no DNA de uma única célula, por meio de uma técnica que desenovela a molécula de DNA, e que após ser submetida a uma corrida eletroforética, permite a visualização da frequência de quebras (FIGURA 3) (AZQUETA et al., 2020). O ensaio cometa alcalino permite identificar compostos com mecanismo de ação direto, pois esta versão da técnica detecta danos causados por quebras de fita simples e sítios alcali-lábeis (FAIRBAIRN, OLIVE e O'NEILL, 1995; KOPPEN et al., 2017).

Assim como outras macromoléculas, o DNA pode ser danificado pelas ROS (XIANG et al., 2017; AMARA et al., 2018; SONG et al., 2019; ZHANG et al.; 2019b). A presença de danos ao DNA é um indicador biológico útil devido ao seu papel na carcinogênese, mas vale ressaltar que as lesões observadas pelo ensaio cometa são passíveis de reparo (AZQUETA et al., 2020).

Os ftalatos podem causar genotoxicidade por meio de diferentes mecanismos. Os danos ao DNA podem ocorrer por meio de mecanismos indiretos, devido a presença de ROS, como observado em células da linhagem HepG2 (LI, YIN e ZHAO, 2017) e em minhocas (SONG et al., 2019) expostas ao DBP e em tecidos cardíacos de ratos expostos ao DEHP (AMARA et al., 2018). Dessa forma, os ftalatos causariam um dano tão severo que a célula não teria capacidade de acionar os mecanismos de reparo, causando a genotoxicidade (LI, YIN e ZHAO, 2017).

Além dos danos causados pelo ROS, os ftalatos podem danificar diretamente o DNA, como observado na linhagem celular embrionária do peixe *Dicentrarchus labrax* exposta a DEHP, o qual causou quebras na fita de DNA e também efeitos aneugênicos (MOLINO et al., 2019). Ademais, ftalatos ainda podem modular os mecanismos epigenéticos, modificando os padrões de metilação e desmetilação do DNA, mudando a condensação das histonas e alterando os padrões da fosforilação de proteínas (BENJAMIN et al, 2017).

FIGURA 3. Nucleóides pertencentes a larvas de *Rhamdia quelen* detectados por meio do ensaio cometa.



FONTE: A autora (2020).

3. MATERIAIS E MÉTODOS

3.1 REAGENTES E SOLUÇÕES TESTE

Os ftalatos utilizados foram o Di-n-butil ftalato (DBP), registro CAS (*Chemical Abstract Service*) 84-74-2 (99%, Sigma-Aldrich®, Darmstadt, Alemanha) e o Di-iso-pentil ftalato (DiPeP), registro CAS 605-50-5 (99%, Petrom® - Petroquímica Mogi das Cruzes SA, Brasil).

O reagente 3,4-dicloroalanina, registro CAS 95-76-1 (99%, Sigma-Aldrich®, Darmstadt, Alemanha) foi utilizado como controle positivo. As soluções foram preparadas nas concentrações de 4 $mg.L^{-1}$ para *Danio rerio* (OECD 236/2013) e 2 $mg.L^{-1}$ para *Rhamdia quelen*, definido após um teste preliminar de toxicidade aguda, devido a maior sensibilidade da espécie nativa a esse composto.

A chamada água de manutenção é a água do sistema, com características físico-químicas controladas, na qual os peixes matriz foram mantidos. A água de manutenção permaneceu em temperatura de 27 ± 1 °C; condutividade de 750 ± 50 µS/cm; pH 7,0 ± 0,5, oxigênio dissolvido acima de 95% de saturação e em ciclo de fotoperíodo de 12:12h (claro: escuro).

A água de manutenção foi utilizada no controle interno da placa, no controle negativo e no preparo das soluções teste de todos os ensaios de toxicidade. A água de manutenção e as soluções teste foram renovadas nos poços das placas após 96 horas de exposição. As soluções estoque dos ftalatos foram preparadas na concentração de 10 $mg.L^{-1}$ de ftalato e utilizou-se o Metanol 0,1% v/v como solvente (CHEN et al., 2014). As soluções de misturas entre DBP e DiPeP foram preparadas na proporção de 1:1 em todas as concentrações, para que cada ftalato contribuísse igualmente para os efeitos em cada concentração da mistura.

Para definir as concentrações dos ftalatos a serem utilizadas, foi inicialmente realizado um teste preliminar de toxicidade aguda, utilizando o DBP e o DiPeP nas concentrações de 0,001; 0,01; 0,1; 1 e 10 $mg.L^{-1}$. De acordo com o resultado desse teste definimos avaliar concentrações nos intervalos de 0,001 a 0,1, as quais causaram efeitos, mas não levaram a 100% de mortalidade.

Os tratamentos foram: controle negativo (NC); controle de solvente com 0,1% de metanol v/v (SC); controle positivo (PC); DBP nas concentrações de 0,001; 0,003; 0,007; 0,015; 0,031; 0,062 e 0,125 $mg.L^{-1}$, DiPeP nas concentrações de 0,001; 0,003;

0,007; 0,015; 0,031; 0,062 e 0,125 mg.L^{-1} e a mistura (MIX) nas concentrações de 0,001; 0,003; 0,007; 0,015 e 0,031 mg.L^{-1}.

3.2 OBTENÇÃO DOS OVOS DE *Danio rerio*

A obtenção de ovos de *Danio rerio*, para o FET e o FEET teste foram realizados no Laboratório de Genética Toxicológica, na Universidade de Brasília (UnB), em Brasília, DF. Os peixes adultos foram mantidos no sistema de recirculação ZebTEC (Tecniplast®, Itália), dentro de tanques com água filtrada com carvão ativado e em osmose reversa (FIGURA 4).

FIGURA 4. Sistema de recirculação ZebTEC contendo os peixes matriz da espécie *Danio rerio*.

FONTE: A autora (2020).

A obtenção dos ovos se deu por meio de cruzamento entre as matrizes no sistema de criação iSpawn, Tecniplast® (FIGURA 5), sendo coletados imediatamente após o acasalamento e inspecionados sob um estereomicroscópio (SMZ 1500, Nikon Corporation®). Os ovos não fertilizados e aqueles com lesões, irregularidades de clivagem ou com nível de divisão celular considerado avançado para o FET teste foram descartados.

FIGURA 5. Sistema de criação iSpawn para obtenção de ovos de *Danio rerio*.

FONTE: A autora (2020).

3.3 OBTENÇÃO DOS OVOS DE *Rhamdia quelen*

A obtenção de ovos de *Rhamdia quelen*, bem como os testes de toxicidade na espécie foram realizados no Laboratório de Tecnologia da Reprodução de Animais Aquáticos Cultiváveis (LATRAAC), na Universidade Estadual do Oeste do Paraná (UNIOESTE), no município de Toledo – PR.

Foram selecionados três machos e três fêmeas com abdômen arredondado e papila urogenital avermelhada para cada réplica do experimento, que foram pesados e separados por sexo. Eles foram mantidos em dois tanques (250 L), com temperatura de 26 ± 1°C, aeração e renovação constantes. Nessa espécie, foi realizada a reprodução induzida e a fertilização *in vitro*, a qual já é bem estabelecida nesse laboratório (BOMBARDELLI et al., 2006; GOES et al., 2016).

As fêmeas receberam uma injeção intramuscular na região dorsal contendo 0,5 mg de extrato de hipófise da carpa (CPE) por kg do peso corporal e após 12 horas receberam uma segunda dose de 5 mg de CPE por kg, com a finalidade de induzir a maturidade sexual. Já os machos receberam uma única dose 2,5 mg de CPE por kg do peso corporal, simultaneamente à segunda dose das fêmeas (FIGURA 6).

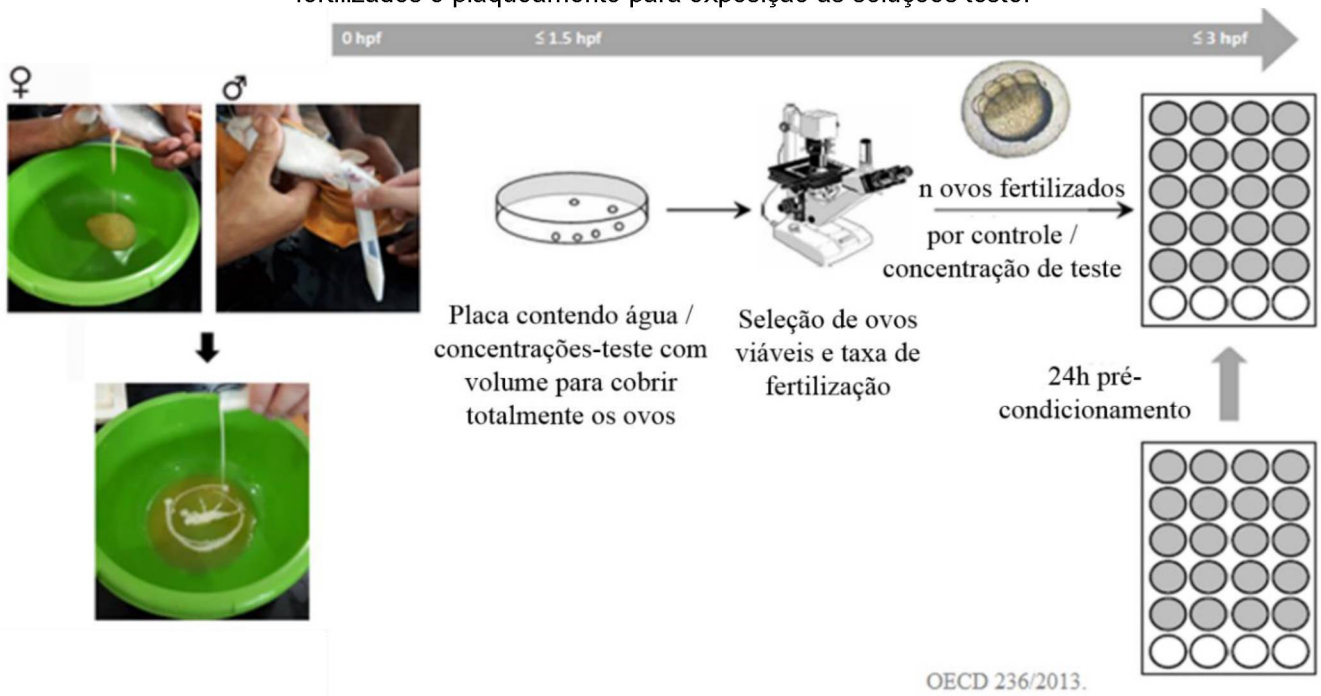
FIGURA 6. Indução hormonal por meio da injeção intramuscular de extrato de hipófise da carpa em *Rhamdia quelen*.

FONTE: A autora (2020).

A temperatura foi mantida em 26 °C e frequentemente monitorada para cálculo da hora-grau (HG). A HG é utilizada para estabelecer o tempo que as fêmeas levarão para desovar, esse valor é calculado em função da temperatura, no qual quanto maior a temperatura, mais rápido ocorrerá a liberação dos gametas (BOMBARDELLI et al., 2006). Para exemplares de *R. quelen* mantidos a 26 °C, HG foi de 240, assim a desova ocorreu nove horas após a segunda aplicação hormonal.

Para a coleta dos gametas, os animais foram anestesiados em solução de benzocaína (1 g de benzocaína em 10 mL de álcool, para 10 L de água), retirado o excesso de água e então foi realizada a massagem na região ventral no sentido encéfalo-caudal do peixe. A primeira porção de ovócitos e sêmen foram descartados para evitar contaminação. Os ovócitos foram coletados separadamente em bacias secas e o sêmen foi coletado em tubos Falcon (FIGURA 7).

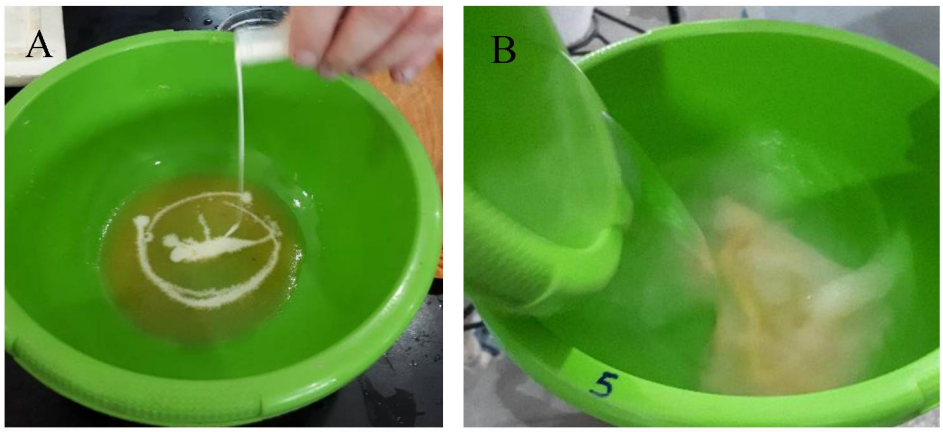
FIGURA 7: Obtenção dos ovos de *Rhamdia quelen* por meio de reprodução induzida e fertilização *in vitro* artificial, seguido do procedimento adotado para FEET teste, incluindo coleta, seleção de ovos fertilizados e plaqueamento para exposição às soluções teste.



FONTE: A autora. Adaptado de OECD (2013).

Os gametas femininos foram inseminados com um *pool* obtido do sêmen dos três peixes machos selecionados. Foi adicionada água de manutenção para hidratação do ovócitos e ativação espermática, na razão de diluição de 1:70 (sêmen: água), e homogeneizado suavemente por 60 segundos (BOMBARDELLI et al., 2006; GOES et al., 2016) (FIGURA 8).

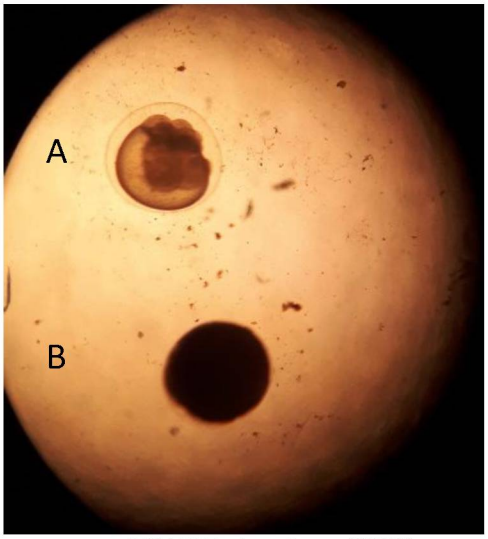
FIGURA 8. Fertilização *in vitro* de *Rhamdia quelen*. A. Inseminação dos ovócitos utilizando o pool de sêmen. B. Ativação espermática.



FONTE: A autora (2020).

Uma parte dos ovos foi coletada para a realização do FET e FEET teste e inspecionada em um estereomicroscópio, no qual os ovos não fertilizados e com irregularidades de clivagem ou lesões foram descartados (FIGURA 9). Outra parte dos ovócitos foram selecionados para avaliação dos biomarcadores e o restante foi levado à incubadora.

FIGURA 9. Diferença entre um ovo fertilizado (A) e um ovo coagulado (B) de *Rhamdia quelen*.



FONTE: A autora (2020).

3.4 *FISH EMBRYO EXTEND TOXICITY* (FEET) TESTE

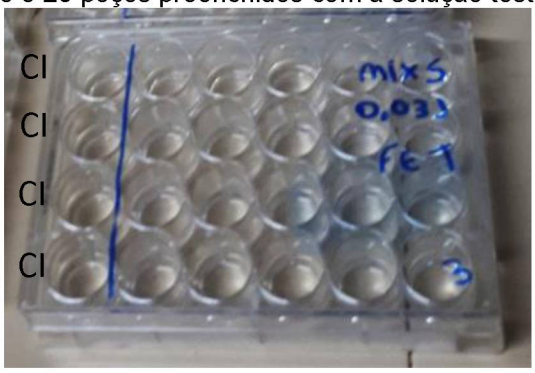
Os experimentos com *D. rerio* e *R. quelen* foram aprovados pelo Comitê de Ética em Uso Animal da Universidade Federal do Paraná, Brasil, com certificado nº 1138/2017.

O ensaio de toxicidade com embriões seguiu os critérios da OECD 236/2013, com o tempo de exposição padrão de 96 horas (FET teste) e com adaptação no tempo de exposição para 168 horas, o então chamado *Fish Embryo Extend Toxicity* (FEET) teste e aplicado nas espécies *Danio rerio* e *Rhamdia quelen*. Os ovos recém fecundados e com desenvolvimento embrionário normal foram separados, contados e transferidos aleatoriamente um por poço em placas de cultivo de 24 poços.

Em cada placa, haviam quatro poços de controle interno da placa (CI), preenchidos com 2 mL da água de manutenção (FIGURA 10), e vinte poços preenchidos com 2 mL da solução teste (solvente, DBP, DiPeP, MIX ou 3,4-dicloroalanina). Os testes foram realizados em triplicata, totalizando 60 ovos por

concentração. As placas foram mantidas em fotoperíodo 12h / 12h claro-escuro, em câmara climática ou sala climatizada a 27 ± 1 °C.

FIGURA 10. Montagem da placa de 24 poços para o FET e FEET teste com *Danio rerio* e *Rhamdia quelen*, sendo os quatro primeiros poços o controle interno da placa (CI), preenchido com água de manutenção e 20 poços preenchidos com a solução teste.



FONTE: A autora (2020).

O desenvolvimento embrionário das espécies *Danio rerio* e *Rhamdia quelen* foi analisado em estereomicroscópio nos períodos de 24, 48, 72, 76, 120, 144 e 168 horas após a fertilização e contabilizando os parâmetros de letalidade descritos pela OECD 236/2013: coagulação dos ovos, ausência de formação de somitos, ausência do destacamento da cauda do saco vitelino e a presença de batimentos cardíacos.

Também foram avaliados parâmetros de subletalidade nas duas espécies, como: deformações estruturais no córion, atraso na eclosão, alterações na pigmentação dos olhos e corpo, presença de hemorragias, edemas nas regiões pericárdica e no saco vitelino, não absorção do saco vitelino, microcefalia, olhos pouco ou não desenvolvidos, deformações no esqueleto, crescimento da larva, defeitos na bexiga natatória e ausência de equilíbrio, e também foi observado a presença de barbilhões reduzidos em *R. quelen*.

3.5 BIOMARCADORES BIOQUÍMICOS

Os biomarcadores bioquímicos foram analisados em 96 e em 168 hpf. Foram utilizados 10 *pools* por tratamento, sendo o NC, SC, grupos expostos ao DBP, DiPeP e a MIX. Em *Danio rerio* cada *pool* continha 13 larvas e em *Rhamdia quelen* cada *pool* continha 10 larvas, as quais continham a quantidade de proteínas suficientes para as análises.

Os *pools* foram congelados em ultrafreezer a -80°C até o momento da análise. Cada *pool* foi homogeneizado em 300 µL de tampão fosfato de potássio (0.1 M, pH 7,0), em seguida centrifugado por 20 minutos a 10.000xg a 4 °C. O sobrenadante foi utilizado nas medições das atividades enzimáticas e da peroxidação lipídica. A concentração de proteína total foi determinada pelo método de Bradford (1976), com albumina soro bovino como padrão.

3.5.1 Acetilcolinesterase

A atividade da acetilcolinesterase foi mensurada pelo método de Ellman et al. (1961) com modificações detalhadas a seguir: na microplaca, foram adicionados 25 µL de amostra, 200 µL de DTNB (5.5 - Ditio-bis-2-nitrobenzoato) a 0,75 mM e 50 µL do substrato acetiltiocolina a 7,5 mM. A placa foi incubada durante 5 min. Após esse período foi realizada a leitura em espectrofotômetro a 405 nm a cada 30 s durante 5 min e a atividade enzimática foi expressa em $nmol.min^{-1}.mg\ proteína^{-1}$.

3.5.2 Peroxidação lipídica (LPO)

A quantificação da LPO foi realizada por meio da avaliação da concentração de hidroperóxidos pelo ensaio FOX (*Ferrous Oxidation / Xylenol Orange Method*), de Jiang, Hunt e Wolff (1992). O sobrenadante das amostras foi ressuspendido em metanol (proporção 1:2 v/v) e centrifugado durante 5 minutos a 10.000x g a 4 ºC. Em seguida, 100 µL do sobrenadante foi adicionado a microtubos e incubado com 900 µL da solução reação (laranja de xilenol 100 µM, H_2SO_4 25 mM, BHT 4 µM, sulfato ferroso amoniacal 250 µM, metanol 90%) por 30 min. A leitura foi realizada em espectrofotômetro a 570 nm e os resultados foram expressos em µmol de $hidroperóxidos.mg\ proteina^{-1}$.

3.5.3 Glutationa-S-transferase (GST)

A atividade da GST foi mensurada pelo método de Keen, Habig e Jakoby (1976), com modificações detalhadas a seguir: adicionou-se 20 µL da amostra e 180 µL de solução reação (GSH a 1,5 mM, CDNB a 1,5 mM) em uma microplaca que foi

incubada por 5 min. A leitura foi realizada a 340 nm por 5 min a cada 30 s e a atividade enzimática foi expressa em $nmol.min^{-1}.mg\ proteína^{-1}$.

3.6 BIOMARCADOR GENÉTICO

O ensaio cometa na versão alcalina, descrito por Singh et al. (1988) e modificado por Ramsdorf et al. (2009) para tecidos sólidos, foi adaptado a fim de permitir a avaliação da genotoxicidade em larvas de peixe. Foi realizado o ensaio cometa em larvas de *D. rerio* e *R. quelen* nos tratamentos NC, SC, ao DBP, DiPeP e MIX, além do controle positivo da técnica nos tempos 96 e 168 hpf.

Foram montadas placas extras para a realização do ensaio cometa com 96 hpf, as quais ficaram nas mesmas condições das placas do FEET teste. Para o ensaio cometa de 168 hpf, foram utilizadas as larvas sobreviventes ao FEET teste. Para o controle positivo do ensaio cometa (PCC), foram selecionadas aleatoriamente 20 larvas pertencentes ao controle interno de cinco placas.

As placas foram mantidas durante 20 min em freezer à -20 ºC como forma de eutanásia das larvas (MATTHEWS e VARGA, 2012). Após a eutanásia, as larvas pertencentes a cada tratamento formaram um *pool* de larvas ($n \cong 20$). Cada *pool* foi colocado em um frasco de microcentrífuga do tipo eppendorfs, contendo 500 µL de soro bovino fetal, armazenadas sob refrigeração e na ausência de luz e então foram desagregadas mecanicamente em micro-homogeinizador. Após desagregadas, as larvas do PCC foram expostas à luz ultravioleta do tipo C durante 5 minutos (KOSMEHL et al., 2006).

Para montagem das lâminas, homogeinezou-se 120 µL de agarose de baixo ponto de fusão (LMP) com 120 µL da amostra e então dispensou-se 100 µL do homogeinizado em cada uma das duas lâminas previamente cobertas com agarose normal (totalizando 6 lâminas por réplica para todos os tratamentos) e mantidas em solução de lise por três horas. Após esse período, as lâminas foram imersas em tampão alcalino por 25 minutos, e as lâminas pertencentes ao PCC foram aleatoriamente distribuídas entre todas as corridas eletroforéticas. A eletroforese durou 25 minutos, a 25 V e 300 mA, e depois foram neutralizadas em tampão de neutralização (pH 7,5) e fixadas em álcool absoluto.

Antes da análise, as lâminas foram coradas com 20 µL de brometo de etídio. A análise foi em teste cego de 100 nucleóides por lâmina, em microscópio de epifluorescência com aumento de 400x (Leica®, modelo DMLS2). Os nucleóides foram classificados por análise visual em classes de 0 a 4, sendo: 0 = sem dano aparente, 1 = dano pequeno, 2 = dano médio, 3 = dano extenso e 4 =dano máximo, para posterior atribuição de escores (COLLINS et al., 1997; KOPPEN et al., 2017). Os escores são obtidos através da multiplicação do número de cometas encontrados em cada classe pelo valor da classe:

{(0 x nº dano 0)+(1 x nº dano 1)+(2 x nº dano 2)+(3 x nº dano 3)+(4 x nº dano 4)}

3.7 ANÁLISE ESTATÍSTICA

O Software BioEstat® 5.0 foi utilizado para a realização das análises. Utilizou-se o teste de normalidade *Kolmogorov-Smirnov* para verificar a normalidade da distribuição das variáveis analisadas e assim determinar a utilização de testes paramétricos ou não paramétricos.

No FEET teste, a regressão não-linear foi utilizada no cálculo da CL_{50} para estimar em qual concentração cada ftalato causaria 50% de mortalidade nos embriões e larvas de *D. rerio* e *R. quelen*. O teste ANOVA, seguido pelo teste de Múltiplas comparações de *Dunnett* foi utilizado para comparar as médias do controle negativo com os tratamentos, para cada uma das malformações.

Utilizou-se o Teste T de *Student* para analisar se houve diferença entre as médias dos ftalatos isolados com a mistura, dentro de uma mesma concentração. O *fold change* foi utilizado para mensurar a diferença entre a média da MIX com as médias de todas as concentrações dos ftalatos isolados. Para cada malformação obteve-se uma média geral para os ftalatos isolados (DBP com DiPeP) e dividiram-se as médias, assim, obtivemos uma razão que gerou os valores de *fold change*.

O teste ANOVA, seguido pelo teste de Múltiplas comparações de *Dunnett* também foram utilizados para comparar as médias entre os tratamentos com o controle na análise da atividade das enzimas GST e AChE e na quantificação da LPO. Já o teste não paramétrico de *Kruskal-Wallis*, seguido pelo teste de *Student-Newman-Keuls* foram utilizados para comparar as médias entre os tratamentos com o controle na análise do ensaio cometa.

Para todas as análises estatísticas serão considerados significativos os valores de $p < 0.05$.

4. RESULTADOS E DISCUSSÃO

CAPITULO I

DBP and DiPeP phthalates mixture increases oxidative stress and embryo-larval malformations in zebrafish

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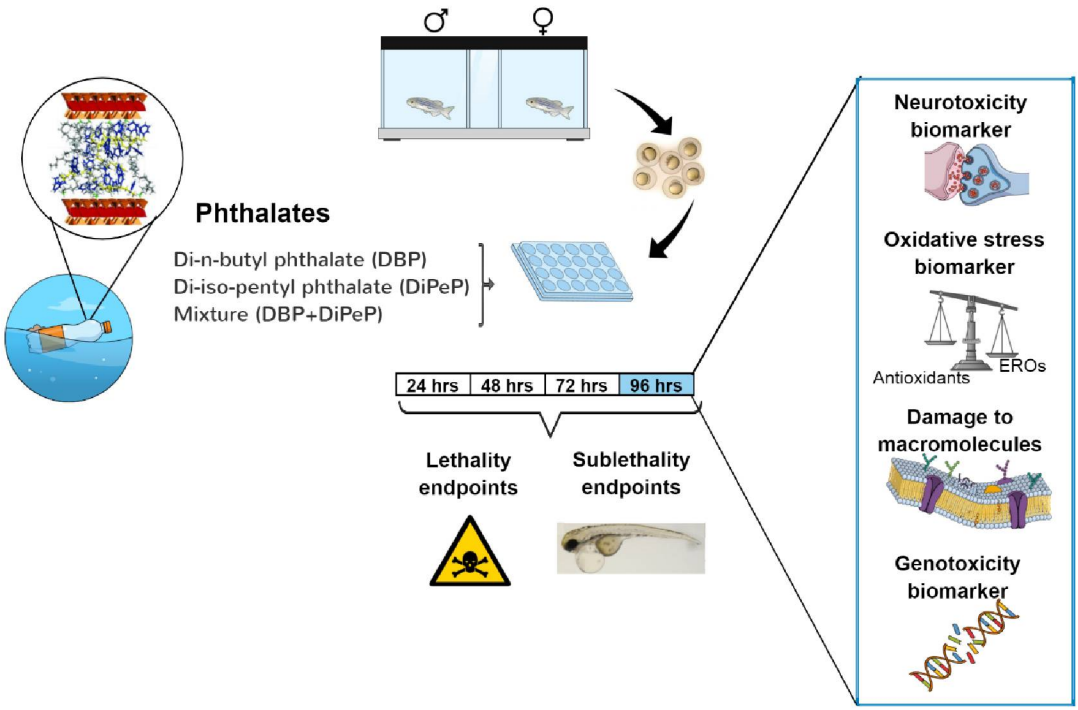

Graphical abstract 1.

ABSTRACT

Di-n-butyl phthalate (DBP) and di-iso-pentyl phthalate (DiPeP) are additives used to increase the flexibility of plastics. The majority of studies using isolated phthalates assess the disruption of the endocrine system. Thus, the objective of this study was to evaluate the toxicity of isolated DBP and DiPeP, and to evaluate whether the mixture of these contaminants causes higher toxicity. Fish Embryo Acute Toxicity (FET) test and biomarkers of neurotoxicity, oxidative stress, lipid peroxidation and genotoxicity in the *Danio rerio* larvae were performed. This research method followed the Organization for Economic Co-operation and Development (OECD) protocol 236/2013. Treatments were exposed to individual DBP and DiPeP in the ranges from 0.001 to 0.125 $mg.L^{-1}$ and combined groups exposed to a combination of DBP + DiPeP (MIX). The DBP caused mortality from 0.062 $mg.L^{-1}$, DiPeP at 0.125 $mg.L^{-1}$ and MIX at 0.031 $mg.L^{-1}$, indicating the toxicity potential of these phthalates. Phthalates isolated and mixed also induced neurotoxicity, oxidative stress and lipid peroxidation at low concentrations in the zebrafish larvae. We concluded that DBP was more toxic than DiPeP in aquatic organisms and MIX was more toxic than the isolated phthalates. Therefore, it is important to know the combined effects of phthalates.

Key words: acetylcholinesterase; comet assay; *Danio rerio*; glutathione S-transferase; lipid peroxidation.

Introduction

Phthalates or esters of phthalic acids are organic compounds widely used as additives and plasticizers in order to improve the flexibility, strength, and durability in various commercial products.[1,2] These compounds are not covalently bonded to the plastic matrix [3 4] easily reaching the environment.[5] The increasing use of phthalates resulted in the presence of these compounds in the soil, air, and water.[6,7,8] The presence of certain phthalates in the aquatic environment has been detected at concentrations of up to 0.05 $mg.L^{-1}$, which emphasizes the need of toxicological studies analyzing concentrations similar to those found in the environment.[2,9,10,11]

In humans and non-aquatic organisms, phthalates are absorbed orally or after inhalation and dermal contact and then metabolized and conjugated in the liver into more soluble metabolites for urinary excretion.[5,12] Phthalates are potent endocrine disrupters, with the ability to bind to hormone receptors and thus mimic or antagonize the action of the natural ligand hormone or to inhibit the biosynthesis of hormones, such as testosterone.[13,14] The toxic potential combined with the extensive use of phthalates has attracted much attention from the scientific community and regulatory agencies.[8,13]

The toxicity of phthalates is directly related to the side chain length of the ester molecule ranging from C1 to C13, in which the esters with 3 to 7 carbons in the linear portion of the side chain display higher antiandrogenic activity and reproductive toxicity.[7,13,15,16] Di-n-butyl phthalate (DBP) is a low molecular weight phthalate (C4) classified the European Agencies as reproductive toxicant category 1B, i.e., presumed to have reproductive toxicity potential for humans, based largely on experimental animal data.[6,8,13] DBP is among the most used phthalates in industry, and its applications range from medical products, automotive parts, toys, food packaging and personal hygiene merchandise.[6,7,17,18]

Di-iso-pentyl phthalate (DiPeP), also known as diisoamil phthalate (DiAP), is used in the production of propellants, nitrocellulose explosives and in PVC products, in addition to other polymers.[19] In Brazil, the local production of DiPeP is possibly favored by the high availability of isoamyl alcohol, a building block of DiPeP production that is also a byproduct of sugar fermentation in the ethanol production process[8]. In Brazil, DiPeP use was banned in personal care products, since 2016, but it is possibly used in several other industrial applications.[20] In addition, its presence has been

identified and quantified on the soil[21], in slurry samples present in landfills,[22] and its metabolites were found in the urine of pregnant Brazilian women.[8] DiPeP, which presents an intermediate side chain length with five carbons (four linear and a methyl branch), has been reported as a potent antiandrogenic phthalate, displaying higher endocrine disrupting activity than more commonly used phthalate esters like DBP.[8]

In a realistic situation, humans and animals are constantly exposed, through different routes, to mixtures composed of various phthalates.[23,24] Thus, experimental studies testing mixtures of different chemicals simultaneously might better represent the real exposure scenarios, since the risks of multiple exposures are usually unknown.[4,23,25] However, most studies use isolated compounds investigating their effects on the reproduction of mammals.[2,4,6,7,8,23,26]

The negative impacts of chemical contamination on the survival, growth and reproductive success should be evaluated on populations and communities.[13,27] Aquatic organisms are particularly vulnerable to the exposure of phthalates.[7] However, the ecotoxicological impact of these compounds in the embryonic development of fish is still unclear.[26] *Danio rerio* (zebrafish) have been used to study the effects of drugs and environmental contaminants in embryonic development.[28,29]

In addition, there are standard protocols available, such as the fish embryos acute toxicity test (FET) carried out by the Organization for Economic Co-operation and Development - OECD 236/2013.[28,30,31,32,33] The use of the FET test enables the exposure to multiple toxic agents simultaneously, as well as the analysis of biochemical biomarkers, thus providing information at the molecular level.[28,34] The addition of the FET biochemical and genetic biomarker test contributes to a better understanding of the mode of action of these compounds.[34]

Therefore, the aim of this study was to evaluate the toxicity of the mixture (MIX) of di-n-butyl phthalate (DBP) and di-iso-pentyl phthalate (DiPeP) as well as the isolated effects of each of these emerging contaminants. Toxicity was evaluated in the embryos and larvae of *Danio rerio*, by analyzing the developmental malformations (FET test), the possible changes in the activity of enzyme involved in neurotoxicity and in oxidative stress, lipid peroxidation and genotoxicity.

Material and methods

The experiments were approved by the Ethics Committee on Animal Use at the Federal University of Parana, Brazil (certificate No. 1138/2017).

Chemical products and test solutions

Di-n-butyl phthalate (DBP) CAS registry (Chemical Abstract Service) 84-74-2 (99%, Sigma-Aldrich®, Darmstadt, Germany) and di-iso-pentyl phthalate (DiPeP), CAS 605-50-5 record (99% Petrom® - Petrochemical Mogi das Cruzes SA, Brazil) were utilized in the experiments. The stock solutions were prepared at a concentration of 10 $mg.L^{-1}$ phthalate and used a Methanol solvent 0.1% v/v.[35]

All solutions were prepared using the fish system water (maintenance water). The concentrations were defined after testing for preliminary acute toxicity. The test solutions used were: DBP at concentrations of 0.001, 0.003, 0.007, 0.015, 0.031, 0.062 and 0.125 $mg.L^{-1}$; DiPeP at the same concentrations and MIX concentrations of 0.001, 0.003, 0.007, 0.015 and 0.031 $mg.L^{-1}$. Experiment controls were prepared as follows: negative control (NC, maintenance water), solvent control (SC, 0.1% methanol v/v) and positive control (PC, 4 $mg.L^{-1}$ 3.4 dicloroalanina [CAS 95-76-1, 99%, Sigma-Aldrich®, Darmstadt, Germany]).

Considering the results of preliminary toxicity tests, five lower concentrations of phthalates for the MIX composition have been defined. The mixtures were prepared at a 1:1 ratio DBP + DiPeP, ensuring that each phthalate contributes equally to the potential effects at each concentration of the mixture.

Zebrafish maintenance and embryo collection

Adults were kept in a recirculating zebrafish facility established at the Department of Genetics and Morphology in the University of Brasilia, Brazil (ZebTec - Tecniplast®, Italy) in water tanks filtered with activated carbon and reverse osmosis. The fish were kept in a cyclical photoperiod of 12:12h (light: dark); water temperature of 27 ± 1 °C; conductivity 750 ± 50µS/cm; pH 7.0 ± 0.5 and a saturation rate of dissolved oxygen at or above 95%. This maintenance water was used in the internal control, negative control and for preparation of the test solutions of all toxicity tests.

The embryos were obtained immediately after natural mating in the iSpawn system (Tecniplast®), and inspected under a stereomicroscope (Stemi 2000 Zeiss, Germany). The unfertilized eggs and those embryos with cleavage irregularities or injuries were discarded.

Toxicity test in zebrafish embryos

The FET test was performed according to OECD 236/2013.[30] The experiments were conducted in 24-well plates. Each plate contained four wells as an internal control plate (filled with 2 ml of water maintenance) and twenty wells were filled with 2 ml of the test solution. Fertilized eggs were randomly selected and carefully distributed, one per well, on the plate. Plates were kept in a climatic chamber at 27 ± 1 °C in a 12h light-dark cycle. Assays were performed in triplicate; totaling 60 eggs per treatment.

Embryo development was evaluated using a stereomicroscope (Carl Zeiss® Stemi 2000-C) for periods of 24, 48, 72 and 96 hours post fertilization (hpf). The following lethality parameters (according to OECD) were analyzed: coagulated embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat. Sublethal parameters were also analyzed: chorion structural deformations, the presence of hemorrhages and edema, yolk sac absorption, skeletal deformities, pigmentation of the eyes and body, reduced larva growth, inflation of the swim bladder and loss of equilibrium.

Biochemical biomarkers: neurotoxicity and oxidative stress

To quantify the activity of acetylcholinesterase (AChE), glutathione S-transferase (GST), and the levels of lipid peroxidation (LPO), pools consisting of 13 larvae each were utilized, which contained a sufficient amount of protein for the analyses of the biomarkers.

For the each group/treatment (NC, SC and DBP, DiPeP and MIX treatments) 10 pools were used, totaling 130 larvae. Each pool was homogenized in a 300 µL of 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged at 10.000xg for 20 minutes at 4 °C. The supernatant was used to measure the enzyme activities and lipid peroxidation. The total protein concentration was determined using Bradford method,[36] using bovine serum albumin as the standard.

Acetylcholinesterase activity was measured by the method of Ellman,[37] with the following modifications: in the microplate were added 25 µL of sample, 200 µL DTNB (5.5 - dithiobis-2-nitrobenzoate 0.75 mM) and 50 µL of 7.5 mM acetylthiocholine. The microplate was incubated for 5 min and reading was performed at 405 nm for 5 min every 30 s. Enzymatic activity was expressed in nmol. min^{-1}.mg $protein^{-1}$.

Glutathione-S-transferase activity was measured according to Keen et al,[38] with the following modifications: 20 µL sample was added to a microplate along with a 180 µL reaction solution (GSH 1.5 mM CDNB the 1.5 mM). The plate was incubated for 5 min and the reading was performed at 340 nm for 5 min every 30 s. Enzymatic activity was expressed in nmol. min^{-1}.mg $protein^{-1}$.

The analysis of lipid peroxidation was accomplished by evaluating the concentration of hydroperoxides using the FOX assay (ferrous oxidation / Xylenol Orange Method).[39] The supernatant was resuspended in methanol at 1:2 (v/v) and centrifuged for 5 minutes 10.000xg at 4 °C. Next, 100 µL of the supernatant was put in microtubes and incubated with a 900 µL reaction solution (100 µM xylenol orange, 25 mM H_2SO_4, BHT 4 µM, 250 µM ferrous ammonium sulfate, 90% methanol) for 30 min. The reading was performed at 570 nm and the results were expressed as µm hydroperoxides.mg. protein.

Genetic biomarker

To analyze the genotoxicity in larvae of *Danio rerio*, the alkaline comet assay was used,[40] modified by Ramsdorf,[41] with the following detailed changes: after the FET test, the surviving *D. rerio* larvae after 96 hours of exposure were euthanized for 20 min at 20 °C, and placed in microtubes containing 500 µL of fetal bovine serum. They were then stored in a cool dark environment.

The larvae belonging to the internal control of the plates were used as the positive control of the comet assay technique, and were exposed to an ultraviolet light of type C (UV-C, wavelength 320 nm) for 5 minutes.[42] The larvae pool (n = 20) were disaggregated in a microhomogenizer (Potter type) and then, were taken 120 µL and mixed and homogenized in 120 µL of agarose at a low melting point (LMP) and dispensed 100 µL on each of the two slides. The slides were then placed in a lysis solution for three hours. After this period, they were covered with an alkaline buffer for 25 minutes and then subjected to electrophoresis (25 V and 300 mA) for 25 minutes.

The slides were prepared in duplicate, for each replica of the FET test, totaling six slides per treatment. The slides were stained with 20 µL of ethidium bromide (10 µL/mL). The analysis was performed as a blind test with 100 nucleoids per slide, using an epifluorescence microscope at 400x magnification (Leica®, DMLS2 model). The nucleoids were ranked through a visual inspection and assigned scores for each class of DNA damage; 0 = no apparent damage, 1 = little damage, damage 2 = medium, 3 = extensive damage and 4 = maximum damage.[43,44] Score was obtained by sum of nucleoids number of of comets each class multiplied by its respective class.

Statistical analysis

The Software BioEstat® 5.0 was used for the analysis. The threshold significance used was 5% (p values <0.05). The Kolmogorov-Smirnov test was used to test the normality of the distribution of variables and to determine the use of parametric or non-parametric tests. The FET test recorded the presence of each of the developmental malformations and the mean value was then compared among treatments using ANOVA, followed by the Dunnett multiple comparison test. The same procedure was done for the analysis of biomarkers relating to neurotoxicity and oxidative stress. Student t-test was used to compare the means for each concentration of isolated phthalates and the mixture. Fold change to measure the increase in these means. For each malformation, a general means was obtained for the isolated phthalates (DBP + DiPeP) and the averages were divided, thus obtaining a ratio that generated the fold change values. To analyze the comet assay data Kruskal-Wallis one-way analysis of variance followed by the Student-Newman-Keuls test were used.

Results

Toxicity test in zebrafish embryos

The embryonic mortality in the negative control (NC) and in the solvent control (SC) groups were less than 10%. The positive control caused a mortality rate higher than 30%, in accordance with the quality standards set forth by the OECD, 236/2013.[30] The organisms belonging to the NC and SC groups showed normal embryonic development.[45]

The phthalates induced higher mortality rates of *Danio rerio* larvae after 96 hours of exposure in comparison to NC. DBP increased the mortality at concentrations of 0.062 $mg.L^{-1}$ and 0.125 $mg.L^{-1}$, while for DiPeP a higher mortality rate was induced only at the highest concentration (0.125 $mg.L^{-1}$). However, the MIX showed a higher toxicity than the two isolated phthalates, thus, causing a significant increase in mortality at a concentration of 0.031 $mg.L^{-1}$ (FIGURE 1).

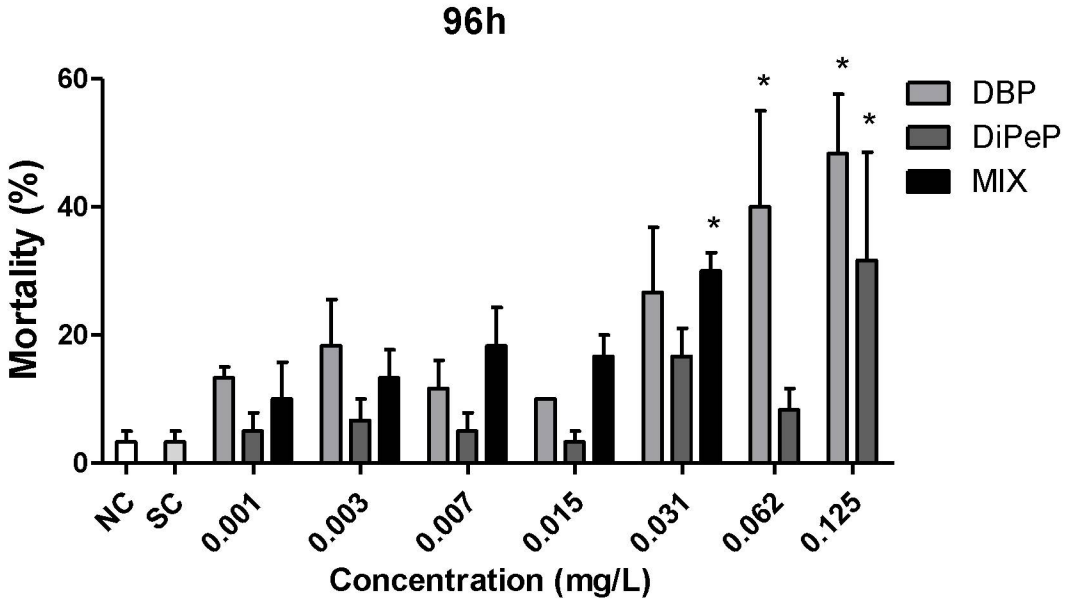

FIGURE 1. Percentage of mortality from FET test in *Danio rerio* embryos and larvae recorded at 96 hours of exposure to di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and the mixture (MIX). NC (negative control), SC (solvent control - 0.1% methanol v/v) and 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 mg.L-1 phthalate. MIX concentrations from 0.001 to 0.031 mg.L-1. (*) Indicates statistical differences compared to the negative control ($p < 0.05$).

The isolated phthalates caused a delay in the hatching of larvae exposed to all the tested concentrations of DBP and DiPeP at 48 hpf. In the mixture, the delay in

onset occurred only at concentrations of 0.003 and 0.007 mg.L^{-1} (FIGURE 2). The hatching rate was normal from 72 hpf.

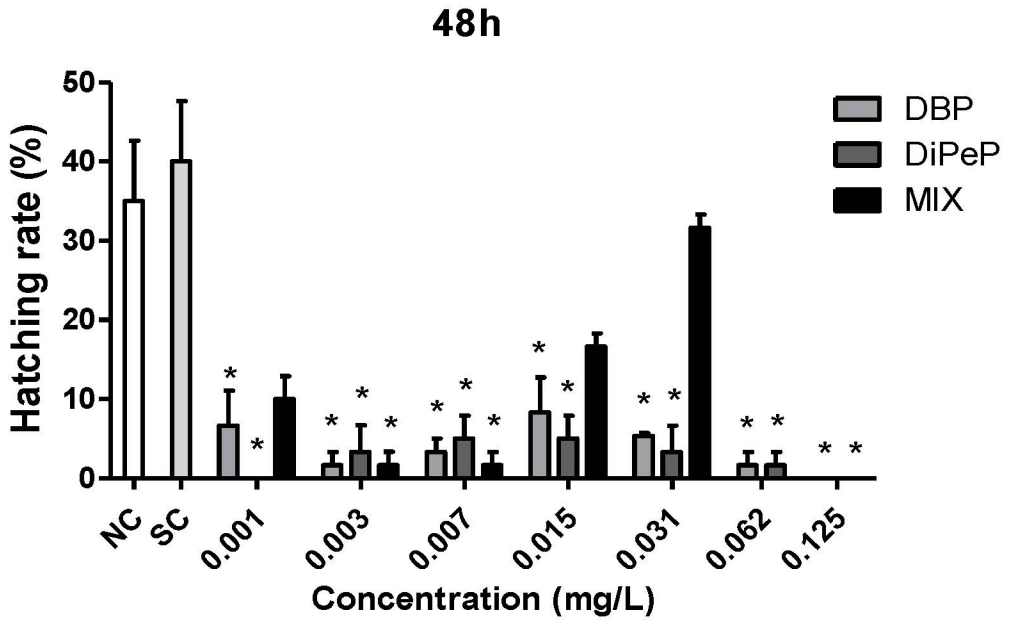

FIGURE 2. Hatching rate of *Danio rerio* larvae within 48 hours post fertilization exposed to di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and the mixture (MIX): NC (negative control); SC (solvent control - 0.1% methanol v/v) and 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 mg.L^{-1} phthalate. MIX concentrations from 0.001 to 0.031 mg.L^{-1}. (*) Indicates statistical difference compared to the negative control ($p < 0.05$).

At all times throughout the experiment, various malformations were observed by the exposure of isolated phthalates, and phthalates as a mixture (FIGURE 3).

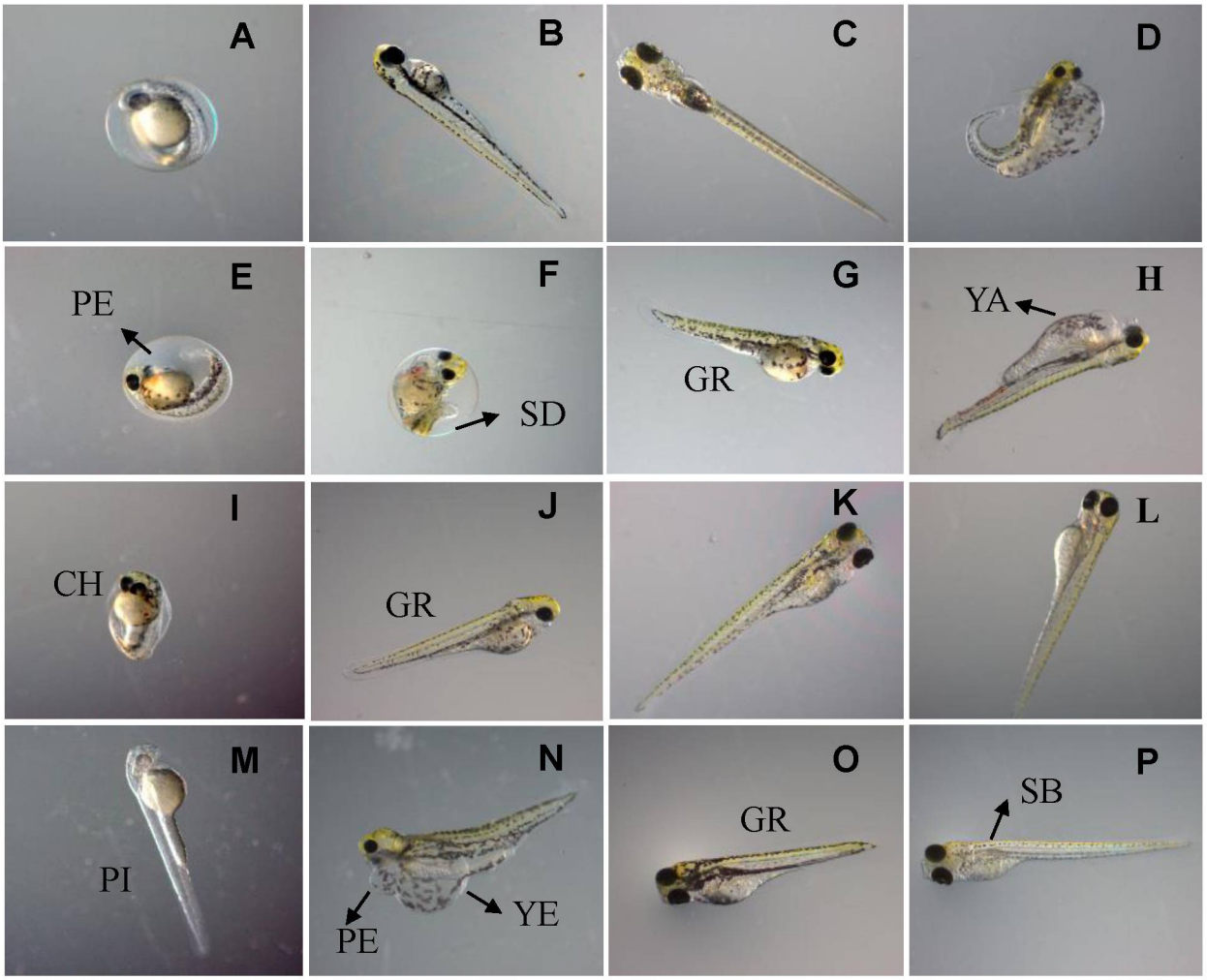

FIGURE 3. Embryonic development of *Danio rerio* observed in 24, 48, 72 and 96 hours post fertilization (photo enlargement at 3 x). Embryos and larvae belonging to the negative control group (A, B and C); Larvae belonging to the positive control group (D); Embryos and larvae exposed to di-n-butyl phthalate (DBP) (E, F, G and H); Embryos and larvae exposed to di-iso-pentyl phthalate (DiPeP) (I, J, K and L); Larvae exposed to the mix group (MIX) (M, N, O and P). PE = pericardial edema; SD = skeletal deformities; GR = reduced larva growth; YA = non yolk sac absorption; CH = chorion structural deformations; PI = altered pigmentation; YE = yolk sac edema; SB = uninflated swim bladder.

Deformations in the chorion structure were observed only after exposure to 0.125 mg.L^{-1} DiPeP at 48 hpf (TABLE 1). The DBP and MIX did not induce these deformations.

DBP induced pericardial edema (PE) from the concentration of 0.062 mg.L^{-1} at 48, 72 and 96 hpf. DiPeP caused PE from 48 hpf in the concentration of 0.125 mg.L^{-1}, after 72 hpf these malformations occurred appeared in the concentrations of 0.062 mg.L^{-1} and at 96 hpf DiPeP caused PE from 0.015 mg.L^{-1}.

Pericardial edema was observed in the MIX from 0.015 $mg.L^{-1}$ at 48 hpf, and from 0.001 $mg.L^{-1}$ at 72 and 96 hpf, in which the larvae belonging to all the exposure concentrations of MIX had such malformations (TABLE 1).

The PE malformations shown to have combined effects when the phthalates were in the mixture. On average, there was an increase of pericardial edema by 2.10 times in embryos and larvae exposed to the MIX when compared to those exposed to isolated phthalates (FIGURE 4 - A).

With the exposure to DBP at 0.125 $mg.L^{-1}$, yolk sac edema (YE) was observed at 24, 48, 72 and 96 hpf (TABLE 1). The exposure to DiPeP caused YE only 24 hpf in *D. rerio*. MIX induced YE with 0.031 $mg.L^{-1}$ at 24, 48 and 72 hpf, and at 96 hpf, YE was observed in the 0.003 $mg.L^{-1}$ MIX. The YE malformations have demonstrated combined effects in the MIX, exhibiting a mean increase of 2.83 times when compared to the isolated phthalates (FIGURE 4 - B).

Non yolk sac absorption (YA) was the most common malformation found with the exposure to isolated phthalates and the mixed group. YA was observed at 24 and 48 hours of exposure to the 0.125 $mg.L^{-1}$ of DBP at 72 hpf with the concentration of 0.031 $mg.L^{-1}$. In addition, at 96 hpf the exposed larvae at concentrations as low as 0.015 $mg.L^{-1}$ DBP also showed YA (TABLE 1). At 48 hpf, the concentration of 0.125 $mg.L^{-1}$ of DiPeP induced YA and after 72 hpf, YA was observed in larvae exposed to 0.031, 0.062 and 0.125 $mg.L^{-1}$ of DiPeP. In the MIX, YA defects were observed at 72 hpf and 96 hpf at all tested concentrations (TABLE 1). The combined effects of MIX were also observed in YA, at an increase of 2.42 times, in comparison to isolated phthalates (FIGURE 4 - C).

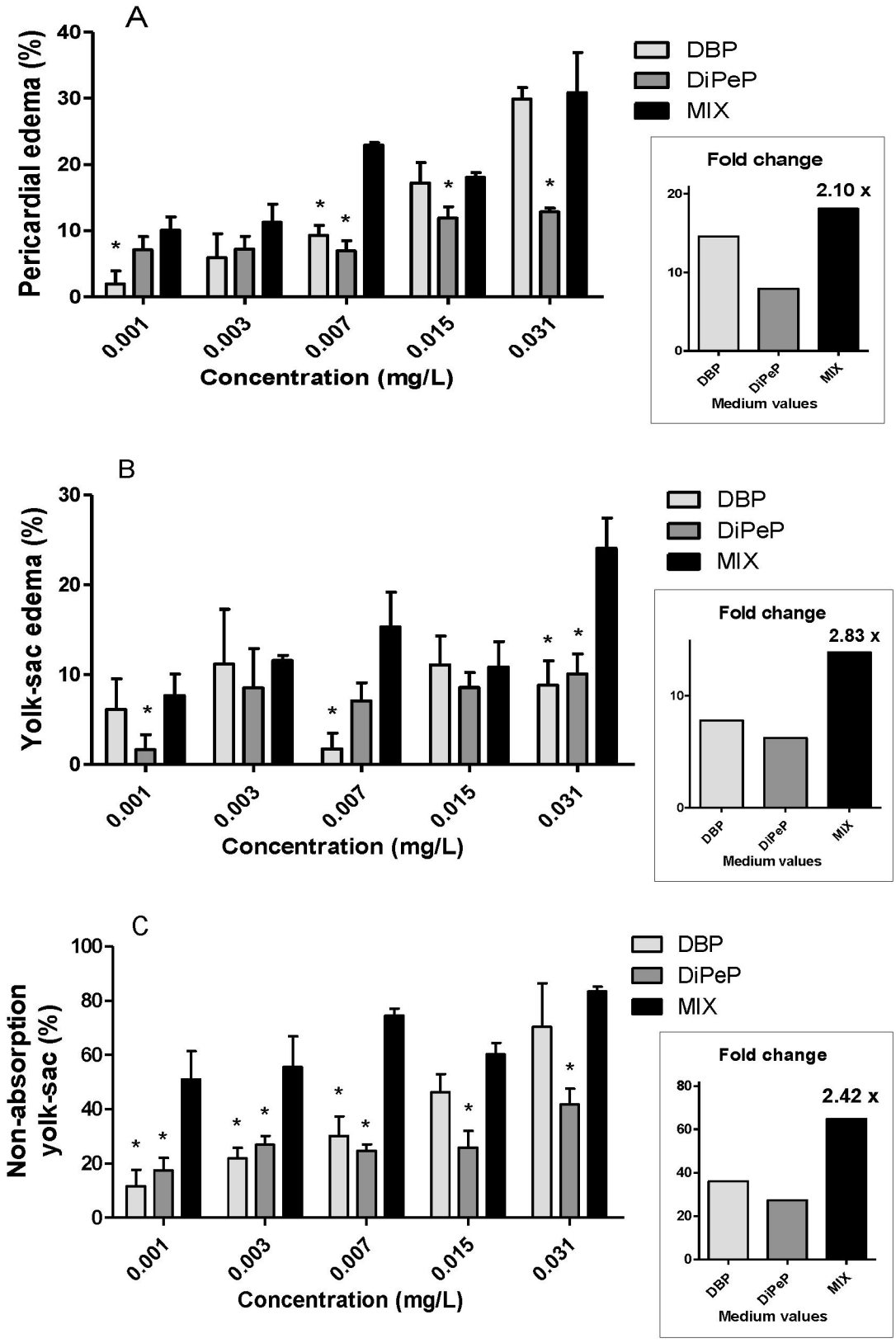

FIGURE 4. Combined effects of a mixture (MIX) between di-n-butyl phthalate phthalate (DBP), di-iso-pentyl phthalate (DiPeP) observed by an increase in the average malformations in *Danio rerio* larvae within 96 hours post fertilization. Analyses were carried out in triplicate. Student's t test, with $p < 0.05$. (*) indicates a statistical difference between the DBP and the MIX in the same concentration, and between DiPeP and the MIX in the same concentration ($mg.L^{-1}$). To calculate the fold change, was used a general

mean, considering all concentrations of isolated phthalates and a ratio was made with the average of the MIX. Fold change shows the mean increase in damage induced by the MIX when compared to isolated phthalates within each malformation: A - pericardial edema, 2.10 fold increase; B- yolk sac edema, an increase of 2.83 times; C- non yolk sac absorption, an increase of 2.42 times.

The reduction in the larva growth (GR) was observed at 96 hours of exposure to 0.125 $mg.L^{-1}$ of DBP, and at 72 hours of exposure to 0.031 $mg.L^{-1}$ of MIX. GR was not observed with the exposure to DiPeP. Apart from a decrease in body size, skeletal deformations were caused by DBP concentrations at 0.031, 0.062 and 0.125 $mg.L^{-1}$, and the concentrations of MIX at 0.015 and 0.031 $mg.L^{-1}$ (TABLE 1).

The altered pigmentation (PI) occurred in larvae exposed to the highest concentrations of DBP (0.125 $mg.L^{-1}$) and DiPeP (0.125 $mg.L^{-1}$) at 48, 72 and 96 hpf. The MIX caused less intense pigmentation in the concentration of 0.031 $mg.L^{-1}$ at 96 hpf (TABLE 1).

An uninflated swim bladder was observed at 96 hours of exposure to 0.031 $mg.L^{-1}$, 0.062 $mg.L^{-1}$, and 0.125 $mg.L^{-1}$ of DBP; 0.125 $mg.L^{-1}$ of DiPeP and 0.003 $mg.L^{-1}$, 0.007 $mg.L^{-1}$, 0.015 $mg.L^{-1}$ and 0.031 $mg.L^{-1}$ of the MIX concentration (TABLE 1).

Behavioral changes assessed by loss of equilibrium during swimming was observed in the exposed larvae only at 96 hpf: in the DBP concentration of 0.031 $mg.L^{-1}$; 0.125 $mg.L^{-1}$ of DiPeP, and from 0.003 $mg.L^{-1}$ of the MIX (TABLE 1).

TABLE 1: Sublethal effects caused by di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and the mixture (MIX) of di-n-butyl phthalate + di-iso-pentyl phthalate in *Danio rerio* larvae during 96 hours post fertilization (hpf).

Sublethal Endpoints	HPF	DBP ($mg.L^{-1}$)	DiPeP ($mg.L^{-1}$)	MIX ($mg.L^{-1}$)
Chorion structural deformations	48	ns	0.125	ns
Pericardial edema	48	0.062	0.125	0.015
	72	0.062	0.062	0.001
	96	0.062	0.031	0.001
Yolk-sac edema	24	0.125	0.125	0.031
	48	0.125	ns	0.031
	72	0.125	ns	0.031
	96	0.125	ns	0.003
Non yolk sac absorption	24	0.125	ns	ns
	48	0.125	0.125	ns
	72	0.031	0.031	0.001
	96	0.015	0.031	0.001
Skeletal deformities	48	0.031	ns	ns
	72	0.031	ns	0.015
	96	0.031	ns	0.015
Altered pigmentation	48	0.125	0.125	ns
	72	0.125	0.125	ns
	96	0.125	0.125	0.031
Reduced larva growth	72	ns	ns	0.031
	96	0.125	ns	0.031
Uninflated swim bladder	96	0.062	ns	0.031
Loss of equilibrium	96	0.031	0.125	0.003

Malformation observed 24, 48, 72 and 96 hours post fertilization (hpf) of exposure to di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and the mixture (MIX). Values indicate from which concentrations (mg.L-1) DBP, DiPeP, and MIX malformations were observed significantly different from the negative control. Analyses carried out in triplicate. ANOVA followed by Dunnett's test, where $p < 0.05$ significant. ns did not differ significantly from control. ns = not significant from the negative control.

Biochemical biomarkers: neurotoxicity and oxidative stress

Increased AChE activity was observed in the exposed larvae at 0.007 $mg.L^{-1}$ of DBP, 0.003 $mg.L^{-1}$ of DiPeP and at 0.003 $mg.L^{-1}$ and 0.007 $mg.L^{-1}$ in the MIX concentration (FIGURE 5 -A) compared to NC. Thus, demonstrating that the effects observed in biochemical biomarkers behave in a non-monotonic dose-response manner.

GST enzyme activity was significantly higher in the larvae exposed to 0.031 $mg.L^{-1}$ of the MIX (FIGURE 5 - B), while isolated DBP and DiPeP did not alter the activity of this enzyme.

There was an increase in the levels of LPO concentrations at 0.007 and 0.015 mg.L^{-1} of DBP, at the concentration of 0.003 mg.L^{-1} of DiPeP, and at 0.007 mg.L^{-1} and 0.015 mg.L^{-1} in the MIX (FIGURE 5 - C). The effect on LPO also appeared at low concentrations, again demonstrating that the standard dose-response is non-monotonic.

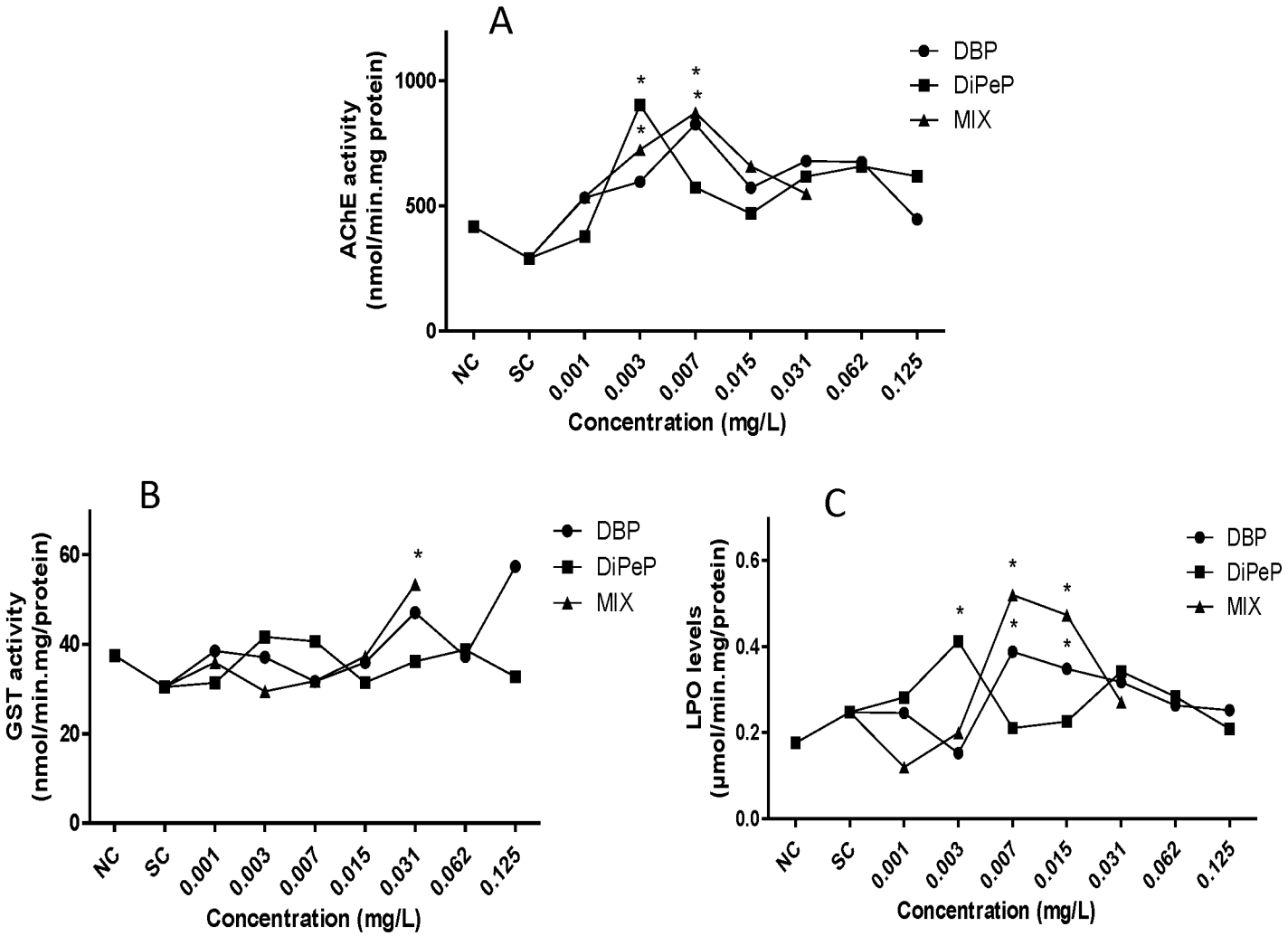

FIGURE 5. Biochemical biomarkers in *Danio rerio* larvae. Enzyme activity A- Acetylcholinesterase (AChE); B- Glutathione S-transferase (GST) and C- Lipid peroxidation (LPO), showing the pattern of nonmonotonic dose-response. AChE: nmol/mg.protein/min; GST: nmol/min.mg/protein; LPO: µmol/min.mg.protein. Comparison between treatments: negative control (NC), SC (solvent control - 0.1% methanol v/v) and the groups exposed to di-n-butyl phthalate, to di-iso-pentyl phthalate (0.001; 0.003; 0.007; 0.015; 0.031; 0.062 and 0.125 mg.L^{-1}) and the groups exposed to the mixture of DiPeP + DBP (0.001; 0.003; 0.007; 0.015; 0.031 mg.L^{-1}). Kruskal-Wallis followed by Dunn test, $p < 0.05$ considered significant. * indicate a significant difference with the negative control.

Genetic biomarker

No genotoxicity was observed in the larvae exposed to phthalates, in any of the tested concentrations of DBP, DiPeP and MIX in 96 hours post fertilization.

Discussion

In this study we evaluated the toxicity of different concentrations of a mixture of two phthalates, which proved to be more toxic than the isolated phthalates. It is important to consider that both humans and other animals are daily exposed to a mixture of phthalates,[4,8,23] and that the mixtures tested in our study, even at lower concentrations than those usually found in the aquatic environment (0.05 $mg.L^{-1}$),[2] caused mortality in zebrafish embryos.

The mortality observed in larvae exposed to DBP, DiPeP, and the MIX was significantly higher than the negative control at 96 hpf. According to Carvalho and Learmonth,[46] newly hatched larvae at 72 and 96 hpf, showed greater sensitivity than the embryos. As the gills have already been formed at 96 hpf, the uptake is more efficient in relation to xenobiotics by the larvae of *D. rerio*,[46,47] and thus, may increase the mortality rates as observed in the present study.

In this study, various sublethal endpoints were altered after the exposure to phthalates, which may due to the potential endocrine disrupting activities of these compounds. Considering the assessment of these parameters, the FET test is more sensitive to estimate the acute toxicity of the tested xenobiotics. Many of the sublethal parameters observed respond to neurotoxic compounds, and may have implications in the long term.[48,49,50]

In our study, we observed a delay in the hatching of larvae exposed to all DBP and DiPeP concentrations and to lower concentrations of the MIX. The hatching is the result of osmotic and enzymatic processes, and embryo movements.[33,51] The effect of these toxic substances on the hatching process can be derived from dysfunction, or even inhibition of corionase enzyme, thus, preventing the breaking of the chorion and thereby delaying the hatching of larvae.[51,52]

Some substances have the ability to cross the chorion barrier and reach the embryo.[53,54] After 26 hpf, the embryo enters the pre-hatching stage, and the chorion becomes more permeable due to an increase in proteolytic enzyme activity.[55] This increase in permeability appears to be related to the emergence of deformities in the corium, which were observed only at 48 hpf with DiPeP in this study. Interestingly, the chorion barrier is more efficient in relation to chemical compounds with higher molecular weights.[56] We observed that DiPeP had more difficulty in crossing the

chorion barrier in comparison with DBP, which is probably due to the fact that DiPeP has five carbon atoms (C5) in its ester side chain while DBP has only four (C4).

Pericardial edema (PE) is characterized by an increase in the accumulation of pericardial fluid, which can lead to serious changes in cardiovascular development and cause lasting effects to organisms.[48,51] The occurrence of pericardial edema coupled to the yolk sac edema (YE), which was observed in the present study, is known as blue sac syndrome, a non-infectious disease caused by exposure to chemicals.[57] The YEs are important toxicological endpoints and because of their role in the early stages of development of *D. rerio*, they may cause coagulation and embryonic death.[31]

YEs are associated with the non yolk sac absorption (YA). The yolk consists of vitamins, proteins and lipids, serving as a nutrient reserve until the larvae begin the feeding period. As this is a critical period, any abnormality in the consumption of yolk can affect the entire larval development and survival.[58,59] In our study, the YA appeared in embryos and larvae exposed to phthalates in a manner dependent on the concentration and exposure time. It is known that certain xenobiotics, including pharmaceuticals,[33,60] metals,[51,53,59] and DBP[58,61] can cause YA in fish embryos.

This delay in the yolk absorption can lead to a reduction in metabolic activity, and may affect the larva growth of the *D. rerio*.[59] Thus, we observed that the larvae had a reduction in body size (GR) when exposed to DBP and the MIX. The mechanisms of action that cause growth inhibition after phthalate exposures are still not completely known. However, studies suggest that phthalates may interact with growth hormones and delay development.[26,62,63] GR is an important variable for toxicity assessment[26] and the reduction in larval body size observed in our study may be linked to persistent growth deficits throughout the development and consequently long term effects up to adulthood.

The pigmentation in zebrafish embryos begins at 24 hpf with the emergence of melanophores,[45,64] which is important for fish survival, by helping to camouflage and avoid predators. Exposure to chemicals can lead to changes in hormonal control and cause abnormalities in the production of melanin[64,65,66] as it happened in the present work with the exposure to phthalates.

The swim bladder is responsible for adjusting the body density and fluctuation in fish.[26] The inflating of the swim bladder occurs at 72 hpf in *Danio rerio*.[45] Following exposures to high concentrations of DBP and the MIX, a delay in the development of the organ was observed. Defects in this organ impair vital functions of the animal such

as swimming, buoyancy and the search for food,[31] which in turn, may affect the equilibrium and the survival of the animals.

Swimming is essential for the survival of fish and changes in this pattern of larvae, are considered behavioral changes that may affect the search for food and reproduction. *Danio rerio* larvae are known as a reliable model for studying the behavioral effects caused by exposure to various pharmaceutical drugs, including mixtures of chemicals.[32] The loss of equilibrium occurs when swimming larvae at 96 hpf are located at the bottom of the microplate.[33] A previous study had shown that an exposure to 0.6 $mg.L^{-1}$ of di-ethyl-hexyl phthalate (DEHP), besides leading to a loss of equilibrium, caused the slowing of movements in the larvae.[1] In our study, the loss of equilibrium in the larvae exposed to DBP, DiPeP and to the lowest concentrations of the MIX, caused the larvae to stay at the bottom of the well in the microplate.

There are few studies analyzing ecotoxicological effects of phthalates in aquatic animals, particularly in the early stages of fish development.[2,26,33] Our results obtained through the FET test for lethal and sublethal parameters indicated the following scale with regards to potential toxicity: MIX> DBP> DiPeP. This is in contrast to the reported endocrine toxicity in rodents, which display higher susceptibility to DiPeP than DBP for the inhibition of testosterone production by fetal rat testes.[8,67] These observations support the notion that the susceptibility to environmental chemicals in a certain species cannot fully predict the toxicity to other organisms.

This work was the first to describe the ecotoxicological effects of a mixture of DBP and DiPeP in *Danio rerio* at environmentally relevant concentrations. Studies assessing the toxicity of mixtures of compounds are of great relevance, since the experimental conditions are more similar to an actual environmental setting, in which animals are exposed daily to a mixture of phthalates and other xenobiotics.[4,8,23,25] It is worth considering that each mixture will have a different combination effect, but in general we can deduce that mixtures between phthalates can be more toxic than isolated compounds.[4]

In general, exposure to low doses of xenobiotics initially results in subtle changes at the molecular level, posing difficulties in the identification and characterization of the exposure effects.[33] In our study, we found that changes in the biomarkers of neurotoxicity and lipid peroxidation have appeared in low concentrations, demonstrating that these biomarkers may be the first line of defense when exposure to phthalates occurs. The appearance of non-monotonic dose-

response curves or an inverted U curve, has already been described for endocrine disrupters such as phthalates, with different doses producing different effects.[68,69]

AChE is an enzyme important in neurotransmission and is an important biomarker of neurotoxicity of environmental compounds.[34,70] In association with the FET it can assist in identifying test compounds that alter the neurological development in fish.[48,71] In our study we observed an increase in AChE activity with exposure to DBP, DiPeP and MIX, however, the mechanism of action of phthalates that caused this increase, is not yet described. This increase in enzyme activity may result in cell apoptosis or cause changes in the autonomic nervous system, and consequently affect behavioral responses,[72,73] like the loss of equilibrium of *D. rerio* larvae exposed to DBP, DiPeP and MIX.

Phthalates can have multiple sites of interaction and interfere in different biological processes that can result in a wide range of effects, including androgen deficiency, altered cell cycles, changes in metabolic functions, neurotoxicity, behavioral changes and oxidative stress.[2,17,26,74,75] It has already been described that the exposure to DEHP and di-ethyl phthalate (DEP) can cause toxicity mediated by oxidative stress in fish embryos.[2,26] Oxidative stress occurs when there is an imbalance in the formation of reactive oxygen species (ROS), and the organism's ability to eliminate them.[17]

The main biological role of antioxidant enzymes such as glutathione S-transferase (GST) is to protect cells from harmful effects caused by ROS and other cell toxic molecules.[76,77] GST is also one of the most important metabolic enzymes and an increase in its activity is found in tissues such as the liver and gills, from oysters and birds exposed to DEHP.[76,78] The same occurred in our study, in which we found an increase in GST activity, but only when embryos were exposed to the highest concentrations of MIX, which may indicate a counter regulatory response[78] to the harmful effects of the phthalate association. Therefore, it is once again demonstrated that the MIX has higher toxicity than the isolated phthalates.

These results demonstrate that in *Danio rerio* exposed to phthalates can modulate this antioxidant defense system in order to prevent damage to macromolecules such as proteins, lipids and DNA.[26,78,79] ROS are highly reactive compounds and their accumulation can cause damage to macromolecules,[79] such as lipid peroxidation enzyme (LPO). The induction of LPO in phthalate exposed *Danio rerio* embryos indicates that LPO is a sensitive exposure biomarker. Other studies

confirm our results, in which phthalate exposure increased LPO levels in *Pimephales promelas* embryos exposed to DEP (10 mg.L^{-1}) and DEHP (1 mg.L^{-1}),[75] in *Xenopus laevis* embryos exposed to DBP (1.1 mg.L^{-1})[18] and in spermatocytes exposed to DEP, DEHP and DBP.[74]

The ecotoxicological impact of phthalate exposure during the early stages of fish life at environmentally relevant concentrations remains uncertain.[26] Considering that larval stage is a period of higher sensitivity to the action of these xenobiotics,[23] and normal development requires that critical events occur properly, the observation of malformations in embryos emphasizes the importance of understanding the mechanisms of toxicity of these compounds.

It is important to consider that different phthalates have different mechanisms of toxicity, which can result in a heterogeneity of responses to isolated compounds or mixtures[75] according to the experimental settings, life stage, and animal model. In our study, mortality and developmental abnormalities were observed in embryos and larvae exposed to the two isolated phthalates and their association at environmentally relevant concentrations. Some malformations appeared in both groups of isolated phthalates, DBP and DiPeP, while other were only seen after exposure to one of them. In general, the MIX was more toxic, thus causing these negative effects at concentrations below the isolated phthalates.

Conclusions

The tested phthalates showed differences in toxicity, where DBP was more toxic than DiPeP in *Danio rerio* larva, which is in contrast to rodent toxicity data. The mixture was more toxic than the isolated phthalates concerning the mortality rate, but also using the sublethal FET test parameters and assessing the activity of the GST. The evaluation of isolated compounds independently, can lead to an underestimation of the adverse effects of phthalates.

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CAPITULO II

Embryo-larval toxicity elicited by phthalates: Evaluation of the Fish Embryo Extend Toxicity (FEET) test in *Danio rerio* and transpose the test to *Rhamdia quelen*

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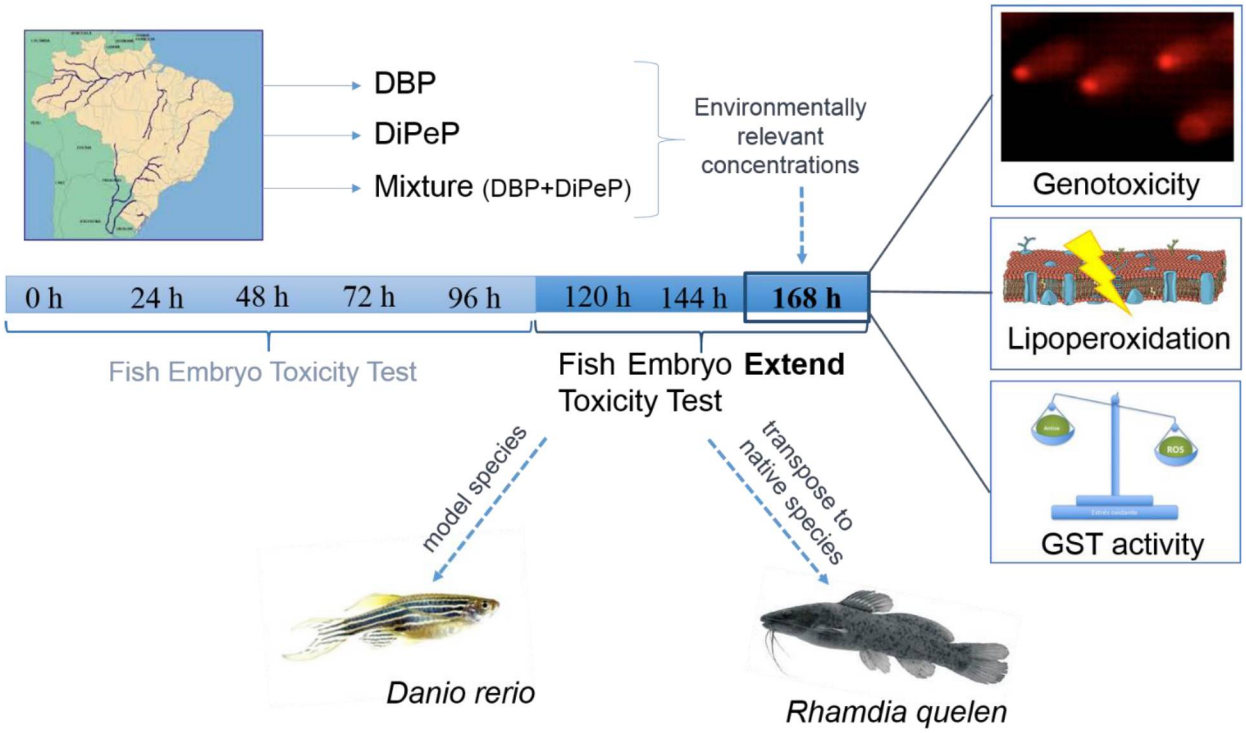


Graphical abstract 2.

ABSTRACT

The use of native species in toxicity tests should be considered, as they have greater ecological relevance. However, the protocols that evaluate toxicity indicate the use of model species, such as *Danio rerio*. In addition, the extended exposure period can be advantageous in assessment of sub lethal effects. Thus, the objective of this study was to transpose the Fish Embryo Extend Toxicity (FEET) test in native species *Rhamdia quelen*, evaluating endpoints of lethality, sublethality, as well as genetic and biochemical biomarkers, after 168 hours of exposure to di-n-butyl phthalate (DBP) and di-iso-pentyl phthalate (DiPeP), isolated and in a mixture. The methodology followed the OECD test guideline 236, with an adaptation in the time of exposure. The embryos were exposed to phthalates in concentrations ranging from 0.001 to 0.125 $mg.L^{-1}$. Phthalates induced mortality, embryonic development malformations, genotoxicity, lipid peroxidation and changes in GST activity. *R. quelen* was more sensitive to exposure to phthalates when compared to *D. rerio*. The embryonic malformations were observed in lower concentrations in MIX tham isolated phthalates. We conclude that the transpose of FEET test to *R. quelen* was feasible, and can be an alternative for embryos and larvae toxicity tests.

Key-words: biomarkers; di-n-butyl phthalate; di-iso-pentyl phthalate; genotoxicity; glutathione-S-transferase; lipid peroxidation.

1. Introduction

The fish embryo toxicity (FET) test is based in the test guideline N°. 236 of the Organization for Economic Co-operation and Development (OECD, 2013), and was proposed as a substitute test for acute toxicity in fish (Oliveira de Farias et al., 2019). FET is considered a refinement within the 3Rs principle in the alternatives to animal testing, which was proposed by Russell and Burch in 1959, being one of the most rigorously validated available methodologies, utilized as an alternative to fish *in vivo* exposure (Braunbeck et al., 2014).

Some adaptations to FET test allow for better evaluation, as extension of the exposure time from 96 to 168 hours after fertilization (hpf) (Oliveira de Farias et al., 2019), was called Fish Embryo Extend Toxicity - FEET test. The extended exposure period enables the assessment of sub lethal effects on growth, locomotor activity and survival, and in some cases, observe the mode of action of xenobiotics, such as those with neurotoxicants (Norberg-King et al., 2018; Oliveira de Farias et al., 2019).

The FET test has been used to evaluate endocrine disrupting compounds (EDC), because it is can harm animal health (Segner, 2009, Fraser et al., 2017). EDC, as phthalates, have received great attention from researchers, due to toxic effects on the development of mammalian reproductive organs (Benjamin et al., 2017; Machtinger et al., 2018; Seyoum and Pradhan, 2019). Phthalates have a high production volume, used mainly as plasticizer and found in several industrialized products (Machtinger et al., 2018; Seyoum and Pradhan, 2019), like sealants, adhesives, automotive parts, paints, clothing, toys, food packaging, medical and personal care products (Martino -Andrade et al., 2006; Ventrice et al., 2013; Benjamin et al., 2017; Rocha et al., 2017; Xu and Gye, 2018).

Di-n-butyl phthalate (DBP) is one of the most widely used plasticizers in the world, being widely used in many consumer products (Ventrice et al., 2013; Rocha et al., 2017; Xu and Gye, 2018). Di-iso-pentyl phthalate (DiPeP) use as plasticizer is common in Brazil, because is a secondary product of ethanol fuel production (Souza et al., 2018). Whereas, has been reported only in Brazil in environmental samples and the presence of DiPeP metabolites was already quantified in biological samples (Do Nascimento Filho et al., 2003; Ferreira and Morita, 2012; Rocha et al., 2017; Souza et al., 2018).

Phthalates are not chemically linked to the polymeric structure of plastics, thus, they are easily released into the environment (Christen et al., 2012; Rocha et al., 2017; Molino et al., 2019). In addition, the large production of phthalates causes water contamination, threatening aquatic life (Katsikantami et al., 2016; Seyoum and Pradhan, 2019). These compounds can precipitate in slow flow water bodies, such as lakes (Cheng et al., 2019), places where fish live, like the brazilian native species *R. quelen* (Guiloski et al., 2017).

Danio rerio (zebrafish) has been used in toxicological studies for more than 60 years due to characteristics such as abundant spawning, rapid embryonic development (TABLE 1) and transparent embryos (Gamse and Gorelick, 2016; Rodrigues de Oliveira et al., 2016; Andrade et al., 2018; Oliveira de Farias et al., 2019). The use of *D. rerio* in tests is advantageous because of its tolerance to environmental changes (Lawrence, 2007). However, is important to consider that these characteristics can make this species more resistant to the action of some xenobiotics, and so, don't reveal the real impact of this contamination.

The species sensitivity must be considered in monitoring and predicting the effects of contaminants on the aquatic environment (Connon et al., 2012). Exotic species have ecological relevance restricted to their place of origin (Schreiber et al., 2017), whereas native species are more representative in ecotoxicological tests (Martins and Bianchini, 2011). Thus, it is important to develop the FET test for other fish species and validate these methods according to OECD criteria (Lammer et al., 2009; Dang et al., 2017). The transposition of FET test guideline to other fish species such as *Misgurnus fossilis* native to Europe (Schreiber et al., 2017), *Oryzias latipes* native to Asia (Lammer et al., 2009; Schiller et al., 2014), *Pimephales promelas* native to North and Central America (Lammer et al., 2009) and *Clarias gariepinus* native of Africa (Dang et al., 2017) has proved to be viable and ecologically relevant.

Little is known about the effects of xenobiotics on the embryonic development of native fish species such as *Rhamdia quelen* (Brito et al., 2017). *R. quelen* is a freshwater fish popularly known as Jundiá, native to South America and of great economic importance in the southern region of Brazil (Bombardelli et al., 2006; Azevedo-Linhares et al., 2018). This species has potential for aquaculture because it has easy reproduction, high rate of induced spawning fertilization and rapid embryonic development (TABLE 1) and growth (de Amorim et al., 2009, (Rodrigues-Galdino et al., 2009; Brito et al., 2017). This catfish is a species susceptible to contaminants effect,

as shown in the studies that evaluated multiple biomarkers (Mela et al, 2013; Pereira et al., 2016; Perussolo et al., 2019).

Thus, the aim of this study was to evaluate whether *Rhamdia quelen*, as a brazilian native species, is a viable option to replace the model *Danio rerio*, given the importance of native species to ecotoxicological studies. To reach this aim were compared parameters obtained from FEET test in both especies, such as mortality rate, embryonic development malformations, genotoxicity, lipid peroxidation and antioxidant enzyme. Both species were evaluated after 168 hours of exposure to DBP and DiPeP isolated and in a mixture.

TABLE 1. Comparison between the start time and duration of the main stages of the embryonic development in time (in hours and minutes) of *Danio rerio* at temperature 28.5 °C and *Rhamdia quelen* at temperature 24 and 27 °C.

Stage	Characteristics	Time after fertilization	
		Danio rerio	*Rhamdia quelen*
Zygote	Animal pole without segmentation	0 t - 45min	0 t - 1h
Cleavage	Morula	45min - 2h	1h - 1h 45min
	Blastula	2h 20min - 3h 15min	2h - 4h 45min
Gastrula	Epiboly	5h 15min	5h 15min
Segmentation	Cephalic organization and differentiation	10h	9h
	Notochord	17h	9h
	Kupffer's vesicle	18h	10h
	Otic vesicle	18h	11h
	Neural groove	19h	7h
	Detachment of the tail	18h	11h
	Spontaneous movements	35h	11h
Pharyngula	Heart beats and blood circulation	36h	18h
	Eye and yolk pigmentation	36h	30h
	Mandible	42h	19h
Larvae	Hatching	48h	20h
	Digestive system	60h	79h 03min
	Swim bladder inflates	72h	39h
	Gill	72h	54h 50min
	Exogenous feeding	72h	90h
	Larva presenting the three pairs of barbels	-	66h
	Body anatomy similar to that of adult fish	120h	123h 15min

Adapted from Kimmel et al. (1995), Pereira et al. (2006) and Rodrigues-Galdino et al. (2009).

2. Material and methods

Experiments were approved by the Ethics Committee on Animal Use at the Federal University of Parana (CEUA/UFPR), Brazil, at certificate number 1138/2017.

2.1 Danio rerio maintenance and embryo collection

Adults fish were kept in a controlled photoperiod cycle (12: 12 light and dark) in the ZebTEC recirculation system (Tecniplast®, Italy), in tanks with filtered water at a temperature of 27 ± 1 °C; conductivity of 750 ± 50 µS/cm; pH 7.0 ± 0.5 and dissolved oxygen above 95% saturation (water maintenance – *D. rerio* tests). The embryos were obtained in an iSpawn system (Tecniplast®) after being laid, they were immediately collected and inspected under a stereomicroscope (Stemi 2000, Zeiss® 3.5x).

2.2 Induced reproduction and in vitro fertilization

Three females and three males specimens of *Rhamdia quelen* were selected for each experiment. The fish were weighed, sexed and kept in two tanks (250 L) at a temperature of 26 ± 1 °C, with constant refining and aeration (water maintenance – *R. quelen* tests). In order to induce sexual maturity, females specimens were subjected to an intramuscular injection in the dorsal region containing 0.5 mg of carp pituitary extract (CPE)/kg of body weight. After 12 hours, the same specimens received a second dose of 5 mg CPE/kg of body weight. Male specimens received a single dose of 2.5 mg of CPE/kg of body weight, simultaneously with the second dose of CPE administered to females (Bombardelli et al., 2006).

The hour-degree calculate is performed to know how long the females will spawn. The higher the temperature, the faster the oocytes will be released, for specimens of *R. quelen*, the spawning occurs after 240 our-degree (Bombardelli et al., 2006). Given its importance to the reproduction, the temperature was maintained at of 26 ± 1 °C and frequently monitored, thus, the gametes were collected dry after nine hours of second hormonal application.

The animals were anesthetized with benzocaine solution (1 g benzocaine/ 10 mL alcohol/ 10 L water). The gametes were colleted by abdominal pressure with movements in the craniocaudal direction. The first collection of oocytes and semen

obtained were discarded to avoid contamination. Finally, the oocytes were collected into a Petri dish (100x20, Kasvi®, vidro) and the semen was also collected separately in graduated tubes (Falcon, polipropileno, 15 mL).

After collection, each female gametes were inseminated with a pool of semen from the three male specimens. Sperm activation was dilution ratio 1:70 dilution ratio (semen: water) was used and gently homogenized for 60 seconds (Bombardelli et al., 2006; Goes et al., 2016). A portion of the embryos were collected for the FEET test and inspected on a stereomicroscope (ES-200, Marte Científica® 3.5x).

2.2 Test substances

3.4-Dichloroaniline , [CAS registry N° 95-76-1 (99%)] was obtained from Sigma-Aldrich®, Darmstadt, and used as a positive control (PC) of the FEET test. The concentrations used were of 4 $mg.L^{-1}$ for *Danio rerio* (as recommended by OECD 236/2013) and 2 $mg.L^{-1}$ for *Rhamdia quelen*, defined by preliminary acute toxicity test. Di-n-butyl phthalate (DBP) [CAS registry N° 84-74-2 (99%)] was obtained from Sigma-Aldrich®, Darmstadt, Germany. Di-iso-pentyl phthalate (DiPeP) [CAS registry N° 605-50-5 (99%)] was obtained from Petrom® - Petrochemical Mogi das Cruzes S.A., Brazil.

The maintenance water is the system water, where the fish were kept and were used in all test solutions. After 96 hours of exposure the solutions were renewed on the plates, in order to maintain test concentrations and water oxygenation. Preliminary assays were done to define the concentrations exposure. Methanol 0.1% v/v was chosen as a solvent control (SC) (Chen et al., 2014). The treatments were: negative control (NC); SC; DBP in concentrations 0.001, 0.003, 0.007, 0.015, 0.031, 0.062 and 0.125 $mg.L^{-1}$, DiPeP at the same concentrations and mixture of compounds at a dilution ratio of 1 DBP: 1 DiPeP in concentrations as follows: 0.001, 0.003, 0.007, 0.015 and 0.031 $mg.L^{-1}$.

2.4 Fish Embryo Extend Toxicity (FEET) test

The fish embryo toxicity test followed the guideline of OECD nº 236/2013, with adaptation in the exposure time to 168 hours, which is called *Fish Embryo Extend Toxicity test* (FEET) and applied in *Danio rerio* and *Rhamdia quelen* species.

The newly fertilized embryos with normal embryonic development were separated, counted and randomly transferred, one per well in 24-well culture plates, filled with 2 mL of the test solution in each well. Each plate contained 20 wells of the test solution and 4 wells as an internal control for plate filled with 2 mL of water maintenance. The assays were performed in triplicate, adding up to 60 embryos per treatment. After setting up the experiment, the plates were maintained in a climatic chamber (at 27 ± 1 °C, *D. rerio*) or in an air-conditioned room (at 26.5 ± 1 °C, *R. quelen*) with a controlled photoperiod (12h light/ 12h dark).

Embryonic and larval development of the species *Danio rerio* and *Rhamdia quelen* were analyzed in stereomicroscope (Stemi 2000, Zeiss®, 2x) for 168 hours post fertilization (hpf), accounting for the lethality parameters described by OECD guideline number 236/2013, as follows: coagulated embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat. Sublethal parameters were also evaluated, as follows: absence of hatching, altered pigmentation, dwarfism, defect in gas bladder, loss of equilibrium, heart malformation, hemorrhage, microcephaly, non-yolk sac absorption, pericardial edema, reduced barbels, skeletal deformities, undeveloped eyes and yolk-sac edema.

2.3 Genetic biomarker: alkaline comet assay

Alkaline comet assay, described by Singh et al. (1988) and modified by Ramsdorf et al. (2009) for tissue assay, was adapted to allow the evaluation of genotoxicity in fish larvae. The surviving larvae at 168 h of the FEET test were euthanized in a freezer at -20 ºC (Matthews and Varga, 2012). Then, each larvae pool (n = 20) were placed in microtubes containing 500 µL of fetal bovine serum and mechanically disaggregated in a micro homogenizer in a cool and dark environment.

For the positive control of the technique, 20 larvae belonging to the internal control of the plates were exposed for 5 minutes to ultraviolet light of type C (UV-C, wavelength 320 nm) (Kosmehl et al., 2006). For slide mounting was used 120 µL of the samples and were homogenized with 120 µL of agarose at low melting point, dispensed 100 µL on each of the two slides previously covered with normal agarose. The slides were kept in a lysis solution [NaCl (2.5 M), EDTA (100 mM), Tris (10 mM), NaOH, N-lauryl-sarcocinate (1%); Triton X 100 (1%), DMSO (10%)] for three hours and then immersed in alkaline buffer for 25 minutes, and subjected to electrophoresis

for 25 minutes, at 25 V and 300 mA. After that, slides were neutralized (Tris – HCl, 0.4 M, pH 7.5), and fixed in absolute ethanol.

The slides were stained with ethidium bromide (2 $\mu g.mL^{-1}$). The analysis of slides was performed as a blind test with 100 nucleoids in epifluorescence microscope at 400x magnification (Leica®, DMLS2 model). The nucleoids were classified through a visual analysis of DNA damage from 0 to 4 and subsequent attribution of scores from 0 to 4 (Collins et al., 1997; Koppen et al., 2017). The slides were prepared in duplicate for each replica of the FEET test.

2.6 Biochemical biomarkers

The analysis of biochemical biomarkers was performed with 10 pools of larvae. Pools contained 13 larvae for *D. rerio* and 10 larvae for *R. quelen*, which contained sufficient protein for analysis. Pools of larvae was homogenized in a 300 µL of 0.1 M potassium phosphate buffer (pH 7.0), then centrifuged for 20 minutes at 10.000xg at 4 °C. The supernatant was used to measure enzyme activities and lipid peroxidation. The total protein concentration was determined with bovine serum albumin as a standard, using the Bradford method (1976).

The analysis of lipid peroxidation (LPO) was performed by assessing the concentration of hydroperoxides using the FOX assay (Ferrous Oxidation / Xylenol Orange Method), described by Jiang et al. (1992). The supernatant was resuspended in methanol (1: 2 v/v ratio) and centrifuged for 5 minutes at 10.000x g at 4 ºC. Then, 100 µL of the supernatant was put in microtubes, incubated with 900 µL of the reaction solution (100 µM xylenol orange, 25 mM H_2SO_4, BHT 4 µM, 250 µM ferrous ammonium sulfate, 90% methanol) during 30 min. The reading was performed at 570 nm. The results were expressed in µmol hydroperoxides.mg $protein^{-1}$.

Glutathione-S-transferase (GST) activity was measured by the method of Keen et al. (1976), with some modifications: 20 µL of the sample was added in a microplate and 180 µL of the reaction solution (GSH 1.5 mM, CDNB 1.5 mM) and the plate was incubated for 5 min. The reading was performed at 340 nm for 5 min every 30 s. The enzymatic activity was expressed in nmol. min^{-1}.mg $protein^{-1}$.

2.7 Statistical analysis

The Kolmogorov-Smirnov normality test was applied to determine the use of parametric or non-parametric tests. The non-linear regression was used to estimate the concentration of the phthalate that causes mortality of 50% of the larvae, the LC_{50} (Lethal concentration). The ANOVA test followed by the Dunnett multiple comparison test was done to evaluate the lethality and sublethality endpoints in the FEET test, in the analysis of GST enzyme activity and in lipid peroxidation. The analysis of CAT activity as well as the comet assay analysis, the Kruskal-Wallis test was used, followed by the Student-Newman-Keuls test. The threshold significance used was 5% (p values < 0.05).

3. Results

3.1 Transpose of FEET test to Rhamdia quelen

Rhamdia quelen matrix had an average weight of 253.7 g for females specimens and 175.7 g for males specimens. The percentage of fertilization was greater than 70% in all artificial reproductions.

The induced reproduction and in vitro fertilization was adequate to obtain a large number of embryos of *R. quelen*. The embryos were kept at constant temperature 26.5 ± 1 ºC and embryonic development occurred normally, according to Pereira et al., 2006 and Rodrigues-Galdino et al., 2009 in animals belonging to the internal, negative and solvent control treatments.

3.2 Fish Embryo Extend Toxicity (FEET) test

Embryonic and larval mortality in NC and SC controls were less than 10% (FIGURE 1) for both species during the 168 hours. The quality standards set forth by OECD guideline n° 236/2013 fulfilled for both species utilized: *Danio rerio* and *Rhamdia quelen*.

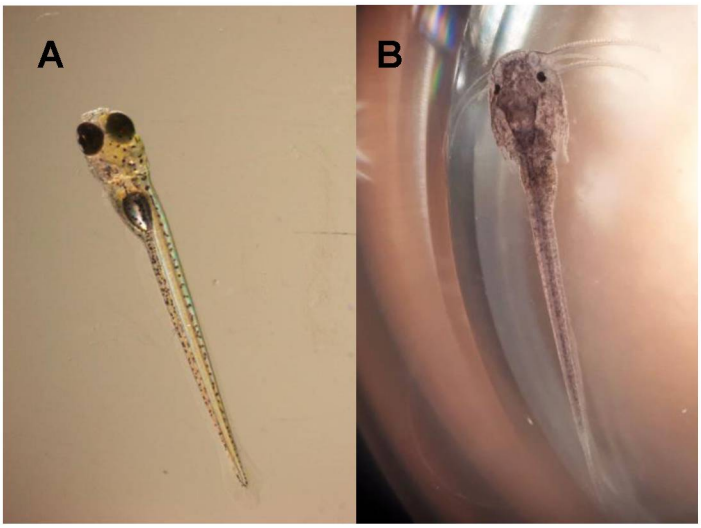


FIGURE 1. A: *Danio rerio* larvae belonging to negative control (NC), 168 hours post fertilization. B: *Rhamdia quelen* larvae belonging to negative control (NC), 168 hours post fertilization. Photo enlargement at 4x.

Positive control caused mortality greater than 30% in both species, however, for *D. rerio* we used the concentration 4 mg.L^{-1} of 3.4-Dichloroaniline, defined by OECD guideline nº 236/2013, whereas for *R. quelen*, PC concentration utilized was 2 mg.L^{-1}, due to the greater sensitivity of the species to this compound.

The LC_{50} value are estimated for both species at 96, 120, 144 and 168 hpf. LC_{50} values decreased over time, demonstrating that the toxicity increased proportionally with time (TABLE 2). The LC_{50} values at 168 hours of DBP exposure were 0.135 mg.L^{-1} for *Danio rerio* and 0.065 mg.L^{-1} for *Rhamdia quelen*. For DiPeP, LC_{50} values found were 7.735 mg.L^{-1} and 1.277 mg.L^{-1} for *D. rerio* and *R. quelen*, respectively. For the mixture (MIX), the LC_{50} at 168 hpf for *D. rerio* was 0.038 mg.L^{-1}, for the native species *R. quelen*, the LC_{50} was 0.031 mg.L^{-1}, demonstrating a higher toxicity of MIX in relation to isolated phthalates for both species (TABLE 2).

TABLE 2. Values referring to lethal concentration (LC50) after 96, 120, 144 and 168 hours of exposure to phthalates Di-n-butyl phthalate (DBP), Di-iso-pentyl phthalate (DiPeP) and mixture (MIX) in larvae of *Danio rerio* and *Rhamdia quelen*.

LC_{50} (mg.L^{-1})		96 h	120 h	144 h	168 h
Danio rerio	DBP	0.296	0.211	0.141	0.135
	DiPeP	14.61	12.01	11.02	7.735
	MIX	0.056	0.046	0.039	0.038
Rhamdia quelen	DBP	0.071	0.069	0.069	0.065
	DiPeP	2.253	1.64	1.636	1.277
	MIX	0.042	0.039	0.038	0.031

Values expressed in mg.L^{-1}. Sample size: 60 larvae per treatment. Analyzes performed in triplicate. Nonlinear regression.

DBP was more toxic than DiPeP for both species. At 168 hpf, DBP caused significant mortality in *Danio rerio* in the 0.062 mg.L^{-1} concentration and in *Rhamdia quelen* in the 0.007 mg.L^{-1} concentration (FIGURE 2). There was mortality of all specimens of *R. quelen* in the higher concentration tested in this study (0.125 mg.L^{-1}). DiPeP caused mortality to *D. rerio* only at the highest concentration tested (0.125 mg.L^{-1}). In addition, for *R. quelen* species, DiPeP caused mortality at the two highest concentrations (0.062 mg.L^{-1} and 0.125 mg.L^{-1}) (FIGURE 2). MIX of both phthalates caused mortality from 0.007 mg.L^{-1} to 0.125 mg.L^{-1} for both species (FIGURE 2).

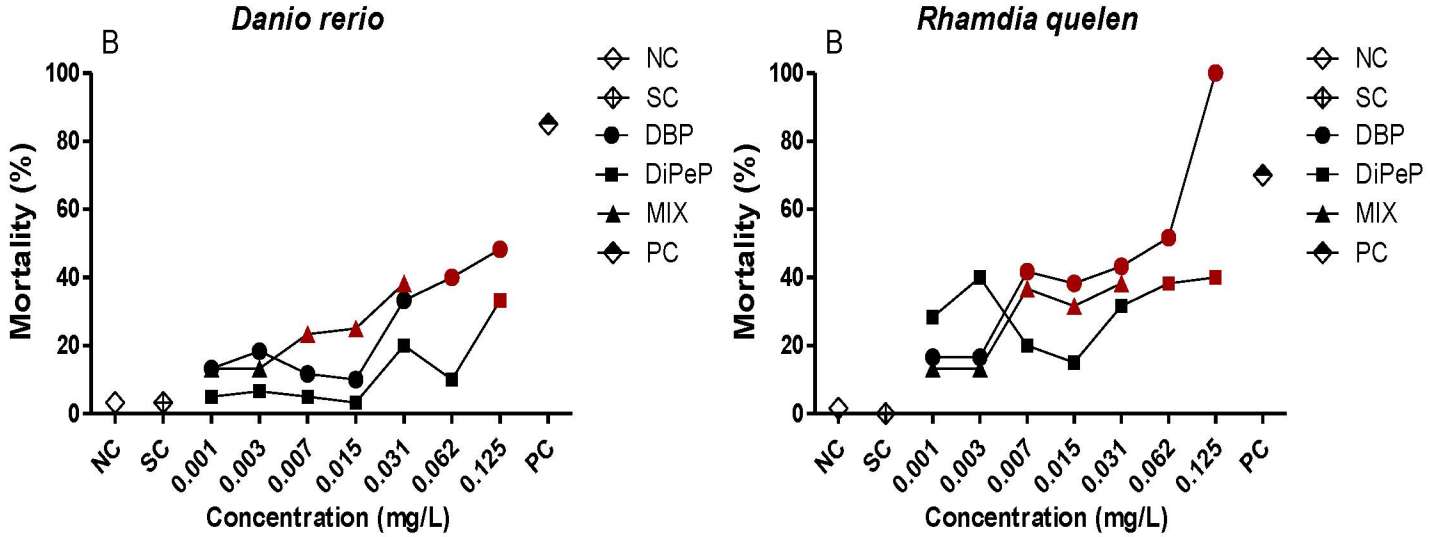


FIGURE 2 Percentage of mortality observed in A: *Danio rerio* and B: in *Rhamdia quelen*. Larvae fish after 168 hours of exposure to Di-n-butyl phthalate (DBP), Di-iso-pentl phthalate (DiPeP) and mixture (MIX). The treatments were: NC (negative control), SC (solvent control - 0.1% methanol v/v); 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 mg.L-1 of the phthalates and positive control (PC). Red symbols indicate a significant difference with the negative control ($p < 0.05$), after ANOVA, followed by the Dunnett Test. Sample size: 60 larvae per treatment.

Sub lethality endpoints were observed in the larvae after 168 hours of exposure to phthalates in both species: *D. rerio* and *R. quelen* (FIGURE 3):

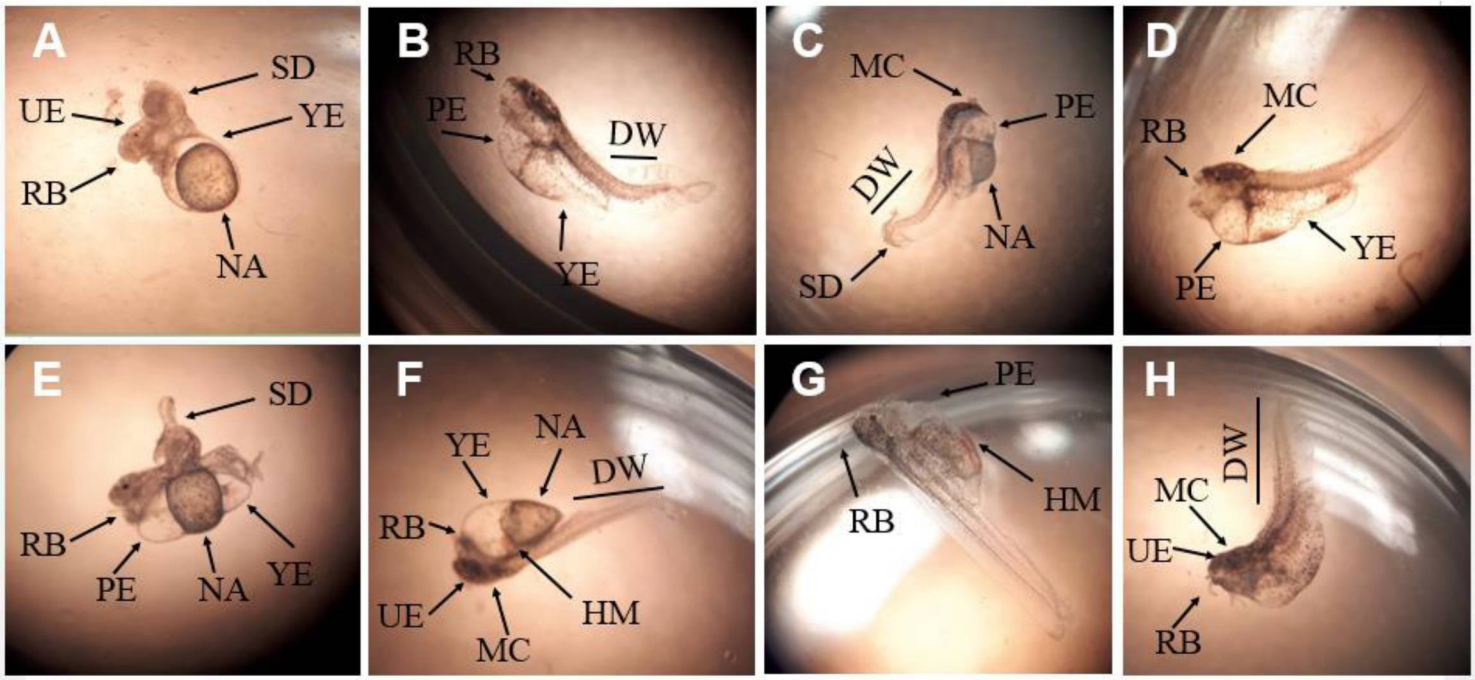


FIGURE 3. Embryonic development malformations of *Rhamdia quelen* after 168 hours of exposure to Di-n-butyl phthalate (DBP), Di-iso-pentyl phthalate (DiPeP) and mixture (MIX). Photo enlargement at 3x. Dwarfism (DW); hemorrhage (HM); microcephaly (MC); non-absorption yolk-sac (NA); pericardial edema (PE); reduced barbels (RB); skeletal deformities (SD); undeveloped eyes (UE) and yolk-sac edema (YE).

The absence of hatching persisted up to 168 hpf in *Rhamdia quelen* exposed to 0.125 mg.L^{-1} of DBP (TABLE 3). Moreover, less intense pigmentation was observed only in *Danio rerio* larvae exposed to 0.125 mg.L^{-1} of DiPeP and 0.031 mg.L^{-1} of MIX (TABLE 3).

Dwarfism (DW) occurred in both species. In *D. rerio*, DW occurred in exposed larvae at concentrations of 0.125 mg.L^{-1} of DBP and 0.031 mg.L^{-1} of MIX (TABLE 3). In *R. quelen* we observed a significant increase in DW in larvae exposed to 0.125 mg.L^{-1} of DBP, 0.007 mg.L^{-1} of DiPeP and 0.015 mg.L^{-1} of MIX (TABLE 3).

Defects in swimming bladder prevented to swim bladder inflation up to 168 hpf in *Danio rerio* larvae exposed to DBP (from the concentration 0.062 mg.L^{-1}) and to 0.031 mg.L^{-1} of MIX (TABLE 3). The same occurred with behavioral changes assessed by equilibrium disturbances in *D. rerio* larvae exposed to 0.062 and 0.125 mg.L^{-1} of DBP, 0.125 mg.L^{-1} of DiPeP and 0.031 mg.L^{-1} of MIX (TABLE 3).

Presence of hemorrhages appeared in *D. rerio* and *R. quelen* larvae exposed to 0.125 mg.L^{-1} of DBP and 0.031 mg.L^{-1} of MIX. In *R. quelen*, DiPeP also induced hemorrhages at concentration of 0.062 mg.L^{-1} (TABLE 3).

Non-yolk sac absorption (NA) was the most frequent sub lethality endpoint in exposure to phthalates. In *D. rerio*, NA occurred in larvae exposed to the concentrations: 0.003 mg.L^{-1} of DBP, 0.031 mg.L^{-1} of DiPeP and 0.001 of MIX (TABLE 3). In *R. quelen*, NA occurred to larvae exposed to all phthalate concentrations (from 0.001 mg.L^{-1}) of DBP, DiPeP and MIX (TABLE 3).

Pericardial edema (PE) in *Danio rerio* occurred at the concentrations: 0.062 mg.L^{-1} of DBP, 0.015 mg.L^{-1} of DiPeP and 0.007 mg.L^{-1} of MIX. In *Rhamdia quelen*, the presence of PE was observed at the concentrations: 0.007 mg.L^{-1} of DBP, 0.062 mg.L^{-1} of DiPeP and 0.007 mg.L^{-1} of MIX (TABLE 3).

Barbels are structures present in *R. quelen* and their shortening was observed in larvae exposed to a higher MIX concentration (0.031 mg.L^{-1}) (TABLE 3).

Skeletal deformities occurred in *D. rerio* larvae exposed to 0.062 and 0.125 mg.L^{-1} of DBP, whereas *R. quelen* larvae presented this malformation only when exposed to 0.125 mg.L^{-1} DBP (TABLE 3).

The undeveloped eyes were observed only in the species *Rhamdia quelen* species at 0.062 mg.L^{-1} of the DBP and DiPeP (TABLE 3).

Yolk-sac edema (YE) appeared in larvae of both species in the phthalate exposure. In *D. rerio* YE occurred in larvae exposed to 0.125 mg.L^{-1} of DBP, 0.062

mg.L^{-1} of DiPeP and 0.003 mg.L^{-1} of MIX. In *R. quelen*, the presence of YE occurred at the concentrations 0.007 mg.L^{-1} of DBP, DiPeP and MIX (TABLE 3).

TABLE 3. Sublethal effects in *Danio rerio* and *Rhamdia quelen* larvae after 168 hours of exposure to di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and mixture (MIX).

Sublethal endpoints	*Danio rerio*			*Rhamdia quelen*		
	DBP	DiPeP	MIX	DBP	DiPeP	MIX
Absence of hatching	n.s.	n.s.	n.s.	0.125	n.s.	n.s.
Altered pigmentation	n.s.	0.125	0.031	n.s.	n.s.	n.s.
Dwarfism	0.125	n.s.	0.031	0.125	0.007	0.015
Defect in gas bladder	0.062	n.s.	0.031	n.s.	n.s.	n.s.
Equilibrium disturbances	0.062	0.125	0.031	n.s.	n.s.	n.s.
Hemorrhage	0.125	n.s.	0.031	0.125	0.062	0.031
Non-yolk sac absorption	0.003	0.031	0.001	0.001	0.001	0.001
Pericardial edema	0.062	0.015	0.007	0.007	0.062	0.007
Reduced barbels	-	-	-	n.s.	n.s.	0.031
Skeletal deformities	0.062	n.s.	n.s.	0.125	n.s.	n.s.
Undeveloped eyes	n.s.	n.s.	n.s.	0.062	0.062	n.s.
Yolk-sac edema	0.125	0.062	0.003	0.007	0.007	0.007

Embryonic development malformations and behavioral changes in larvae of *Danio rerio* and *Rhamdia quelen* after 168 hours of exposure to treatments: 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 mg.L^{-1} of di-n-butyl phthalate (DBP); 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 mg.L^{-1} of di-iso-pentyl phthalate (DiPeP) and 0.001; 0.003; 0.007; 0.015; 0.031 mg.L^{-1} of MIX. Values indicate in which concentrations (mg.L^{-1}) DBP, DiPeP, and MIX malformations were significantly different from the negative control. ns did not differ significantly from control. Analyses carried out in triplicate. Sample size: 60 larvae per treatment. ANOVA followed by Dunnett's test, where $p < 0.05$ significant.

3.3 Genetic biomarker

Using the alkaline comet assay, it was possible to verify that phthalates caused genotoxicity. However, this effect in isolated in some phthalates and it was not dependent on concentration; behaving in a non-monotonic dose-response manner.

In *Danio rerio*, DNA damage occurred in the concentration 0.015 mg.L^{-1} of DBP (FIGURE 4-A), 0.001 mg.L^{-1} of DiPeP (FIGURE 4-B) and 0.015 and 0.031 mg.L^{-1} concentrations of MIX (FIGURE 4-C). In *Rhamdia quelen*, DBP was genotoxic at concentrations of 0.015, 0.031, 0.062 and 0.125 mg.L^{-1} (FIGURE 4-D), DiPeP at concentrations of 0.015 and 0.125 mg.L^{-1} (FIGURE 4-E) and MIX at concentration of 0.031 mg.L^{-1} (FIGURE 4-F).

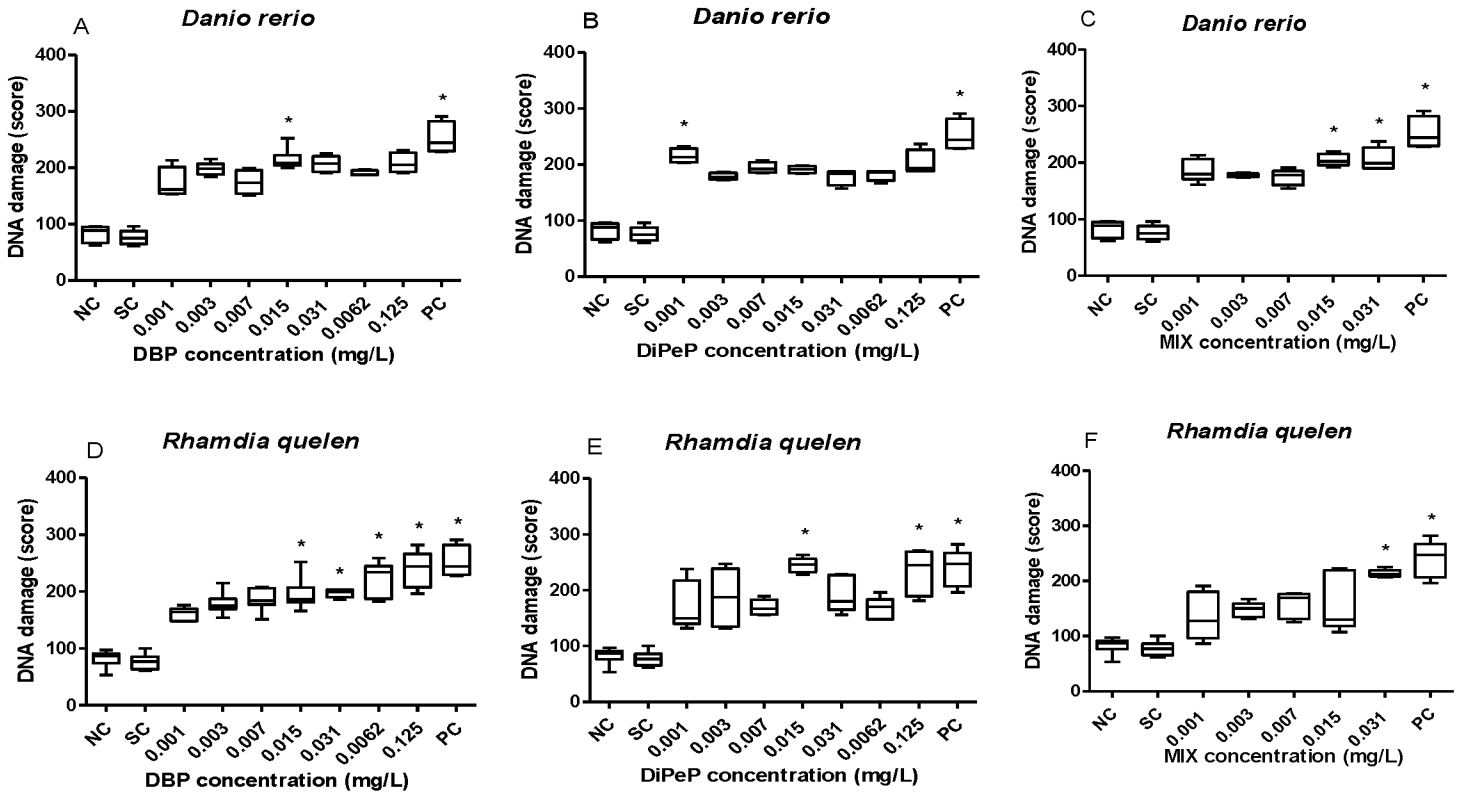
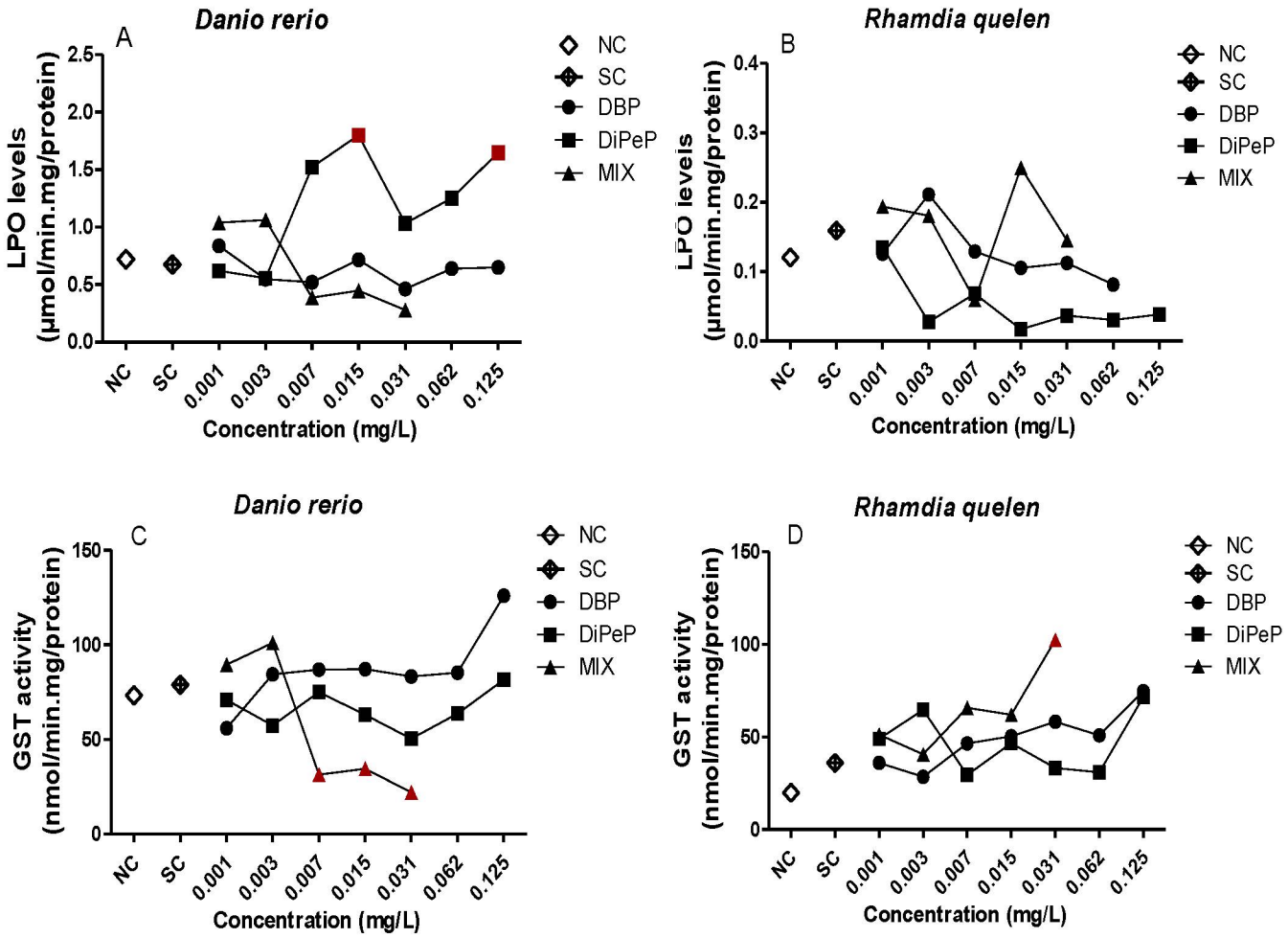


FIGURE 4. DNA damage in: A, B and C: *Danio rerio* larvae and D, E and F: *Rhamdia quelen* larvae evaluated using the comet assay. Larvae fish after 168 hours of exposure to di-n-butyl phthalate (DBP), di-iso-pentyl phthalate (DiPeP) and mixture (MIX). the treatments were: NC (negative control), SC (solvent control - 0.1% methanol v/v); 0.001; 0.003; 0.007; 0.015; 0.031; 0.062; 0.125 $mg.L^{-1}$ of the phthalates and comet assay's positive control (PC). * Indicate a significant difference with the negative control ($p < 0.05$). Kruskal-Wallis test, followed by the Student-Newman-Keuls test.

3.4 Biochemical biomarkers

The LPO levels increased only in larvae of *Danio rerio* exposed to 0.015 and 0.125 $mg.L^{-1}$ of DiPeP, again demonstrating a non-monotonic dose-response pattern (FIGURE 5-A). DBP and MIX did not cause an increase in LPO levels.

GST activity altered only in the exposure to MIX in both species. GST activity in *Danio rerio* was significantly reduced at concentrations of 0.007, 0.015 and 0.031 $mg.L^{-1}$ (FIGURE 5-C). However, in *R. quelen* the highest MIX concentration (0.031 $mg.L^{-1}$) increased the GST activity (FIGURE 5-D).



FIGUREm 5. Biochemical biomarkers A, C and E: in *Danio rerio* and B, D and F: in *Rhamdia quelen*. A and B- Lipid peroxidation – LPO (µmol/min/ mg protein); C and D- Glutathione S-transferase – GST activity (nmol/ min/ mg protein). Larvae fish after 168 hours of exposure to Di-n-butyl phthalate (DBP), Di-iso-pentyl phthalate (DiPeP) and mixture (MIX). The treatments were: NC (negative control), SC (solvent control - 0.1% methanol v/v), 0.001; 0.003; 0.007; 0.015; 0.031; 0.062 and 0.125 mg.L^{-1} of phthalates. Red symbols indicate a significant difference in relation to negative control ($p < 0.05$). Kruskal-Wallis test, followed by the Student-Newman-Keuls test.

4. Discussion

The transpose of FEET test to native species *Rhamdia quelen* proved to be effective for toxicity testing of chemical compounds in embryos. The use of induced reproduction and in vitro fertilization allowed a large number of oocytes and high fertilization rate (de Amorim et al., 2009), in which the control embryos presented normal development. Another advantage is that *R. quelen* species has naturally low mortality (Gubiani et al., 2012), thus, the fish used as matrix survived after spawning.

Rhamdia quelen has transparent embryo's membrane (Gomes et al., 2000; Pereira et al., 2011) as well as *Danio rerio*, allowing the visualization of various structures during ontogeny. This characteristic enabled the observation of embryonic development as well as following OECD standards for validation of guideline number 236, such as: fertilization rate of all eggs collected should be ≥ 70%; overall survival of embryos in the negative control and solvent control should be ≥ 90% until the end of the 96 h exposure, hatching rate in the controls should be ≥ 80% and a minimum mortality of 30% at positive control (OECD, 2013). In addition, the physical/chemical standards required by guideline 236 (OECD, 2013) were followed, thus, the transposition of the FEET test to *R. quelen* was successful.

In general, *Rhamdia quelen* embryonic development occurs faster than *Danio rerio.* The initial stages such as zygote, cleavage and gastrula differ temporally in minutes or a few hours between species (TABLE 1). During the segmentation and pharyngula stages, important structures are formed and we observed that most of the characteristics appear in up to 19 hpf in *R. quelen* (Pereira et al., 2006; Rodrigues-Galdino et al., 2009) while in *D. rerio* its appear only in 42 hpf (Kimmel et al., 1995). In larvae stage, hatching occurs 48 hpf in *D. rerio* (Kimmel et al., 1995), whereas in *R. quelen* it occurs around 20 hpf (Rodrigues-Galdino et al., 2009). The *R. quelen* develops the main organs and structures in less time than *D. rerio*, however, both species complete their organogenesis and reach the similar anatomy of an adult around 120 hpf (Kimmel et al., 1995; Pereira et al., 2006; Rodrigues-Galdino et al., 2009).

There is a tendency to carry out tests with a longer exposure time to xenobiotic and using ecologically relevant species, which have applicability in toxicity tests (Schreiber et al., 2017; Norberg-King et al., 2018). Exposure time extension to 168 hpf (FEET test) proved to be adequate for the evaluation of endocrine disruptors

compounds (ED), such as phthalates. In our study, larvae have been shown to be more sensitive than embryos, and decreases in LC_{50} values over time have already been described in the literature (Dang et al., 2017). This may be explained because the chorion acts as barrier for chemical compounds, protecting the embryo. In addition, when the gills have not being completely formed, there is less absorption of the compounds and, as a consequence, there is a limitation in the metabolic capacity of fish embryos (Braunbeck et al., 2014; Stelzer et al., 2018; Oliveira de Farias et al., 2019).

The OECD guideline n° 236 is applicable only to *Danio rerio*, however, there is need to develop for other species, as there is a difference in sensitivity among them (Dang et al., 2017; Stelzer et al., 2018). It has been shown that endemic species such as *Misgurnus fossilis* native to Europe and *Rhamdia quelen* native to South America can be more sensitive than exotic species, traditionally used in toxicity testing protocols, such as *D. rerio* (Schreiber et al., 2017; Azevedo-Linhares et al., 2018). This can be observed in our work, in which the LC_{50} estimated values were average 6 mg lower in *R. quelen* in comparison with LC_{50} values obtained for *D. rerio*.

DBP was more toxic than DiPeP for both species, did not surprise us because the toxicity of phthalates is directly related to its side chain length (Staples et al., 1997; Bradlee and Thomas, 2003; Benjamin et al., 2017). Phthalates with low molecular weight (C1 to C4), such as DBP, are more toxic than phthalates with higher molecular weight (Bradlee and Thomas, 2003). In a study using *Danio rerio* embryos, DBP has shown to be the most toxic among six phthalates evaluated, causing the highest mortality rate (Pu et al., 2019). However, it is important to know the toxicity of DiPeP, especially in endemic species, because Brazil is the only country where the presence of DiPeP has been reported (Souza et al., 2018).

In the aquatic environment, organisms are constantly exposed to a mixture of compounds, among them phthalates, which can act through the same biological pathway, and in some cases, causing greater toxicity (Christen et al., 2012; Zhou, Gao and Flaws, 2017; Luo et al., 2018; Neier et al., 2019). Such results were also observed in our study, in which lethality and sublethality endpoints appeared in lower concentrations in MIX when compared to isolated phthalates. In Brazil, the presence of DBP and DiPeP is simultaneous (Rocha et al., 2017), which makes extremely important to evaluate the toxicity of these compounds mixture, especially in endemic species.

The analysis of larvae malformations is important to assess the risk of exposure to compounds, because some of them can be permanent and consequently induce to fish death (Azevedo-Linhares et al., 2018). Some malformations appeared in only one species when exposed to phthalates, such as altered pigmentation and uninflated swimming bladder, observed only in *D. rerio*. It is already known that the change in the color pattern is fish is closely related to hormonal control (Logan, Burn and Jackson, 2006). For this reason, phthalates can cause this change due to strongly potential of EDC, as has already been demonstrated by bisphenol F (Mu et al., 2019).

Swimming bladder has the function of fluctuation and adjustment of the body density, allowing the animal to start free swimming phase (Rodrigues de Oliveira et al., 2016; Yang et al., 2018). Defects such as uninflated swim bladder in *Danio rerio*, caused by exposure to phthalates can harm the swimming behavior, causing loss of equilibrium, as observed in larvae exposed to DBP, DiPeP and MIX. Loss of equilibrium is a behavioral change defined as the larva's permanence at the bottom of the microplate well (Oliveira de Farias et al., 2019). This condition has already been observed in *D. rerio* larvae exposed to phthalates (Staples et al., 1997; Poopal et al., 2020). However, the loss of equilibrium was not observed in *R. quelen*, because this fish species lives at bottom of rivers, so it is a natural tendency for the fish larvae to remain at the bottom of microplate well, and cannot be considered a behavioral change.

Embryonic development malformations were observed, demonstrating that phthalates can interact with different tissues and organs (Ventrice et al., 2013). Both species showed dwarfism, hemorrhages, non-absorption of the yolk sac, presence of edema and skeletal deformities. In general, embryonic development malformations appeared in the lowest concentrations of phthalates tested in the native species *R. quelen* whereas in *Danio rerio* embryos the malformations appeared in highest concentrations of phthalates tested.

Dwarfism happens when the larvae has a shortened body length and is an important index to be evaluated (Yang et al., 2018). Therefore, for the growth of larvae to occur correctly, a lot of energy is needed (Bagatto, Pelster and Burggren, 2001). However, when the larvae are exposed to xenobiotics a lot of energy is spent in the detoxification process, leaving a small amount of energy to be used for growth. The yolk is the nutritive reserve used by the embryo until it starts exogenous feeding, thus,

the non-absorption of the yolk sac also caused to reduction in larva's metabolism, affecting its growth (Lourenço et al., 2017).

It has been described that phthalates can lead to cardiovascular impairment due to increase of production of reactive oxygen species (ROS) (Amara et al., 2019). The presence of hemorrhages (HM) is associated with ruptures and leakage of the endothelial vessels of the pericardium (Hallare et al., 2005). The appearance of HM can lead to a deterioration of cardiac function, and consequently cause edema and death (Krzykwa, Saeid and Jeffries, 2019; Zhu et al., 2019), as observed in the exposure to DBP, DiPeP and MIX.

Skeletal deformities (SD) were observed in larvae exposed to DBP, corroborating previous studies with DBP and the species *Danio rerio* (Ortiz-Zarragoitia et al., 2006; Pu et al., 2019). However, this was the first study to demonstrate that DBP cause this malformation in *Rhamdia quelen* larvae. The presence of SD can lead to large spine deformations, impeding embryo movement inside the egg, thus, hatching can not occur properly (Mu et al., 2019), as described larvae of *R. quelen* larvae exposed to DBP.

The development of tissues and organs in embryos can be easily stopped due to exposure to toxic substances (Sehonova et al., 2016). In *R. quelen*, exposure to phthalates resulted in undeveloped eyes and small size barbels. Decrease in the size of the eyes has been associated with reduction in the size of brain (Wold et al., 2017), and may have long-term impacts on fish lives (Krzykwa, Saeid and Jeffries, 2019). *R. quelen* species has three pairs of barbels that grow around the mouth, mainly with sensory function, helping to locate prey (Casatti et al., 2001). Consequently, damage to barbels, as occurred in the exposure to phthalates mixture, can impair the search for food and reproduction (Azevedo-Linhares et al., 2018), being an important endpoint to be evaluated in this species.

Assessment of lethality effects along with sub lethality provides greater sensitivity to the FET test (Stelzer et al., 2018; Krzykwa, Saeid and Jeffries, 2019). Nevertheless, these damages are irreversible to the organism, whereas an early response could prevent these damages to reach high levels of biological organization (Hook, Gallagher and Batley, 2014). Thus, the use of biomarkers is adequate in toxicological assessment, because they are more sensitive in the detection of EDC (Rivero-Wendt et al., 2016; Fraser et al., 2017; Poopal et al., 2020).

The exposure to phthalates can cause damage to macromolecules such as DNA and lipids, generated mainly by oxidative stress (Mankidy et al., 2013; Jeng et al., 2014; Li, Yin and Zhao, 2017; Rocha et al., 2017; Yang et al., 2018; Amara et al., 2019; Song et al., 2019; Park et al., 2020; Poopal et al., 2020). Oxidative stress is caused by the imbalance between the endogenous formation of reactive oxygen species (ROS) and the cell's ability to eliminate them (Sehonova et al., 2016; Rocha et al., 2017).

The alkaline comet assay allows to detect single strand breaks and alkali-labile sites in DNA molecule (Koppen et al., 2017), caused by compounds with direct mode of action. Besides to the damage caused by ROS, phthalates can directly damage DNA, such as the di (2-ethylhexyl) - phthalate (DEHP), a compound that causes DNA strand breaks (Amara et al., 2019; Molino et al., 2019). In our study we observed that DBP and DiPeP isolated and in a mixture can cause genotoxicity in the larvae of *D. rerio* and *R. quelen*. The pattern of DNA damage caused by phthalates was different between species.

For *D. rerio* species, genotoxicity was not dependent on concentration, something that can happen when animals are exposed to some contaminants (Lourenço et al., 2017), such as EDC compounds, so the appearance of non-monotonic dose-response curves, or inverted U curve may be possible (Martino - Andrade et al., 2006; Vandenberg et al., 2012). For *R. quelen* species, genotoxicity was concentration-dependent, as occurred in rat cardiac cells exposed to DEHP (Amara et al., 2019), in rat liver exposed to di-heptyl phthalate (Jin et al., 2009) and in HepG2 cells exposed to DBP (Li, Yin and Zhao, 2017).

The present study showed that only DiPeP increased the levels of LPO, possibly because DiPeP generated more severe oxidative stress. Some studies suggest that DBP, despite being toxic, causes less oxidative stress than other phthalates, such as DEHP and di-ethyl phthalate (DEP) (Mankidy et al., 2013). As already mentioned, ROS are highly toxic molecules that can attack polyunsaturated fatty acids and cause an increase in lipoperoxidation (Jeng et al., 2014; Wang et al., 2018; Yang et al., 2018; Amara et al., 2019; Zhang et al., 2019). Consequently, DiPeP could generate more ROS, causing lipoperoxidation in *D. rerio*.

Antioxidant enzymes are involved in cell defense mechanism against the harmful effects of ROS (Du et al., 2014; Kroon, Streten and Harries 2017; Xiang et al., 2017; Wang et al., 2018). GST is phase II enzyme that conjugates compounds or their metabolites, making them hydrophilic in order to facilitate their excretion (Souza et al.,

2013; Song et al., 2019; Severo et al., 2020). Phthalates can modulate the activity of enzymes such as GST, causing an increase (Xiang et al., 2017; Wang et al., 2018; Song et al., 2019; Zhang et al., 2019) or a decreased in their activity (Du et al., 2014; Wang et al., 2018; Song et al., 2019).

GST enzyme activity may differ between species (Kroon, Streten and Harries 2017), as occurred in this study. For *D. rerio* larvae exposed to three highest concentrations of MIX, there was a decrease in GST activity, indicating that the cell may have exceeded its antioxidant capacity (Wang et al., 2018), which means that the rate of enzymatic synthesis was not sufficient to eliminate the amount of ROS produced (Souza et al., 2013; Du et al., 2014; Song et al., 2019). For the *R. quelen* larvae exposed to highest concentrations of MIX, there was an increase in GST activity, being an adaptive response to excess of ROS produced, in order to compensate the damage induced by MIX (Wang et al., 2018; Park et al., 2020).

In our study, we used a standardized species and indicated by regulatory agencies (OECD, 2013) and a endemic species, commonly used in adulthood to assess the toxicity of xenobiotics (Mela et al, 2013; Piancini et al., 2015; Pereira et al., 2016; Guiloski et al., 2017; Mathias et al., 2018; Perussolo et al., 2019). Both species were exposed to the same phthalates and in the same concentrations, in order to compare the sensitivity of *D. rerio* and *R. quelen*. We demonstrated a strong potential of the *Rhamdia quelen* species in attending criteria of use as standard species in toxicity tests (Azevedo-Linhares et al., 2018), as in the FEET test.

5. Conclusions

DBP and DiPeP isolated and in a mixture can cause mortality, embryonic development malformations and genotoxicity in fish larvae. DBP may be considered more toxic, when evaluating parameters like mortality and malformations. However, by analyzing the biochemical biomarkers, DiPeP demonstrated severe toxicity, causing lipoperoxidation. The GST enzyme activity was altered only when exposed to phthalates mixtures. The native species *R. quelen* was more sensitive, with lower LC_{50} values and the presence of malformations in concentrations below that observed in *D. rerio*. Thus, the transpose of *R. quelen* on Fish Embryo Extend Toxicity test proved to be successful and of great ecological relevance.

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5. CONSIDERAÇÕES FINAIS

Os ftalatos demonstraram ser tóxicos aos embriões e larvas de *Danio rerio* e *Rhamdia quelen* causando mortalidade, malformações no desenvolvimento embrionário, alteração comportamental, neurotoxicidade, genotoxicidade e peroxidação lipídica.

O DBP demonstrou-se mais tóxico quando observamos os resultados obtidos pelo FEET teste, apresentando menores valores de CL_{50}. No entanto, o DiPeP causou maiores danos a nível de macromoléculas como os lipídeos, possivelmente por causar maior estresse oxidativo.

Esse foi o primeiro estudo a avaliar uma mistura entre o DBP e o DiPeP. Nossos resultados demonstraram que a MIX entre esses ftalatos intensificou seus efeitos tóxicos, no qual a atividade da enzima GST foi alterada apenas nesses grupos. Dessa forma, a avaliação dos compostos isolados pode levar a subestimação do efeito dos ftalatos.

A extensão do tempo de exposição para 168 horas se mostrou adequada para a avaliação de parâmetros como a mortalidade, alterações comportamentais e biomarcadores genéticos, no qual os danos ao DNA foram observados somente após 168 horas de exposição aos ftalatos.

Esse estudo revelou que a transposição do FEET teste para a espécie nativa da América do Sul *R. quelen* é viável e útil na avaliação dos efeitos tóxicos dos ftalatos. *R. quelen* apresentou mortalidade e malformações em concentrações abaixo do que em *D. rerio*, demonstrando que sua utilização pode ser uma alternativa para testes de toxicidade com embriões e larvas.

A partir dos resultados obtidos, concluímos que *R. quelen* foi mais sensível a exposição ao DBP, DiPeP e a MIX do a espécie modelo *D. rerio*. Destacamos a importância da transposição dos testes de toxicidade com embriões e larvas para outros peixes, devido as respostas específicas de cada espécie. E por fim, sugerimos a avaliação da toxicidade de desreguladores endócrinos, como os ftalatos, bem como a avaliação de misturas entre esses compostos em espécies nativas da América do Sul.

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APÊNDICE 1 –PROJETO APROVADO - CNPq

Esse projeto foi aprovado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq – Universal 2018, processo n° 421809/2018-3.

ANEXO 1 – CERTIFICADO DO COMITÊ DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO PARANÁ

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 1138

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.199318/2017-17

APROVADO/APPROVAL: 17/10/2017 – R.O. 09/2017

TÍTULO: Padronização do FET (Fish Embryo Acute Toxicity) em espécies nativas – Uma abordagem em Ftalatos.

TITLE: Normalization of FET (Fish Embryo Acute Toxicity) in native species - An approach in Phthalates.

AUTORES/AUTHORS: Marta Margarete Cestari, Juliana Roratto Lirola.

DEPARTAMENTO/DEPARTMENT: Genética

Profa. Dra. Katya Naliwaiko
Coordenadora da CEUA