Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais

Toxicidade e efeitos dos contaminantes emergentes diclofenaco e metilparabeno para o zebrafish *Danio rerio*: estudos em larvas e adultos

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Orientadora: Camila de Martinez Gaspar Martins

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Dedicatória

Dedico esta dissertação para:
minha mãe Keylla, minha vó Jocelina e meu amigo irmão Muryllo, pois, sem a ajuda de vocês ao longo da minha estrada eu não teria conseguido. Obrigada!

RESUMO

Produtos farmacêuticos e de cuidado pessoal (PPCPs) como o Metilparabeno (MeP) e o Diclofenaco (DCF) vêm alcançando os corpos d'água naturais de forma crescente nas últimas décadas. O uso contínuo destes compostos adicionados à falta de saneamento e à ineficiência das Estações de Tratamento de Esgoto (ETEs) em remover os mesmos são as principais causas da sua presença nos ambientes aquáticos. No entanto, seus efeitos sobre a biota ainda são pouco conhecidos e eles não são regulamentados em nível mundial. Assim, o objetivo deste estudo foi avaliar os efeitos tóxicos do MeP e DCF em adultos e larvas do zebrafish *Danio rerio*, um modelo biológico consagrado na área de ecotoxicologia. Os valores de CL_{50} de MeP foram estimados em 105,09 mg/L para adultos e 211,12 mg/L para larvas, indicando uma sensibilidade maior dos adultos. Essa diferença na sensibilidade foi corroborada pelos resultados dos ensaios com concentrações subletais do composto (30 µg/L de MeP como ambientalmente relevante e a concentração de efeito não observado – CENO, que foi 60 mg/L para larva e 50 mg/L para adultos). De maneira geral as brânquias de adultos responderam mais que o fígado à presença de MeP. Na concentração de 50 mg/L de MeP houve uma redução na atividade da EROD e aumento da lipoperoxidação (LPO) nas brânquias, ainda, foi detectado um aumento na incidência de micronúcleos (MNs) e de outras anormalidades nucleares nos eritrócitos dos peixes. Já para larvas, o MeP não induziu LPO na concentração de CENO. A concentração ambientalmente relevante de MeP não provocou efeitos nos biomarcadores, exceto na microbiota intestinal de adultos que aumentou o número de fontes de carbono utilizadas, o que sugerimos ser uma adaptação ao estresse ambiental e possível escassez de fontes de carbono, mas isso em si não deve ser considerado como um efeito negativo. A microbiota intestinal foi analisada, pois hipotetizou-se que a diferença na sensibilidade entre larvas e adultos poderia ser decorrente da ação antimicrobiana do MeP que estaria afetando a microbiota intestinal da qual os adultos são muito mais dependentes que as larvas. Com relação ao DCF os valores de CL_{50} para adultos e larvas foram semelhantes, sendo de 5,49 mg/L e 5,22 mg/L, respectivamente. Este composto provocou um aumento nas atividades da GST e dos transportadores ABC, além de LPO nas brânquias dos adultos e aumento de LPO em larvas. Isto ocorreu na concentração de 3 mg/L que corresponde ao CENO do DCF para ambos os estágios de vida. Novamente, as brânquias se apresentaram mais responsivas que o fígado o que

possivelmente ocorre em função do seu contato primeiro e direto com o meio e os contaminantes dissolvidos nele. Os resultados desta dissertação fornecem subsídios para entendermos os alvos da toxicidade do MeP e DCF, bem como, para a regulamentação destes compostos.

Palavras-chave: PPCPs; zebrafish; CL_{50}; biomarcadores; microbiota intestinal.

ABSTRACT

Pharmaceutical and personal care products (PPCPs) such as Methylparaben (MeP) and Diclofenac (DCF) have been reaching natural bodies of water in an increasing manner in the last decades. The continued use of these compounds added to the lack of sanitation and the inefficiency of Wastewater Treatment Plants (WWTPs) in removing them are the main causes of their presence in aquatic environments. However, its effects on biota are still poorly understood and they are not regulated worldwide. Thus, the objective of this study was to evaluate the toxicity and toxic effects of MeP and DCF in adults and larvae of the zebrafish *Danio rerio*, a biological model well established in the area of ecotoxicology. The LC50 values of MeP were estimated at 105.09 mg/L for adults and 211.12 mg/L for larvae, indicating a higher sensitivity of adults. This difference was corroborated by the results of the tests with sublethal concentrations of the compound (30 µg/L of MeP as environmentally relevant and the non-effect concentration - NOEC, which was 60 mg / L for larvae and 50 mg/L for adults). In general, the adult gills responded more than the liver to the presence of MeP. At the concentration of 50 mg/L of MeP there was a reduction in EROD activity and an increase in lipoperoxidation (LPO) in the gills, also an increase in the incidence of micronuclei (MNs) and other nuclear abnormalities in fish erythrocytes was detected. For larvae, MeP did not induce LPO at NOEC. The environmentally relevant concentration of MeP had no effect on biomarkers, except in the intestinal microbiota of adults, increasing the number of carbon sources used by it. We suggested this as an adaptation to environmental stress and possible scarcity of carbon sources, but it does not mean a negative effect. The intestinal microbiota was analyzed, as it was hypothesized that the difference in sensitivity between larvae and adults could be due to the antimicrobial action of MeP that would affect the intestinal microbiota on which adults are much more dependent than larvae. Regarding the DCF, the LC_{50} values for adults and larvae were similar, being 5.49 mg/L and 5.22 mg/L, respectively. This compound caused an increase in the activities of GST and ABC transporters, in addition to LPO in the gills of adults and an increase in LPO in larvae. This occurred at a concentration of 3 mg/L, which corresponds to the DCF NOEC for both lofe stages. Again, the gills were more responsive than the liver, which possibly occurs due to their first and direct contact with contaminants dissolved in the medium. Results of this work provide subsidies to understand the targets of MeP and DCF toxicity, as well as, for the regulation of these compounds.

Key-words: PPCPs, zebrafish; LC50; biomarkers; intestinal microbiota

APRESENTAÇÃO

Esta dissertação contém uma introdução geral, seguida de objetivos e referências bibliográficas. Após, são apresentados dois capítulos em formato de artigo científico, redigidos em inglês e de acordo com as normas das revistas às quais pretende-se submeter os mesmos. Tais normas encontram-se anexas à dissertação. O 1º capítulo traz os resultados referentes ao contaminante metilparabeno e é intitulado "**Toxicity and sublethal effects of methylparaben (MeP) on larvae and adults of zebrafish *Danio rerio***", ele será submetido à revista *Ecotoxicology and Environmental Safety*. O 2ºcapítulo compreende os resultados a respeito do contaminante diclofenaco e é intitulado "**Effects of diclofenac assessed by toxicity tests and biomarkers in adults and larvae of *Danio rerio***", este será submetido para a revista *Environmental Toxicology and Pharmacology*. Por fim, estão as considerações finais da dissertação.

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INTRODUÇÃO GERAL

Contaminantes emergentes e os Produtos Farmacêuticos e de Cuidado Pessoal (PPCPs)

Atividades humanas vêm provocando a contaminação de recursos hídricos e, portanto, afetando a qualidade dos ecossistemas aquáticos (Carpenter, 2011). Dentre os poluentes de origem antrópica estão os contaminantes orgânicos emergentes (COEs) que podem ser definidos como substâncias, na sua maioria sintéticas, que não são comumente monitoradas no ambiente, mas que podem induzir efeitos indesejáveis aos organismos e ecossistemas (Stuart et al., 2012). Os COEs não são necessariamente compostos recém desenvolvidos, mas, sua presença no ambiente e implicações na integridade do mesmo são reconhecidas apenas recentemente (Daughton, 2004). De fato, isso só foi possível em virtude dos avanços tecnológicos que permitiram a detecção dos COEs em concentrações ambientais, geralmente, muito baixas, e análises de marcadores biológicos de alta sensibilidade (Richardson e Ternes, 2011). No entanto, deve-se considerar que a presença de COEs nos corpos de água pode ser atenuada através de processos como diluição, sorção, volatilização e degradação (Lin et al., 2006).

Os Produtos farmacêuticos e de cuidado pessoal, conhecidos como PPCPs, do inglês *Pharmaceuticals and Personal Care Products* fazem parte dos COEs (Bo et al., 2016). Dentre as diferentes classes de compostos que compõem este grupo estão: medicamentos antiinflamatórios, conservantes, hormônios, antibióticos, desinfetantes, repelentes, fragrâncias e os filtros UV (Shannon et al., 2010). A produção anual de PPCPs é estimada em valores superiores a 2 milhões de toneladas, sofrendo aumentos progressivos a cada ano, porém o seu uso não se restringe ao cuidado individual, eles também são aplicados na indústria e no tratamento de plantas e animais (agricultura, aquicultura, pecuária, etc...) (Wang et al., 2016).

O uso extenso e contínuo dos PPCPs tem levado à contaminação de água naturais subterrâneas e superficiais com estes compostos. Esta contaminação pode ocorrer através da lixiviação de solos contaminados, percolação ou por descarga direta de efluentes industriais, hospitais, e, principalmente, domésticos. Cabe

ressaltar que as estações de tratamento efluentes (ETEs) acabam sendo uma fonte adicional da contaminação aquática por PPCPs, uma vez que seus processos não são eficazes na remoção completa dos mesmos (Nakada et al., 2007, Mompelat et al., 2009, Roberts et al., 2016, Yu et al., 2013).

De fato, a eficiência de uma ETE em remover um PPCPs depende basicamente dos processos de tratamento adotados (primário, secundário ou terciário) e das características químicas das moléculas orgânicas retidas na ETE para a produção de um efluente tratado mais limpo (Haman et al., 2015; Kasprzyk-Hordern et al., 2009; Norvill et al., 2016; Tarpani and Azapagic, 2018). Os processos oxidativos avançados, consistem em um método terciário, como por exemplo, injeção de elevadas concentrações de oxigênio, isso promove um segundo processo oxidativo aumentando à eficiência na degradação de matéria orgânica e de outros compostos como os PPCPs (Diamont 2017).

Sun et al., (2014) e Yu et al., (2014) em seus estudos mostraram uma remoção de 80 e 91 % do Diclofenaco e do Metilparabeno respectivamente em tratamentos terciários, no entanto, este tipo de método é mais caro e geralmente restrito a países mais desenvolvidos.

Dessa forma, os PPCPs podem alcançar os ecossistemas aquáticos de maneira generalizada, o que inclui os mananciais de captação de água para o abastecimento humano. Assim, dentre as possíveis formas para minimizar a problemática estão investimentos em saneamento básico, melhoria nos sistemas de tratamento de efluentes, principalmente adoção de tratamentos terciários que são comprovadamente mais eficazes na limpeza do efluente bruto, informar a população quanto o uso conciente e controlodado de medicamentos e formas mais adequadas de seu descarte (Martín et al., 2012).

Estudos sobre os PPCPs com abordagens como identificação e concentrações ambientais, biodegradabilidade, metabólitos, toxicidade, riscos ambientais, riscos para humanos, interações químicas e biológicas e regulametação vêm crescendo substancialmente nos últimos anos (Daughton, 2016; Gao et al., 2014; Gredelj et al., 2018; Minguez et al., 2016; Montes-Grajales et al., 2017; Peng et al., 2014; Wu et al., 2017; Zhao et al., 2017). Efeitos agudos e crônicos dos PPCPs incluem desregulação endócrina, indução de estresse oxidativo, citotoxicidade, genotoxicidade, histopatológias, entre outros, já que se trata de um grupo muito amplo de substâncias. De maneira geral, os dados que se têm até o

momento mostram que muitos PPCPs representam risco para saúde dos ecossistemas (Da Silva et al., 2009; Kasprzyk-Hordern et al., 2009; Zhao et al., 2017).

Com relação a toxicidade dos PPCPs, os ensaios de toxicidade são usados classicamente para que se definir critérios de qualidade de água e, portanto, para regulamentar substâncias químicas lançadas no ambiente. Os PPCPs têm sido ponto focal de agências regulamentadoras como a *Environmental Protection Agency* (USEPA), *Eurpean Chemicals Agency* (ECHA, EU), *Drug Enforcement Administration* (DEA, US) e do Conselho Nacional do Meio Ambiente (CONAMA, Brasil). No entanto, para que os compostos sejam efetivamente regulamentados estudos devem ser feitos para embasar seu controle do ponto de vista ambiental (Lima et al., 2018). Dentre as várias substâncias que compõe este grugo destacamos o Diclofenaco e o Metilparabeno

Diclofenaco (DCF)

O diclofenaco (DCF) ($C_{14}H_{11}Cl_2NO_2$), ácido 2-[2-(2,6-dicloroanilino)fenil]acético, é uma droga anti-inflamatória não-esteroidal com ação analgésica comumente utilizada para o tratamento da dor em humanos e animais, sendo enquadrado na categoria de produto farmacêutico. O mecanismo de ação desse fármaco atua na inibição da a ciclo-oxigenase-1 (COX-1) e ciclo-oxigenase-2 (COX-2), isoenzimas da ciclo-oxigenase, que assim agem como inibidores diretos da síntese de prostaglandina e tromboxano a partir do ácido araquidônico. COX-1 é a forma constitutiva da COX, encontrada em plaquetas, células endoteliais vasculares, estômago e rins, onde está envolvido na produção de prostaglandinas, responsáveis pela proteção da parede do estômago (prostaglandina E2), a agregação plaquetária (tromboxano A2) e função renal (prostaglandina I2) (Vane et al., 1998) . COX-2 é a forma induzida na presença de inflamação, mas pode também ser encontrada em tecidos cerebrais e renais, na ausência de inflamação. A suprarregulação da COX-2, em algumas áreas do sistema nervoso central (SNC) leva à produção de prostaglandinas (tais como PGE2) que estão envolvidas na dor, febre e inflamação (Cashman, 1996). Peixes, aves e mamíferos possuem os genes para codificar as COXs (Chandrasekharan e Simmons, 2004).

O DCF apresenta solubilidade em água (23,7 g/L à 25°C), pKa 4,10-4,50 à 25°C e Log Kow 4,50-4,80 `a 25°C (Ziylan e Ince, 2011). Ele é um dos compostos mais consumidos em nível mundial. Seu consumo global um consumo anual global de 1443 ± 58 tons (Acuña et al., 2015).

Apesar da sua curta meia–vida na água, aproximadamente 3,3 horas (Schmitt-Jansen et al., 2007), porém, devido ao seu aporte diário e baixa eficiência de remoção pelas ETES o DCF tem sido detectado em efluentes domésticos e hospitalares e corpos d'água superficiais (Aquino et al., 2013; Ferrari et al., 2004; Glassmeyer e Shoemaker, 2005; Gros et al., 2006; Suárez et al., 2008; Verlicchi et al., 2012; Yan et al., 2014). No que diz respeito a concentrações ambientais do DCF no mundo, Ma et al., (2016) encontrou concentrações de 230 ng/L em um rio na China, Carmona et al., (2014) e Valdés et al., (2014) encontraram também em rios da Espanha e Argentina concentrações de 39 e 34-145 ng/L, respectivamente. No Brasil, concentrações próximas de 20 ng/L de DCF foram encontradas em águas costeiras da cidade de Santos / SP (Pereira et al., 2016).

Com relação a toxicidade do DCF para peixes, alguns valores de CL_{50} foram estimados em 6,1 mg/L para embriões de *Danio rerio* (Praskova et al., 2011), de 10,1 mg/L para juvenis *Oryzias latipes* (Nassef et al*.,* 2009), e de 25,12 mg/L para juvenis de *Clarias gariepinus (*Ajima et al*.,* 2015) todos com duração de 96 h.

O DCF também provoca efeitos subletais como alterações hematológicos, bioquímicas e imunológicas, todas estas causadas em exposições crônicas. Como por exemplo, alterações histológicas em brânquias de *Salmo trutta* na concentração de 50 µg/L (Hoeger et al., 2005), alterações histológicas nas brânquias e rins de *Oncorhynchus mykiss* nas concentrações de 5 µg/L e 500 µg/L (Schwaiger et al., 2004), depleção de glicogênio, alterações histológicas, e necrose em fígado, brânquias e rins de *Cyprinus carpio* na concentração de 500 µg/L (Triebskorn et al., 2004). No entanto, é importante ressaltar importância de exposições prevendo consequências ecológicas decorrente da exposição crônica que se aproxima mais ao regime ao qual os organismos estão expostos ao DCF no ambiente (Fent et al., 2006, Santos et al., 2010).

Metilparabeno (Mep)

O Metilparabeno é um parabeno de com potente ação antimicrobiana. O MeP é o parabeno mais amplamente utilizado na indústria, estando na composição de diferentes produtos de cuidado pessoal como desodorantes, shampoos, cremes e loções. Além disso, é usado na conservação de alimentos. Sua concentração limite em alimentos é até 0,1%, em cosméticos até 1% e em produtos farmacêuticos até 20% (Dambal et al., 2017). O MeP é considerado como um conservante ideal pelo seu amplo espectro de atividade, ele age contra batérias gram-positivas, mofos e leveduras; além disso, sua molécula é altamente estável frente a variações de pH e possui baixo custo (Martín-Villamil et al., 2016). Sua fórmula química é $C_8H_8O_3$, Log Kow é 1,96, apresentando baixa solubilidade em água (2,5 g/L à 25ºC).

O seu mecanismo de ação é pouco conhecido, no entanto, alguns estudos relacionam a capacidade dos parabenos em serem interferentes endócrinos leves, alterando a ação endógena de hormônios, além de modificar sua síntese, metabolismo e transporte em geral. Estes podem competir na ligação com os receptores nucleares (estrogênio, androgênio, progesterona entre outros...), aumentar ou diminuir a produção de esteroides endógenos e a síntese de receptores (Bledzka et al., 2014).

Sendo assim a maior parte dos estudos com MeP Grande são relacionando sua ação como desregulador endócrino alterando a expressão da vitelogenina (Vtg) em peixes. Como exemplo, Dambal et al. (2017) em seu estudo com embriões de *D. rerio* expostos a 15 µg/L de MeP observaram um aumento na expressão de Vtg em relação ao grupo controle. Barse et al., (2010) também observou indução da produção de vitelogenina em machos *Cyprinus carpio* nas concentrações de 0.84 µg/L e 1.68 µg/L. Outros trabalhos relatam sua acumulação na biota aquática por suas características químicas já mencionadas. O estudo de Ramaswamy et al., (2011), por exemplo, mostrou altas concentrações de parabenos em várias espécies de peixes com diferentes hábitos adquiridos em mercados nas Filipinas, sendo que, 80% das amostras de peixes continham o MeP, confirmando a alta ocorrência do composto.

Valores de toxicidade aguda (CL_{50} – 96 h) do MeP foram reportados por Yamamoto et al., (2011) e Hopkins e Blaney (2016) para *O. latipes* e *Pimephales promelas* como 63 e 58 mg/L, respectivamente. Com relação a sua presença no ambiente, Jonkers et al., (2010) encontrou MEP no Rio Aveiro em Portugal em concentrações que variaram de 2,1 a 51ng/L, já no Brasil no estado do Rio Grande

do Sul, o MeP foi encontrado em corpos d'água superficiais em concentrações que variaram de 7,6 a 29,8 µg/L na cidade de Rio Grande, e de < 1 a 134 µg/L na cidade de Morro Redondo (Silveira et al., 2013). Adicionalmente, o estudo de Carmona et al., (2014) realizado na cidade de Valência na Espanha reportou a presença do MeP na concentração de 12 ng/L em amostra de água potável, indicando a presença desse composto no ambiente.

Assim como o DCF, o MeP não está presente nas legislações ambientais vigentes, nacionais ou internacionais, com vistas à sua ocorrência em ambientes aquáticos. Os órgãos ambientais mencionam em suas normativas e resoluções o uso de ensaios de toxicidade como exames comprobatórios para atestar a qualidade de efluentes, bem como, para monitorar ambientes aquáticos naturais. No entanto, há de se notar que os ensaios ecotoxicológicos padronizados indicam toxicidade com base em parâmetros poucas sensíveis e irremediáveis, sendo assim, eles não são a melhor ou única maneira de assegurar a qualidade de uma amostra com vistas à manutenção dos ecossistemas. Neste aspecto, o uso de biomarcadores vem ganhando destaque por determinar efeitos precoces da presença de contaminantes, antes que estes produzam efeitos irreversíveis em níveis hierárquicos maiores, como populacionais ou de comunidades.

Biomarcadores

Biomarcadores são respostas biológicas individuais a condições adversas (presença de contaminantes, por exemplo), que variam de moleculares à comportamentais, e são antecipatórias do ponto de vista ecotoxicológico. Estas ferramentas podem ser aplicadas em organismos coletados *in situ* para se determinar o estado de saúde da biota de um ambiente e monitorá-la, ou em laboratório, para determinação sua especificidade e as relações causa efeito (Peakall 1994; Freire et al., 2008).

Os biomarcadores recebem diferentes classificações, uma delas seria: de exposição e de efeito. O biomarcador de exposição compreende uma resposta biológica frente à um estresse, mas que não significa dano, mostra apenas que o organismo está reagindo ou está sob estresse. Já os biomarcadores de efeito representam um dano em função do estresse gerado no organismo. Dentre

biomarcadores de exposição, ressaltamos aqui enzimas e outras proteínas envolvidas no processo de metabolização e detoxificação de compostos químicos. Estes biomarcadores fornecem informações importantes a respeito do contato do organismo com o xenobiótico, bem como, da sua capacidade para se defender do mesmo (Burgeot et al., 1996).

De forma breve, a via clássica do processo de detoxificação envolve 3 fases, fase 1, 2 e 3, sendo as fases 1 e 2 relacionadas ao processo de biotransformação e a fase 3 ao processo de extrusão celular. Importante ressaltar que o processo de biotransformação pode ser sequencial ou não, ou seja, dependendo do composto este pode passar por apenas uma e ou duas fases.

Na fase 1, um grupo polar reativo é inserido na molécula com potencial para exercer toxicidade tornando-a mais solúvel, bem como, um substrato adequado para as enzimas de reação de fase 2. As monooxigenases microsomais citocromo P450 (CYP) são tipicamente envolvidas na fase 1. Estas enzimas realizam algumas reações como oxidação ou hidrólise (Hodgson, 2008). As CYP são importantes no metabolismo de uma grande variedade de compostos endógenos, mas também participam da metabolização de xenobióticos, incluindo fármacos e outros compostos orgânicos (Nelson, 1993).

Dentre as várias proteínas da família do CYP, a isoforma CYP1A, identificada em peixes, é responsável pela biotransformação de compostos como HPAs, PCBs, dioxinas, dentre outros (Bucheli, Fent, 1995). Assim, a indução da CYP1A em fígado de peixes é reconhecida como um biomarcador útil que representa exposição de peixes à diferentes tipos de poluentes. Sua atividade pode ser quantificada pela transformação da 7-etoxiresorufina-O-desetilase (EROD) em resorufina (conversão de substrato não fluorescente em um produto fluorescente pela CYP1A) (Whyte et al., 2000).

As enzimas que atuam na fase 2 de biotransformação permitem que os compostos sejam mais facilmente excretáveis, sendo representadas pelas transferases que incluem as UDP glucuronosil transferases, sulfotransferases, N-acetiltransferases, glutationa S-transferases (GST) e metiltransferases. A reação das enzimas de fase 2 com a molécula-alvo, que pode ser um substrato oriundo da reação de fase 1, resulta em conjugados mais hidrofílicos e menos reativos do que os compostos originais (Jancova et al., 2010, Malik et al., 2016). Nesta linha, especificamente a enzima GST, compreende uma família de enzimas que catalisa

reações de conjugação entre o xenobiótico e o tripeptídeo glutationa (GSH) (Hayes e Pulford, 1995). No processo, ocorre a ativação do átomo de enxofre da GSH ao ânion tiolato (GS-, um nucleófilo forte que ataca substratos eletrofílicos como carbono, nitrogênio ou enxofre) (Wu e Dong, 2012).

A fase 3, por sua vez, previne acúmulo de xenobióticos e componentes endógenos não desejados no interior das células, removendo-os via transportadores dependente de energia conhecidos como proteínas da família ABCs (ATP-Binding Cassete) (Kroon et al., 2017). As proteínas do tipo ABC funcionam como bombas de efluxo, e estão localizadas nas membranas celulares. Células com alta atividade de proteínas ABC são conhecidas como mais resistentes à múltiplos xenobióticos (MXR) (Cunha et al., 2017).

As principais subfamílias das proteínas ABC são as ABCBs, grupo ao qual pertencem as glicoproteínas – P (Pgp) que atuam principalmente na eliminação celular de metabólitos hidroxilados pelas CYP e de compostos não metabolizados (Loncar et al., 2010); e as ABCCs, que compreendem o grupo das MRPs que realizam o efluxo celular de ânions orgânicos e compostos conjugados com sulfato, glicuronídeo e GSH (Anilloa et al., 2006). Sabe-se que estas proteínas estão envolvidas na extrusão celular de contaminantes como metais e pesticidas, mas poucos estudos foram realizados com PPCPs (Chen et al., 2016).

Com relação aos biomarcadores de efeito, a lipoperoxidação (LPO) tem se destacado por indicar danos às membranas celulares por sua constituição lipídica, afetando diretamente a funcionalidade das células. Apesar da sua inespecificidade a LPO é um indicador claro de um quadro de estresse oxidativo (Monserrat et al., 2007). O estresse oxidativo ocorre quando há um desbalanço entre pró e antioxidantes, dessa forma, há um acúmulo de espécies reativas de oxigênio (EROs) que reagem com biomoléculas (proteínas, DNA e lipídios), danificando-as e/ou inativando-as. De feto, as EROs são produzidas naturalmente durante o processo de respiração mitocondrial e síntese de ATP, onde o oxigênio sofre sucessivos passos de redução, formando espécies intermediárias (ânion superóxido – $O_2^{\cdot -}$, peróxido de hidrogênio – H_2O_2 e radical hidroxila – $HO^{\cdot}$) até reduzir-se completamente à água (Lushchak, 2011). Uma das formas em que a LPO é mensurada a partir da quantificação da fragmentação dos lipídeos e formação de aldeídos, tais como o malondealdeído (MDA).

O aumento de EROS pode levar a modificações no DNA, reagindo com esta molécula provocando quebras na fita e degradação de bases, resultando em genotoxicidade e mutagenicidade (Birben et al., 2012). No entanto, esta ação sobre o DNA não se restringe à EROs, uma vez que existem compostos tóxicos reativos com habilidade para interagir diretamente com o a molécula de DNA.

Danos no DNA também são considerados como biomarcadores de efeito e são utilizados para medir a ação genotóxica de um composto. Neste sentido, o teste do micronúcleo (MN) tem sido bastante aplicado por sua clareza de resposta (fácil visualização) e baixo custo. Em peixes, os MNs são frequentemente analisados em eritrócitos, por serem nucleados (Campana et al, 2003). O princípio do teste baseia-se no fato de que, durante o processo de divisão celular, principalmente na anáfase, as cromátides e os fragmentos cromossômicos acêntricos não são transportados pelas fibras do fuso para os pólos opostos. Após a telófase, os cromossomos sem danos são incluídos no núcleo de cada uma das células filhas, no entanto, alguns elementos não são incluídos nos núcleos formados e permanecem no citoplasma, constituindo as estruturas caracterizadas MNs (Schmid, 1976).

Autores como Hose et al., (1987) e Carrasco et al. (1990) caracterizaram as diferentes deformidades dos MNs, classificando-os em: núcleos lobulados, núcleos segmentados, núcleos com constrição em formato de rim e núcleos binucleados (Figura 1.). Sugere-se que as alterações eritrocíticas nucleares devem ser incluídas nas análises de genotoxicidade em peixes complementando a contagem de micronúcleo, por apresentar resultados mais confiáveis e mais completos (Ayllón e Garcia-Vazquez, 2001). Este tipo de biomarcador pode ser usado de uma maneira preditiva, evitando consequências ecológicas irreversíveis como mutações (Jha, 2008).

Figura.1. Representação das principais alterações eritrocíticas nucleares (ENAs) e micronúcleo encontrados em peixes. Imagem adaptada de Carrola et al. (2014).

Modelo Biológico – zebrafish *D. rerio*

Peixes têm sido usados em ensaios de toxicidade e para avaliar efeitos de contaminantes e monitorar impactos ambientais pelo fato de apresentarem elevada capacidade de absorver e acumular contaminantes dissolvidos na água, e alta sensibilidade aos mesmos, inclusive quando presentes em baixas concentrações ou concentrações ambientalmente relevantes (Çavas et al., 2005; Hill et al., 2005). A espécie escolhida como modelo biológico para o presente estudo foi o peixe *D. rerio*, um teleósteo da família Cyprinidae, ordem dos Cypriniformes, com distribuição natural na Ásia.

Apesar não se tratar de uma espécie nativa do Brasil, a escolha foi feita por esta ser uma espécie muito utilizada na área de ecotoxicologia, para a qual existem protocolos padronizados envolvendo seus diferentes estágios de vida (Ali et al., 2014; ABNT-NBR 15499/2007, ABNT NBR 15088, OECD 2013). Adicionalmente, características que contribuem para o uso deste modelo em pesquisas são: fácil obtenção (comercializada) e manutenção (cerca de 3 a 5 cm), baixos custos de cultivo, alta taxa de fecundidade (um casal pode colocar 200-300 ovos) e genoma sequenciado (Westerfield, 2000).

Sendo assim, o objetivo desse estudo foi avaliar a toxicidade através de CL50 e efeitos subletais através da análise de biomarcadores de exposição e efeito dos PPCPs diclofenaco (DCF) e Metilparabeno (MeP), utilizando *Danio rerio,* adultos e larvas,como modelo biológico.

REFERÊNCIAS BIBLIOGRÁFICAS

[ABNT] - Associação Brasileira de Normas Técnicas. 2016. Ecotoxicologia aquática - Toxicidade aguda Método de ensaio com peixes (Cyprinidae) ABNT NBR 15088, terceira edição, 13.12.2016.

[ABNT] - Associação Brasileira de Normas Técnicas. 2007. Ecotoxicologia aquática – Toxicidade crônica de curta duração – método de ensaio com peixes. Norma ABNT-NBR 15499.

[ECHA] European Chemical Agency, 2019. ECHA [WWW Document]. Methyl 4-hydroxybenzoate.URLhttps://echa.europa.eu/registration-dossier/-/registered-dossier/14310/5/3/2%0D (accessed 1.19.19).

ACUÑA, V. et al. Balancing the health benefits and environmental risks of pharmaceuticals: Diclofenac as an example. Environment international, v. 85, p. 327-333, 2015.

AJIMA, Malachy NO et al. Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, Clarias gariepinus. **Drug and chemical toxicology**, v. 38, n. 4, p. 383-390, 2015.

ALI, Shaukat; AALDERS, Jeffrey; RICHARDSON, Michael K. Teratological effects of a panel of sixty water-soluble toxicants on zebrafish development. **Zebrafish**, v. 11, n. 2, p. 129-141, 2014.

ANNILO, Tarmo et al. Evolution of the vertebrate ABC gene family: analysis of gene birth and death. **Genomics**, v. 88, n. 1, p. 1-11, 2006.

AQUINO, Sergio Francisco de; BRANDT, Emanuel Manfred Freire; CHERNICHARO, Carlos Augusto de Lemos. Remoção de fármacos e desreguladores endócrinos em estações de tratamento de esgoto: revisão da literatura. 2013.

AYLLON, F.; GARCIA-VAZQUEZ, E. Micronuclei and other nuclear lesions as

genotoxicity indicators in rainbow trout Oncorhynchus mykiss. **Ecotoxicology and Environmental Safety**, v. 49, n. 3, p. 221-225, 2001.

BARSE, A. V. et al. Vitellogenin induction and histo-metabolic changes following exposure of Cyprinus carpio to methyl paraben. Asian-Australasian Journal of Animal Sciences, v. 23, n. 12, p. 1557-1565, 2010.

BIRBEN, Esra et al. Oxidative stress and antioxidant defense. **World Allergy Organization Journal**, v. 5, n. 1, p. 9, 2012.

BO, Liu; SHENGEN, Zhang; CHANG, Chein-chi. Emerging Pollutants-Part II: Treatment. **Water Environment Research**, v. 88, n. 10, p. 1876-1904, 2016.

BUCHELI, Thomas D.; FENT, Karl. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. **Critical reviews in environmental science and technology**, v. 25, n. 3, p. 201-268, 1995.

BURGEOT, Thierry et al. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. **Marine ecology progress series**, v. 131, p. 125-141, 1996.

BŁĘDZKA, Dorota; GROMADZIŃSKA, Jolanta; WĄSOWICZ, Wojciech. Parabens. From environmental studies to human health. **Environment international**, v. 67, p. 27-42, 2014.

CAMPANA, Marcela A. et al. Genotoxic evaluation of the pyrethroid lambda-cyhalothrin using the micronucleus test in erythrocytes of the fish Cheirodon interruptus interruptus. **Mutation Research/Genetic Toxicology and Environmental Mutagenesis**, v. 438, n. 2, p. 155-161, 1999.

CARMONA, Eric; ANDREU, Vicente; PICÓ, Yolanda. Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin: from waste to drinking water. **Science of the total environment**, v. 484, p. 53-63, 2014.

CARPENTER, Stephen R.; STANLEY, Emily H.; VANDER ZANDEN, M. Jake. State of the world's freshwater ecosystems: physical, chemical, and biological changes. **Annual review of Environment and Resources**, v. 36, p. 75-99, 2011.

CARRASCO, Kenneth R.; TILBURY, Karen L.; MYERS, Mark S. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. **Canadian Journal of Fisheries and Aquatic Sciences**, v. 47, n. 11, p. 2123-2136, 1990.

CARROLA, João et al. Frequency of micronuclei and of other nuclear abnormalities in erythrocytes of the grey mullet from the Mondego, Douro and Ave estuaries—Portugal. **Environmental Science and Pollution Research**, v. 21, n. 9, p. 6057-6068, 2014.

CASHMAN, Jeremy N. The mechanisms of action of NSAIDs in analgesia. **Drugs**, v. 52, n. 5, p. 13-23, 1996.

CASTILLO, M. D. et al. Ensayos toxicológicos y métodos de evaluación de calidad de aguas: estandarización, intercalibración, resultados y aplicaciones. 2004.

CAVAS, Tolga; GARANKO, Natasha N.; ARKHIPCHUK, Victor V. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. **Food and Chemical Toxicology**, v. 43, n. 4, p. 569-574, 2005.

CHANDRASEKHARAN, N. V.; SIMMONS, Daniel L. The cyclooxygenases. **Genome biology**, v. 5, n. 9, p. 241, 2004.

CHEN, Yi et al. Occurrence, removal and environmental risk assessment of pharmaceuticals and personal care products in rural wastewater treatment wetlands. **Science of the Total Environment**, v. 566, p. 1660-1669, 2016.

Chen, Z., Shi, T., Zhang, L., Zhu, P., Deng, M., Huang, C., ... & Li, J. (2016). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in

multidrug resistance: A review of the past decade. *Cancer letters*, *370*(1), 153-164.

CUNHA, V. et al. Effects of pharmaceuticals and personal care products (PPCPs) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (Danio rerio) embryos. **Ecotoxicology and environmental safety**, v. 136, p. 14-23, 2017.

DA SILVAI LUIS, Diecson Ruy Orsolin et al. Monitoramento de agrotóxicos em águas superficiais de regiões orizícolas no sul do Brasil. **Ciência Rural**, v. 39, n. 9, 2009.

DAMBAL, Vrinda Yatin et al. Developmental toxicity and induction of vitellogenin in embryo-larval stages of zebrafish (Danio rerio) exposed to methyl Paraben. **Ecotoxicology and environmental safety**, v. 141, p. 113-118, 2017.

DAUGHTON, Christian G. Non-regulated water contaminants: emerging research. **Environmental Impact Assessment Review**, v. 24, n. 7-8, p. 711-732, 2004.

DAUGHTON, Christian G. Pharmaceuticals and the Environment (PiE): Evolution and impact of the published literature revealed by bibliometric analysis. **Science of the Total Environment**, v. 562, p. 391-426, 2016.

DE GARCÍA, Sheyla Ortiz; GARCÍA-ENCINA, Pedro A.; MATA, Rubén Irusta. Paper V. Occurrence and effects of pharmaceuticals and personal care products: new contributions in predictive models, potential risks assessments and rankings of hazard, p. 119, 2015.

DIAMOND, Jerome et al. Use of prospective and retrospective risk assessment methods that simplify chemical mixtures associated with treated domestic wastewater discharges. Environmental toxicology and chemistry, v. 37, n. 3, p. 690-702, 2018.

EBELE, Anekwe Jennifer; ABDALLAH, Mohamed Abou-Elwafa; HARRAD, Stuart. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. **Emerging Contaminants**, v. 3, n. 1, p. 1-16, 2017.

FENT, K.; WESTON, A. A.; CAMINADA, D. Erratum to “Ecotoxicology of human pharmaceuticals”[Aquatic Toxicology 76 (2006) 122-159](DOI: 10.1016/j. aquatox. 2005.09. 009). **Aquat. Toxicol**, 2006.

FERRARI, Benoît et al. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. **Ecotoxicology and environmental safety**, v. 55, n. 3, p. 359-370, 2003.

FREIRE, Marina Moreira et al. Biomarcadores na avaliação da saúde ambiental dos ecossistemas aquáticos. **Oecologia Brasiliensis**, v. 12, n. 3, p. 2, 2008.

GAO, Yanpeng et al. Computational consideration on advanced oxidation degradation of phenolic preservative, methylparaben, in water: mechanisms, kinetics, and toxicity assessments. **Journal of hazardous materials**, v. 278, p. 417-425, 2014.

GLASSMEYER, S. T.; SHOEMAKER, J. A. Effects of chlorination on the persistence of pharmaceuticals in the environment. **Bulletin of environmental contamination and toxicology**, v. 74, n. 1, p. 24-31, 2005.

GREDELJ, Andrea et al. Deriving predicted no-effect concentrations (PNECs) for emerging contaminants in the river Po, Italy, using three approaches: Assessment factor, species sensitivity distribution and AQUATOX ecosystem modelling. **Environment international**, v. 119, p. 66-78, 2018.

GROS, Meritxell; PETROVIĆ, Mira; BARCELÓ, Damiá. Development of a multi-residue analytical methodology based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. **Talanta**, v. 70, n. 4, p. 678-690, 2006.

HAMAN, Camille et al. Occurrence, fate and behavior of parabens in aquatic environments: a review. **Water Research**, v. 68, p. 1-11, 2015.

HAYES, John D.; PULFORD, David J. The glut athione S-transferase supergene family: regulation of GST and the contribution of the lsoenzymes to cancer chemoprotection and drug resistance part I. **Critical reviews in biochemistry and molecular biology**, v. 30, n. 6, p. 445-520, 1995.

HILL, Adrian J. et al. Zebrafish as a model vertebrate for investigating chemical toxicity. **Toxicological sciences**, v. 86, n. 1, p. 6-19, 2005.

HODGSON, Ernest et al. Phase 1 metabolism of toxicants and metabolic interactions. **Molecular and Biochemical Toxicology**, p. 173-203, 2008.

HOEGER, Birgit et al. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). **Aquatic Toxicology**, v. 75, n. 1, p. 53-64, 2005.

HOPKINS, Zachary R.; BLANEY, Lee. An aggregate analysis of personal care products in the environment: identifying the distribution of environmentally-relevant concentrations. **Environment international**, v. 92, p. 301-316, 2016.

HOSE, Jo Ellen et al. Elevated circulating erythrocyte micronuclei in fishes from contaminated sites off southern California. **Marine Environmental Research**, v. 22, n. 3, p. 167-176, 1987.

JANCOVA, Petra et al. Phase II drug metabolizing enzymes. **Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub**, v. 154, n. 2, p. 103-16, 2010.

JHA, Awadhesh N. Ecotoxicological applications and significance of the comet assay. **Mutagenesis**, v. 23, n. 3, p. 207-221, 2008.

JONKERS, Niels et al. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. **Environmental Science and Pollution**

Research, v. 17, n. 4, p. 834-843, 2010.

KARADAG, Hasan; FIRAT, Özgür; FIRAT, Özge. Use of oxidative stress biomarkers in Cyprinus carpio L. for the evaluation of water pollution in Ataturk Dam Lake (Adiyaman, Turkey). **Bulletin of environmental contamination and toxicology**, v. 92, n. 3, p. 289-293, 2014.

KASPRZYK-HORDERN, Barbara; DINSDALE, Richard M.; GUWY, Alan J. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. **Water research**, v. 43, n. 2, p. 363-380, 2009.

KLAASSEN, Curtis D.; WATKINS, J. B. Fundamentos em Toxicologia de Casarett e Doull (Lange). **Grupo A. pag**, v. 95, 2012.

KLEYWEGT, S.; SMYTH, S. A.; PARROTT, J. **Pharmaceuticals and personal care products in the Canadian environment: research and policy directions**. National Water Research Institute, 2000.

KROON, Frederieke; STRETEN, Claire; HARRIES, Simon. A protocol for identifying suitable biomarkers to assess fish health: A systematic review. **PloS one**, v. 12, n. 4, p. e0174762, 2017.

LIMA, Eder C. et al. Removal of emerging contaminants from the environment by adsorption. **Ecotoxicology and environmental safety**, v. 150, p. 1-17, 2018.

LIN, Angela Yu-Chen; PLUMLEE, Megan H.; REINHARD, Martin. Natural attenuation of pharmaceuticals and alkylphenol polyethoxylate metabolites during river transport: photochemical and biological transformation. **Environmental Toxicology and Chemistry: an International Journal**, v. 25, n. 6, p. 1458-1464, 2006.

LONAPPAN, Linson et al. Diclofenac and its transformation products: environmental occurrence and toxicity-a review. **Environment international**, v. 96, p. 127-138, 2016.

LONČAR, Jovica et al. Gene expression analysis of the ABC efflux transporters in rainbow trout (Oncorhynchus mykiss). **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, v. 151, n. 2, p. 209-215, 2010.

LUSHCHAK, Volodymyr I. Environmentally induced oxidative stress in aquatic animals. **Aquatic toxicology**, v. 101, n. 1, p. 13-30, 2011.

MALIK, Ajamaluddin et al. Structural and thermodynamic properties of kappa class glutathione transferase from Camelus dromedarius. **International journal of biological macromolecules**, v. 88, p. 313-319, 2016.

MARTÍN, J. et al. Occurrence of pharmaceutical compounds in wastewater and sludge from wastewater treatment plants: removal and ecotoxicological impact of wastewater discharges and sludge disposal. **Journal of hazardous materials**, v. 239, p. 40-47, 2012.

MARTÍN-VILLAMIL, María et al. Evaluación de la toxicidad de metilparabeno en Artemia franciscana: efectos sobre crecimiento, supervivencia y biomarcadores. **Nereis**, n. 8, p. 103-118, 2016.

MOMPELAT, S.; LE BOT, B.; THOMAS, O. Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. **Environment international**, v. 35, n. 5, p. 803-814, 2009.

MONSERRAT, José M. et al. Pollution biomarkers in estuarine animals: critical review and new perspectives. **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, v. 146, n. 1-2, p. 221-234, 2007.

MONTES-GRAJALES, Diana; FENNIX-AGUDELO, Mary; MIRANDA-CASTRO, Wendy. Occurrence of personal care products as emerging chemicals of concern in water resources: A review. **Science of the Total Environment**, v. 595, p. 601-614, 2017.

NAKADA, Norihide et al. Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. **Water research**, v. 41, n. 19, p. 4373-4382, 2007.

NASSEF, Mohamed et al. Pharmaceuticals and personal care products toxicity to Japanese medaka fish (Oryzias latipes). **J Fac Agric Kyushu Univ**, v. 54, n. 2, p. 407-411, 2009.

NELSON, David R. et al. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. **DNA and cell biology**, v. 12, n. 1, p. 1-51, 1993.

NORVILL, Zane N.; SHILTON, Andy; GUIEYSSE, Benoit. Emerging contaminant degradation and removal in algal wastewater treatment ponds: Identifying the research gaps. **Journal of hazardous materials**, v. 313, p. 291-309, 2016.

[OECD] ORGANISATION DE COOPÉRATION ET DE DÉVELOPPEMENT ÉCONOMIQUES. Test No. 236: fish embryo acute toxicity (FET) test. OECD Publishing, 2013.

PEAKALL, DW. Biomarkers: the way forward in environmental assessment. Toxicol Ecotoxicol News 1:55–60, 1994.

PENG, Xianzhi et al. Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal landfills in China. **Science of the Total Environment**, v. 490, p. 889-898, 2014.

PEREIRA, Camilo D. Seabra et al. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. **Science of the Total Environment**, v. 548, p. 148-154, 2016.

PRASKOVA, E. et al. Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (Danio rerio). **Polish journal of veterinary**

sciences, v. 14, n. 4, p. 545-549, 2011.

RAMASWAMY, Babu Rajendran et al. Determination of preservative and antimicrobial compounds in fish from Manila Bay, Philippines using ultra high performance liquid chromatography tandem mass spectrometry, and assessment of human dietary exposure. **Journal of hazardous materials**, v. 192, n. 3, p. 1739-1745, 2011.

RICHARDSON, Susan D.; TERNES, Thomas A. Water analysis: emerging contaminants and current issues. **Analytical chemistry**, v. 83, n. 12, p. 4614-4648, 2011.

ROBERTS, Jenna et al. Pharmaceuticals and personal care products (PPCPs) in Australia's largest inland sewage treatment plant, and its contribution to a major Australian river during high and low flow. **Science of the total environment**, v. 541, p. 1625-1637, 2016.

SANTOS, Lúcia HMLM et al. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. **Journal of hazardous materials**, v. 175, n. 1-3, p. 45-95, 2010.

SCHMID, W. The micronucleus test for cytogenetic analysis. In: **Chemical mutagens**. Springer, Boston, MA, 1976. p. 31-53.

SCHMITT-JANSEN, Mechthild et al. Phytotoxicity assessment of diclofenac and its phototransformation products. **Analytical and bioanalytical chemistry**, v. 387, n. 4, p. 1389-1396, 2007.

SCHWAIGER, J. et al. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. **Aquatic Toxicology**, v. 68, n. 2, p. 141-150, 2004.

SHANNON, Mark A. et al. Science and technology for water purification in the coming decades. In: **Nanoscience and technology: a collection of reviews from nature Journals**. 2010. p. 337-346.

SILVEIRA, Maria Angelis K. et al. Quantification of pharmaceuticals and personal care product residues in surface and drinking water samples by SPE and LC-ESI-MS/MS. **Journal of the Brazilian Chemical Society**, v. 24, n. 9, p. 1385-1395, 2013.

STUART, Marianne et al. Review of risk from potential emerging contaminants in UK groundwater. **Science of the Total Environment**, v. 416, p. 1-21, 2012.

SUÁREZ, Sonia et al. How are pharmaceutical and personal care products (PPCPs) removed from urban wastewaters? **Reviews in Environmental Science and Bio/Technology**, v. 7, n. 2, p. 125-138, 2008.

TARPANI, Raphael Ricardo Zepon; AZAPAGIC, Adisa. A methodology for estimating concentrations of pharmaceuticals and personal care products (PPCPs) in wastewater treatment plants and in freshwaters. **Science of The Total Environment**, v. 622, p. 1417-1430, 2018.

TRIEBSKORN, R. et al. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (Oncorhynchus mykiss). **Aquatic toxicology**, v. 68, n. 2, p. 151-166, 2004.

VALDÉS, María Eugenia et al. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). Science of the Total Environment, v. 472, p. 389-396, 2014.

VANE, J. R.; BAKHLE, Y. S.; BOTTING, R. M. CYCLOOXYGENASES 1 AND 2. **Annual review of pharmacology and toxicology**, v. 38, n. 1, p. 97-120, 1998.

VERLICCHI, Paola; AL AUKIDY, M.; ZAMBELLO, Elena. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and

environmental risk after a secondary treatment—a review. **Science of the total environment**, v. 429, p. 123-155, 2012.

WANG, Jianlong; WANG, Shizong. Removal of pharmaceuticals and personal care products (PPCPs) from wastewater: a review. **Journal of environmental management**, v. 182, p. 620-640, 2016.

WESTFIELD, M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio) Eugene, OR. 2000.

WHYTE, J. J. et al. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. **Critical reviews in Toxicology**, v. 30, n. 4, p. 347-570, 2000.

WU, Baojian; DONG, Dong. Human cytosolic glutathione transferases: structure, function, and drug discovery. **Trends in pharmacological sciences**, v. 33, n. 12, p. 656-668, 2012.

WU, Yang et al. Comparative studies of aerobic and anaerobic biodegradation of methylparaben and propylparaben in activated sludge. **Ecotoxicology and environmental safety**, v. 138, p. 25-31, 2017.

YAN, Qing et al. Occurrence, fate and ecotoxicological assessment of pharmaceutically active compounds in wastewater and sludge from wastewater treatment plants in Chongqing, the Three Gorges Reservoir Area. **Science of the Total Environment**, v. 470, p. 618-630, 2014.

YU, Yong; WU, Laosheng; CHANG, Andrew C. Seasonal variation of endocrine disrupting compounds, pharmaceuticals and personal care products in wastewater treatment plants. **Science of the Total Environment**, v. 442, p. 310-316, 2013.

ZAK, John C. et al. Functional diversity of microbial communities: a quantitative approach. **Soil Biology and Biochemistry**, v. 26, n. 9, p. 1101-1108, 1994.

ZHAO, Wenxing et al. Deriving acute and chronic predicted no effect concentrations of pharmaceuticals and personal care products based on species sensitivity distributions. **Ecotoxicology and environmental safety**, v. 144, p. 537-542, 2017.

ZIYLAN, Asu; INCE, Nilsun H. The occurrence and fate of anti-inflammatory and analgesic pharmaceuticals in sewage and fresh water: treatability by conventional and non-conventional processes. **Journal of hazardous materials**, v. 187, n. 1-3, p. 24-36, 2011.

CAPÍTULO 1

Toxicity and sublethal effects of methylparaben (MeP) on larvae and adults of zebrafish *Danio rerio*

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Abstract

Among the parabens, MeP is the most commonly found in personal care products. Due to the continuous use of MeP and low removal efficiency by wastewater treatment plants (WWTPs), it reaches aquatic environments, being incorporate by organisms. In this sense, this study aimed to evaluate the effects of MeP on larvae and adults of the zebrafish *Danio rerio* through toxicity tests and physiological and biochemical biomarkers. For biomarkers measurements, fish were exposed to the environmental concentration of 30 µg/L of Mep and the non-effect concentration (NOEC) estimated for larvae (60 mg/L) and adult (50 mg/L) in toxicity tests. The LC_{50} of MeP was 105.09 mg/L for adults and 211.12 mg/L for larvae, indicating an unexpected greater sensitivity of adults compared to larvae. In adult fish, exposure to MeP at 50 mg/L induced a significant decrease in phase 1 biotransformation (EROD activity) and an increase in lipoperoxidation (LPO) in gills, as well as, an increase in micronuclei (MNs) incidence in erythrocytes. Sublethal exposure of MeP (30 µg/L and 60 mg/L) did not cause toxicity to larvae, based on LPO quantity. In this sense, we investigated if the difference in sensitiveness between adults and larvae would be associated with the antimicrobial action of MEP that could affect the intestinal microbiota of adults. We found only an increase in the number of carbon sources consumed by them without effects on richness and abundance. Perhaps, this is an adaptation to environmental stress, but it is not a signal of a negative effect. However, the oxidative stress (represented by LPO) and mutagenicity caused by MeP to the adults of zebrafish call direct attention to the importance of regulating the presence of this compound in the environment and investing in the processes adopted by WWTP.

Key words: PPCP, fish; acute tests; biomarkers; microbiota.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are used in individual care, industry, agriculture, and animal production; thus, they becoming ubiquitous in environments (Bo et al., 2016; Daughton and Ternes, 1999). Due to the continued use of PPCPs, improper disposal and inefficiency of Wastewater Treatment Plants (WWTPs) to remove them from urban sewage, they have been found in rivers, water sources for human consumption and marine ecosystems, at increasing concentrations over the years (Nakada et al., 2007; Roberts et al., 2016; Yu et al., 2013). However, the majority of PPCPs are not environmentally regulated and this is one of the reasons why PPCPs are considered as contaminants of emerging concern. In this sense, Agencies as USEPA (Environmental Protection Agency, US), ECHA (European Chemicals Agency, EU), and DEA (Drug Enforcement Administration, USA) are looking for information to control these products from an environmental point of view (Lima, 2018).

Parabens are one of the main groups of chemical compounds found in PPCPs products (Ramaswamya et al., 2011). The extensive use of parabens occurs by its broad spectrum of antimicrobial activity, high stability, and low costs of production (Błędzka et al., 2014). Among parabens, MeP is the most commonly used (Jonkers et al., 2010) and, therefore, one of the most frequently detected in effluents (Haman et al., 2015). Despite the rapid degradation of MeP under aerobic conditions (1mg/L takes 19.3 min to degrade in water) (Wu et al., 2017), processes adopted at WWTP are not efficient enough to remove all input of MeP. Also, the lack of sanitation contributes to the presence of MeP in the environment (only 40% of the world manage sewage safety). About environmental concentrations of MeP, Stuart et al., (2012) reported concentration up to 5 µg/L in groundwater in UK, Peng et al., (2014) reported values between 0.3 to 9 ng/L in the Pearl River Delta in China between 2012 and 2013; and in Southern Brazil,

MeP was detected in concentration varying from 7.6 to 29.8 μg/L inurface water located in two citys in Southern Brazil, between <1 to 134 μg/L in surface water (Silveira et al., 2013).

Toxicity data reported in the literature for MeP and its Predicted Non-Effect Concentration (PNEC) to aquatic life are higher than concentrations found in natural environments (Puerta et al., 2020). However, most of these results were estimated based on acute toxicity tests and little is known about the sublethal effects of this compound, especially, on lower levels of organization such as physiological, cellular and molecular. In this sense, biomarkers are considered as useful tools to access early adverse of effects and contaminants presence provide important information regarding organisms living in polluted environments (Burgeot et al., 1996). There is a lack of studies with biomarkers for MeP and responses like enzymes and proteins involved in the metabolism and the damage that this compound can cause.

Briefly, detoxification comprises biotransformation processes (phase 1 and phase 2) and cell extrusion (phase 3). Phase 1 involves oxidation reactions, reduction and xenobiotics hydrolysis; while phase 2 covers xenobiotic conjugation or its metabolites to an endogenous compound such as glutathione (GSH). Phase 1 reactions (oxidation) are catalyzed by the cytochrome P450 (CYP). Its activity can be measured as the activity of Ethoxyresorufin O-deethylase (EROD), a sensitive indicator for the inductive response of the CYP1A, which, in turn, responds mainly to contamination by organic pollutants (Kroon et al., 2017). Phase 2 biotransformation can be mediated by the enzyme Glutathione S-transferase (GST), which catalyzes xenobiotics conjugation with GSH (Heath, 1995). Both processes make the contaminant more hydrophilic, facilitating their excretion. While, phase 3 prevents xenobiotics accumulation and/or their metabolites inside the cells, removing them via energy-

dependent ABC (ATP-Binding Cassette) transporters which are located in the cell membranes (Kroon et al., 2017).

Therefore, the combination of toxicity tests and biomarkers analysis provides more information about the chemical risk assessmentto aquatic ecosystems. In this sense, the objective of this study was to evaluate MeP toxicity (values of CL_{50}) to larvae and adults of the zebrafish *Danio rerio*, as well as, its sublethal effects through biomarkers of exposure (phase 1, phase 2 and phase 3 detoxification) and damage such as lipoperoxidation (LPO) and DNA anomalies.

2. Materials and methods

2.1 Zebrafish

Adults of zebrafish *D. rerio* were purchased from a commercial supplier and acclimated in a cultivation system for 15 days in a 15 liter aquariums filled with dechlorinated tap water (1 g fish/liter), constantly aerated, at 27°C andphotoperiod of 12D:12L. Fish were fed twice a day with commercial feed (Tetra). To obtain the larvae, zebrafish were put in breeding aquariums in a ratio of 1 female: 1 male, overnight.

After spawning,in the early morning, the eggs were collected to a beaker (250 mL) filled with dechlorinated tap water and incubated at 27º C at a photoperiod of 12D:12L for hatching. Larvae up to 24 h post-hatching (hph) were used for the experiments. Fish cultivation and larvae obtaining were performed at Animal Care Room of the Instituto de Ciências Biológicas – Universidade Federal do Rio Grande – FURG.

Averages of water physic-chemical parameters measured in the acclimation period and over the experiments (sections 2.3 and 2.4) are summarized in Table 1.

Table 1. Physic-chemical parameters of water measured during acclimation and experiments. Values are presented as mean ± standard error (SE).

Parameter	Measurement
Temperature	26.1 ± 0.3ºC
pH	7.1 ± 0.3
Dissolved oxygen	6.00 ± 0.1 mg/L
Conductivity	290.2 ± 3 $\mu S \cdot cm^{-1}$
Nitrite	0.5 ± 0.01ppm
Ammonia	0.5 ± 0.1 ppm

2.2 Toxicity Tests

Toxicity tests were performed with acclimated adults and zebrafish larvae following the guidelines ABNT NBR 15499 (ABNT, 2007) and ABNT NBR 15088 (ABNT, 2016), respectively. For toxicity tests with adults, males and females were randomly exposed (96 h) in aquariums glass to 30, 48, 67.5, 101 and 150 mg/L of MeP and a control group (without MeP in the medium) was kept over the test. It was used 10 organisms per treatment and one replica with 0.39 g ± 0.04g of weight and 3.2 cm of length. Media were totally renewed at 48 h and fish were not fed during the test.

The 24 hph larvae was put into in exposed to 60, 102, 173, 294 and 500 mg/L of MeP and a control group was also maintained over the experiment. It was used 40 larvae per treatment in quadruplicate. Larvae exposure lasted 168 h, with a total renewal of the media every 48 h. Due to the low solubility of MeP in the water, we had to use DMSO as a solvent, thus all treatments were maintained with 0.01% of DMSO. Moreover, MeP (CAS 99-76-3) used in our experiments was 99% purity and commercially obtained from manipulation pharmacy. In both assays, lethality was recorded and used to estimate LC_{50} (median lethal concentration for 50 % of the organisms). Physic-chemical parameters of water are described in Table 1.

2.3 Sublethal exposures

Adult males of *D. rerio* (n = 36, 0.34g ± 0.03g of weight and 2.8 cm ± 0.03 of length) were exposed in triplicate to the Non-Effect Concentration (NOEC) of MeP, established in the toxicity test (section 2.2), that was 50 mg/L, and the relevant environmental concentration of 30 µg/L of MeP (see Haman et al., 2015). Exposure lasted 96 h with the total renewal of the medium at 48 h. Fish have not been fed during the experiment. In the same way, larvae (n = 40 divided into 4 replicates) were also exposed (168 h) to the NOEC of MeP estimated in the toxicity test (section 2.2) as 60 mg/L, and to the environmental 30 µg/L of MeP, plus the control group. All the treatments contained 0.01% of DMSO.

After 96 h of exposure, adults (n = 6 fish of each treatment) were anesthetized with tricaine (MS-222) (200 mg/L) and blood was punctured from the caudal vein using an insulin needle fixed to a syringe. Blood smears collected from each fish were prepared on clean slides and then fixed with methanol (99.8%) for 10 min. Micronucleus (MNs) and other nuclear abnormalities (NAs) were checked in the Red Blood Cells (RBCs). Then, all fish, including those used for RBCs analysis, were euthanized with tricaine (MS-222) (500 mg/L) and their liver, gills and intestine were dissected and submitted to the procedures described in (sections 2.4 and 2.5).

For larvae, after 168 h of the test, they were directly immersed in tricaine (MS-222) solution (300 mg/L) for euthanasia, and storage at -80ºC for further analysis of LPO. Physic-chemical parameters of water can be seen in Table 1.

2.4 RBCs analysis

Slides with fixes smears were stained with acridine orange (0.003%) dissolved in Sorenson's buffer pH 6.8, for 2 to 3 minutes. Then, they were examined under a fluorescence microscope (Opticam O500R FLUO) at 1000x magnification according to Ueda et al., (1992) with modifications. A total of 2000 RBCs were examined for each fish for the presence of

MNs and NAs following the protocol described by Carrasco et al. (1990). A total of 2000 RBCs were accounted for each fish slide.

2.5 Biochemical assays

Gills and liver dissected from the adult fish were used to measure activities of EROD and GST enzymes and ABC transporters, and also LPO content. Only ABC activity was determined in fish tissues right after the end of the experiment. Other measurements were made in tissues stored at – 80ºC.

For the EROD and GST assay, liver and gills (6 pools of 3 fish organs, per treatment) were thawed on ice, homogenized in phosphate buffer (0.1 M, pH 7.0) and centrifuged (20000 g, 20 min, 4°C). EROD activity was determined in the supernatant according to Eggens and Galgani (1992) by the conversion rate of 7-ethoxyresorufin (non-fluorescent) to resorufin (fluorescent). Eleven readings lasted 10 min. Results were expressed as pmol of resorufin/mg protein/min. GST activity was estimated according to Keen et al., (1976) by the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB); the readings lasted 270 seconds and results were expressed in nmol of GSH/mg protein/min.

The ABC transporters activity was analyzed in gills and livers (n = 6 per treatment) according to the method of Kurelec (1992), using Rhodamine B as a fluorescent substrate of these transporters. Tissues were weighed and incubated in Rhodamine B (10 µmol) for 1 h in the dark at room temperature (24º C). After that, gills and liver were washed twice with Phosphate-Buffered Saline (PBS) to remove the unabsorbed fluorescent and, subsequently, 300 µl of distilled water was added to each sample to homogenize the tissues. Then, the homogenates were centrifuged at 7500 g for 5 min. Finally, 100 µL of the supernatant was added to 96-well microplate and fluorescence of Rhodamine B was quantified at 540 nm of excitation and 590 nm of emission in a fluorimeter (F5 Multi-Mode Microplate Reader –

Molecular Devices). The ABC activity was calculated base on the Rhodamine B accumulation and it was relativized by tissue wet weight. Results were expressed as a percentage of ABC protein activity considering activity in the control group as 100%.

For LPO analysis, gills and liver (6 pools of 2 fish per treatment) of adult fish and whole larvae (4 pools of 10 larvae per treatment) were thawed on ice and homogenized in 1.15% potassium chloride buffer with 35 μM of butylated hydroxytoluene (BHT), and centrifuged at 20000 g for 20 min at 4°C. LPO was determined in the supernatant using a TBARS assay kit (Cayman chemical, 10009055). The method is based on the reaction of malondialdehyde (MDA) with acid thiobarbituric (TBA) under conditions of high temperature and acidity that generates a fluorochrome, which is quantified by fluorimetry (F5 Multi-Mode Microplate Reader – Molecular Devices) at 553 nm of emission and 515 nm of excitation. This assay measures MDA, which is one of the final products of LPO. Results were expressed as μM MDA/mg protein.

Protein content in the supernatant was quantified by the biuret method (commercial kit Doles Reagentes Ltda., Brazil).

2.6 Intestinal microbiota analysis

Intestines collected from fish (n = 6 per treatment) were rinsed immediately after dissection with sterile saline; then, they were transferred to 5 ml test tubes filled with the same solution and vortexed for 30 sec for cleaning. The mixture was diluted 150 times and transferred (120 μl) to the Biolog EcoPlate (Insam, 1997).

The ability of microbial communities to use different carbon sources was assessed. The 96 wells plate contained 31 carbon sources (carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids and polymers) or saline solution (control), in triplicate. The utilization of the carbon sources by microbiota was evaluated by reducing the violet redox

tetrazolium dye, which moved from colorless to purple when microorganisms are using the substrate.

The reactions were analyzed in a fluorometer (F5 Multi-Mode Microplate Reader – Molecular Devices) at 590 nm after 120 h of incubation. Readings of samples incubated with different carbon sources were subtracted from the average of control readings.

Besides the number of substrates used by microbiota, we also calculated the index of color development, Shannon diversity and Shannon evenness (Gryta et al., 2014). Average color development (AWCD) was calculated as:

$$AWCD = \sum \frac{ODi}{31}$$

Where ODi is the initial optical density.

The Shannon diversity index was calculated as:

$$H = -\sum pi\ (lnpi)$$

Where, H is Shannon index of diversity and pi is proportional color development

The Shannon evenness (E) was estimated as:

$$E = \frac{H}{lnS}$$

Where, H is the Shannon index of diversity and S is the substrate

2.7 Statistical analysis

Toxicity data expressed as LC_{50} were calculated using *Trimmed Spearman Karber* software. The NOEC value used for the biomarker assays was obtained from the highest concentration that did not affect the survival of the exposed organisms in toxicity tests. Data on biomarker and microbiota analyzes were expressed as mean ± standard error (SE). Differences between treatments were verified by one-way analysis of variance (ANOVA) and, for the significant differences, the *posthoc* Tukey test was performed. Residual analyzes were executed to validate the assumptions of normality (Shapiro-Wilk), homoscedasticity (Levene) and independence (autocorrelation function (acf)) of data. All analyses were performed using the software R-Studio.

3. Results

Toxicity tests from the present study resulted in LC_{50} of 105.09 mg/L (91.68 - 120.45) for adults and 211.12 mg/L (190.82 - 233.60) for the larvae.

Figure 1A shows a tendency (not statistically different) of EROD to be higher in the liver of fish from control groups compared to treatments with MeP. However, EROD was significantly inhibited in gills of fish acutely exposed MeP at 50 mg/L (Figure 1B). Regarding GST activity and ABC proteins, there were no significant differences between treatments in both liver and gills (Figure 2 and Figure 3, respectively). LPO did not defer in the liver, but it was augmented in the gills of fish exposed to the NOEC concentration (50 mg/L of MeP) (Figure 4A and Figure 4B, respectively). Besides adults, this biomarker was also analyzed in larvae of *D. rerio* and no significant difference was observed between treatments (Figure 5). It is worth saying that the NOEC tested for larvae was 60 mg/L, a little higher than the NOEC used in the experiments with adults which was 50 mg/L of MeP.

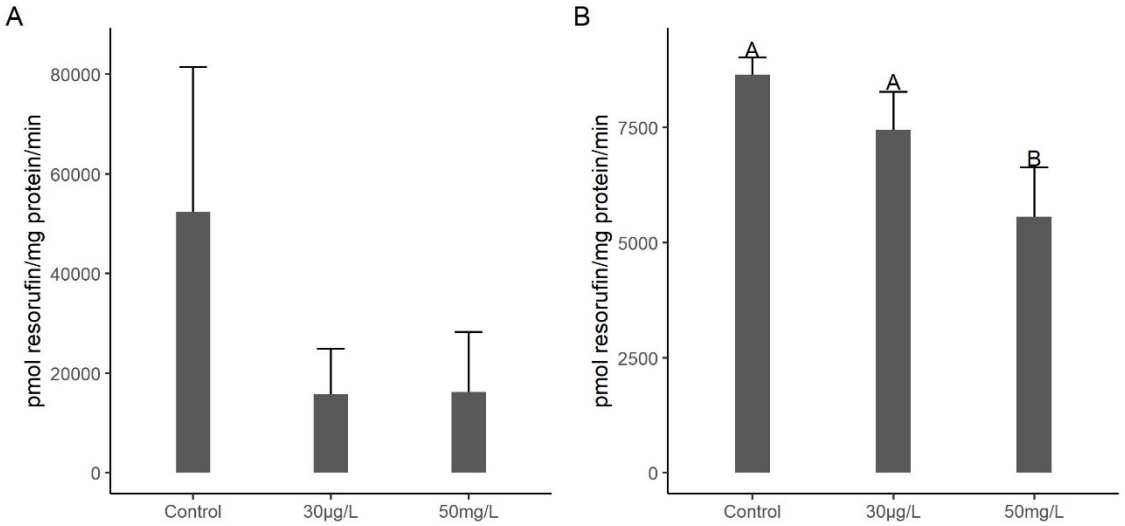

Figure 1. Ethoxyresorufin-O-deethylase (EROD) activity in the liver (A) and gills (B) of adult zebrafish males under control condition or exposed to MeP at 30 µg/L (environmental concentration) and 50 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE. Different letters indicate significant differences between treatments ($p < 0.05$)

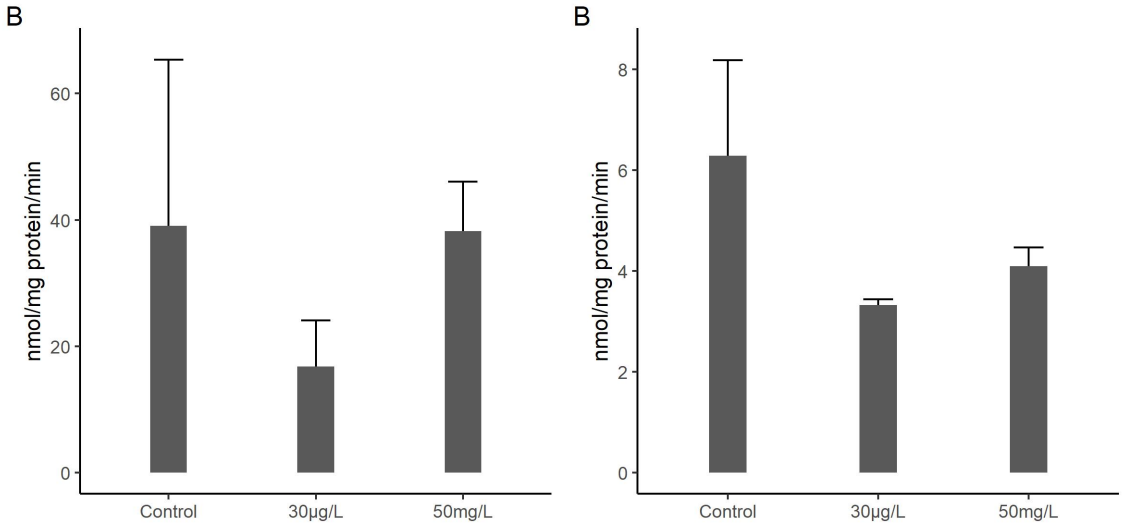

Figure 2. Glutathione S-transferase (GST) activity in the liver (A) and gills (B) of adult zebrafish males under control condition or exposed to MeP at 30 µg/L (environmental concentration) and 50 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE

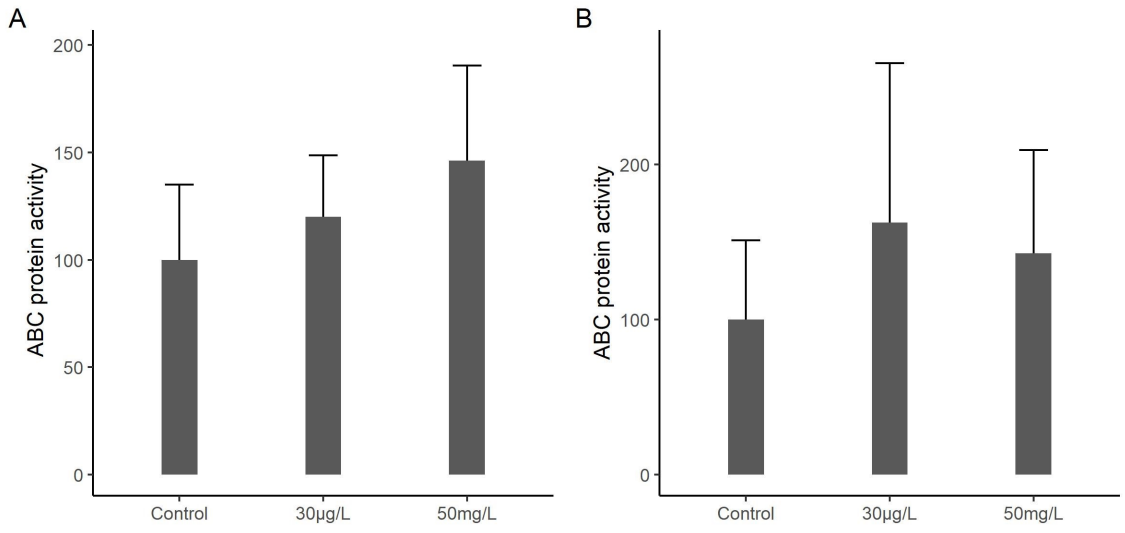

Figure 3. ABC protein activity percentage in the liver (A) and gills (B) of adult zebrafish males under control condition or exposed exposed to MeP at 30 µg/L (environmental concentration) and 50 mg/L of (NOEC) for 96 h. Results were estimated based in the control group which was considered as 100% of activity. Data are expressed as mean ± SE.

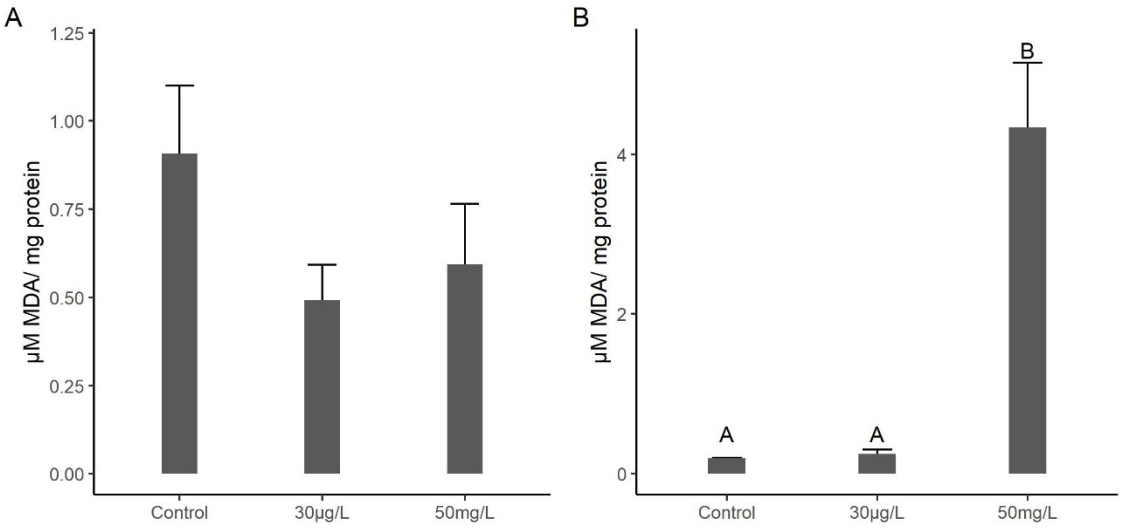

Figure 4. Lipid peroxidation (LPO) expressed as MDA formation in liver (A) and gills (B) of adult zebrafish males under control condition or exposed to MeP at exposed to 30 µg/L (environmental concentration) and 50 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE. Different letters indicate statistical differences between treatments ($p<0.05$).

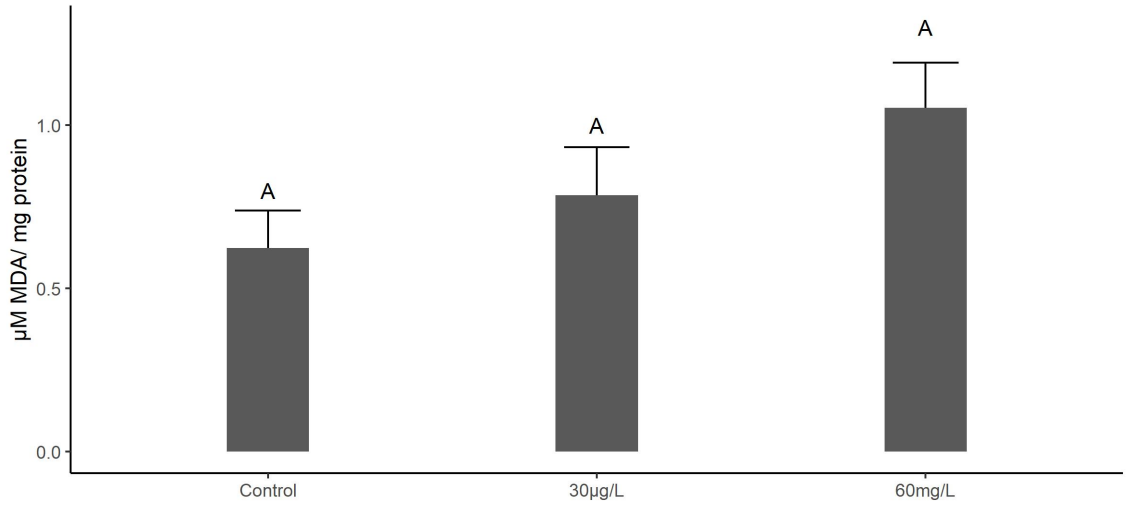

Figure 5. Lipid peroxidation (LPO), expressed as the formation of MDA of larvae zebrafish under control condition or exposed to MeP at 30 µg/L (environmental concentration) and 60 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE

Table 2 summarizes the results noticed for RBCs. The MNs augmented in RBCs of fish exposed to 50 mg/L of MeP, but no at 30 µg/L, in comparison to the control. There was also an increase in the number of kidney-shaped nuclei at the concentration of 50 mg/L of MeP.

Table 2. Results of MNs and NAs in zebrafish RBCs under control conditions or exposed to MeP at 30 µg/L (environmental concentration) and 50 mg/L (NOEC concentration). A total of 2000 RBCs were accounted for each fish for these analyses. Different letters indicate a significant difference between treatments ($p < 0.05$).

Anomalies	Control	30 µg/L MeP	50 mg/L MeP
Segmented	0^a	0^a	0^a
Lobulated	8^a	4^a	8^a
Kidney	3^a	4^a	18^b
Binucleated	0^a	0^a	0^a
Micronucleo	0^a	0^a	4^b

Microbiota of intestine did not vary in terms of the indices of AWCD, Shannon diversity or Shannon evenness, as shown in Table 3. However, the number of substrates used (richness) by them increased in fish exposed to MeP at both concentrations tested. Despite that, there were no significant differences between treatments related to each carbon source (Figure 6).

Table 3. Results of the AWCD, Shannon diversity, Shannon evenness, and the number of carbon sources used by microbiota, that we named richness. They were calculated for the microbiota extracted from the intestine of adult zebrafish under control conditions or exposed to MeP at an of 30 µg/L(environmental concentration) and 50 mg/L (NOEC). Samples were incubated for 120 h. Data are expressed as means ± SE. Different letters indicate significant differences between treatments ($p < 0.05$).

Index	Treatments		
	Control	30 µg/L MeP	60 mg/L MeP

Shannon evenness	0.98 ± 0.05[a]	0.98 ± 0.02[a]	0.97 ± 0.03[a]
Shannon diversity	2.22 ± 0.26[a]	2.48 ± 0.51[a]	2.27 ± 0.40[a]
AWCD	1 ± 0.04[a]	1.37 ± 0.84[a]	1 ± 0.002[a]
Richness	21 ± 3.2[a]	28 ± 2,2[b]	29 ± 2,4[b]

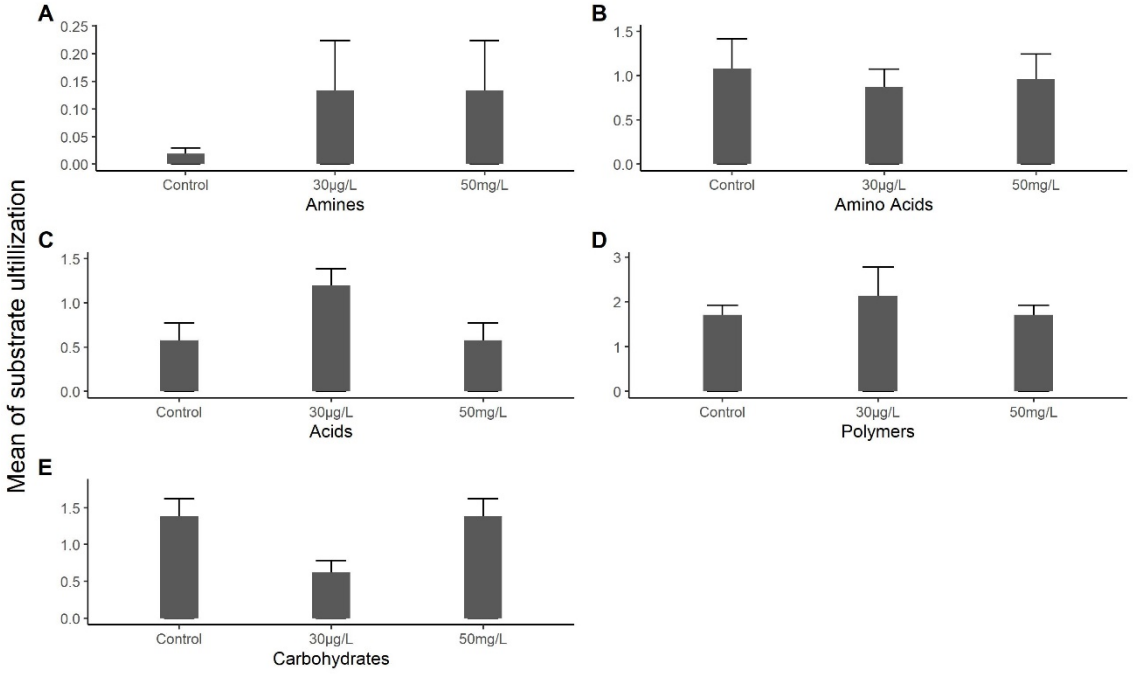

Figure. 6. Use of different carbon sources (A: Amine, B: Amino Acids, C: Carboxylic Acids, D: Polymers, E: Carbohydrates) by the microbiota from the intestine of fish exposed (96 h) to MeP (30 µg/L and 50 mg/L) or under control condition. Data are expressed as means ± SE.

4. Discussion

Toxicity tests showed that MeP is more toxic to *D. rerio* adults than larvae (LC_{50} were 105.09 mg/L and 211.12 mg/L, respectively), what was unexpected, since early stages of fish are commonly most sensitive to pollutants (Freiry et al., 2014; McKim, 1977). Concerning literature about MeP toxicity to fish, most studies founded smaller values compared to our work. Yamamoto et al., (2011), Hopkins and Blaney, (2016), and Silva et al., (2018), reported LC_{50} values, for the japanese rice *Orizias latipes,* fathead minnow *Pimephales promelas,* and

Nile tilapia *Oreochromis niloticus,* as being 63.5, 67 and 11 mg/L, respectively. Moreover, Ates et al. (2018) estimated a LC_{50} of 50 mg/L for *D. rerio* embryos. Taking into account these data and those presented in this study, seemingly, adult and larva of *D. rerio* are more tolerant to MeP than the other mentioned organisms.

Mechanisms of MeP toxicity are not clear, and only a few studies are assessing its effects through biomarkers. Most information available for fish refers to MeP as an endocrine disruptor that causes adverse effects on fish reproduction (Alslev et al., 2005; Barse et al., 2010; Dambal et al., 2017; Terasaki et al., 2009). Additionally, Dobbins et al., (2009) proposed that parabens cause narcosis to invertebrate and fish. Narcoses are reversible changes in membrane integrity and functionality caused by the interaction and/or accumulation of pollutants in the lipophilic portion of the membrane (Escher and Hermens, 2002; Sandermann, 2008). It can cause changes in fish behavior and gills inflammation (redness) (Van Wezel and Opperhuizenb, 1995). In this sense, we observed changes in fish swimming performance and redness in the gills during the acute toxicity test, but only for the higher concentration tested, and because these symptoms were observed and not quantified, we did not show in the results section.

Fish gills are considered an important route for contaminants uptake and therefore, they may react firstly to their presence in comparison to other organs. The causes for this are mainly the wide surface area in contact with the external medium, thin epithelium and presence of several transporters used for ion exchange (Ameur et al., 2015; Azambuja et al., 2011; Brunelli et al., 2011). Inhibition of phase 1 biotransformation, represented by the reduction of EROD activity, with a concomitant increase of LPO in gills of fish exposed to 50 mg/L, indicates that MeP may affect biotransformation pathways, including antioxidants defenses. In this sense, the lack of augmented in GST activity would also contribute to the LPO increase, since GST prevents oxidative damage by conjugation of a xenobiotic to GSH

or by interacting directly with products of LPO (Choi et al., 2008). Another important point was the non-increase of ABC transporters activity following exposure to MeP, which, in turn, could allow an increase in the intracellular concentration of MeP, rising its potential to cause toxicity. Ates et al. (2018) in their study also observed an increase in LPO in zebrafish embryos exposed to 50 mg/L of MeP, reinforcing our results.

Concerning the liver, it was not seen a significant effect of MeP for any parameter or concentration tested. The liver has been considered as the main organ involved in xenobiotic biotransformation towards detoxification (Hinton et al., 2017). Perhaps, the absence of a response was due to short time of exposure or because the liver is better prepared to deal with the presence of contaminants and therefore the tested concentrations of MeP (30µg/L and 50 mg/L) were not potent enough to induce noticeable changes in the biomarkers analyzed in this tissue.

Genotoxic effects observed as the increasing of MNs and ENAs in RBCs of fish exposed to NOEC may be relatedto augment LPO levels and decrease in EROD in the gills. Even if the analyzes were not performed in the same tissue, an effect of MeP on gills may lead to a systemic effect, affecting RBCs, and therefore the general health of the animal. In this way, Samanta et al., (2018) also observed a positive relationship between DNA damage in blood and LPO in gills, liver, and kidney of the goldfish *Carassius auratus*. Damage to lipid membranes as LPO can expose the genetic material to contaminants uptake by the cells. If these alterations are not repaired, mutagenicity may occur (Corredor-Santamaría 2016). The genotoxic potential of MeP was previously observed in human skin and lymphocytes cells (Alnuqaydan and Sanderson, 2016; Bayülken et al., 2017) but for fish is the first time.

The higher tolerance of larvae of *D. rerio* to MeP compared to adults in terms of LC50 was confirmed by the absence of LPO at the equipotent NOEC concentration of 60 mg/L. In fact, NOEC of larvae and adults were similar. Thus, we hypothesized that antimicrobial action

of MeP would affect adults, due to their higher dependence on intestinal microbiota than larvae, which depended basically on the vitelo to feed themselves. We investigated if MeP influenced on characteristics of microbiota present in the intestine of adult zebrafish and according to our results (Table 3), at 30 µg/L and 50 mg/L, the MeP did not affect the bacterial community (increase, decrease or substitution), indicating that intestinal microbiota was preserved.

Moreover, there was no variation in the preference of sources (substrates) chosen by the intestinal microbiota (Figure 8). However, at both treatments, there was an increase in the number of substrates used by intestinal microbiota when compared to the control treatment. Perhaps, this increase may indicate an adaptation to ensure the survival of microbiota in adverse conditions such as polluted environments where quality or quantity of food (carbon) source can be altered. Notwithstanding, this information does not explain differences in sensitivity between adults and larvae.

In this sense, studies evaluating toxicity and sublethal effects of MeP are important to regulate it. In parallel, investments in the processes adopted in WWTPs and sanitation are important to reduce the input of MeP in aquatic ecosystems.

5. Conclusion

MeP is more toxic to larvae than adults of zebrafish. In adults, MeP toxicity was associatedwith a decrease in phase 1 biotransformation and augmentin LPO in gills and also to DNA damages in the RBCs. Negative effects were observed mostly in the NOEC concentration (50 mg/L of MeP). MeP affected intestinal microbiota of adult fish, but only in terms of substrates number used by them; which does not mean a negative effect. Thus, despite MeP has been found at low concentrations in the aquatic environments it may pose a potential risk to aquatic biota. Results presented by this study MeP call attention to the

importance of regulating MeP, of investing in sanitation and improve the processes adopted by WWTP to remove more efficiently the amount of MeP present in the wastewater.

6. Acknowledgements

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7. References

[ABNT] - Brazilian Association of Technical Standards. (2007). Aquatic ecotoxicology - Short-term chronic toxicity - test method with fish. ABNT-NBR 15499 (portuguese).

[ABNT] - Brazilian Association of Technical Standards. (2016). Aquatic ecotoxicology - Acute toxicity Test method with fish (Cyprinidae) ABNT NBR 15088, third edition, 13.12.2016.

Adame, A., Rocha, F.R.P., 2014. A multi-pumping flow system for acute toxicity bioassay using the Vibrio fischeri bacteria. Anal. Methods 6, 7367–7373. https://doi.org/10.1039/C4AY00789A

Ajima, M.N.O., Ogo, O.A., Audu, B.S., Ugwoegbu, K.C., 2015. Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, Clarias gariepinus. Drug Chem. Toxicol. 38, 383–390. https://doi.org/10.3109/01480545.2014.974108

Alnuqaydan, A.M., Sanderson, B.J., 2016. Toxicity and Genotoxicity of Beauty Products on Human Skin Cells In Vitro 6, 1–9. https://doi.org/10.4172/2161-0495.1000315

Alslev, B., Korsgaard, B., Bjerregaard, P., 2005. Estrogenicity of butylparaben in rainbow trout Oncorhynchus mykiss exposed via food and water. Aquat. Toxicol. 72, 295–304. https://doi.org/10.1016/j.aquatox.2005.01.005

Américo-Pinheiro, J.H.P., Isique, W.D., Nádia Hortense Torres, A.A.M., Carvalho, S.L. de, Filho, W.V.V., Ferreira, L.F.R., 2017. Ocorrência de diclofenaco e naproxeno em água superficial no município de Três Lagoas (MS) e a influência da temperatura da água na detecção desses anti - inflamatórios. Eng. sanitária e Ambient. 22, 429–435. https://doi.org/10.1590/S1413-41522017128719

Ameur, W. Ben, El Megdiche, Y., de Lapuente, J., Barhoumi, B., Trabelsi, S., Ennaceur, S., Camps, L., Serret, J., Ramos-López, D., Gonzalez-Linares, J., Touil, S., Driss, M.R., Borràs, M., 2015. Oxidative stress, genotoxicity and histopathology biomarker responses in Mugil cephalus and Dicentrarchus labrax gill exposed to persistent pollutants. A field study in the Bizerte Lagoon: Tunisia. Chemosphere 135, 67–74. https://doi.org/10.1016/j.chemosphere.2015.02.050

Ates, P.S., Alturfan, A.A., Emekli-alturfan, E., 2018. Methylparaben induces malformations and alterations on apoptosis , oxidant – antioxidant status , ccnd1 and myca expressions in zebrafish embryos. J. Biochem. Mol. Toxicol. 1–6. https://doi.org/10.1002/jbt.22036

Azambuja, C.R., Mattiazzi, J., Riffel, A.P.K., Finamor, I.A., Garcia, L. de O., Heldwein, C.G., Heinzmann, B.M., Baldisserotto, B., Pavanato, M.A., Llesuy, S.F., 2011. Effect of the essential oil of Lippia alba on oxidative stress parameters in silver catfish (Rhamdia quelen) subjected to transport. Aquaculture 319, 156–161. https://doi.org/10.1016/j.aquaculture.2011.06.002

Barse, A. V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., Kumar, N., Raman, R.P., Jadhao, S.B., 2010. Vitellogenin induction and histo-metabolic changes following exposure of Cyprinus carpio to methyl paraben. Asian-Australasian J. Anim. Sci. 23, 1557–1565.

https://doi.org/10.5713/ajas.2010.10118

Bayülken, D.G., Tüylü, B.A., Sinan, H., Sivas, H., 2017. Investigation of genotoxic effects of paraben in cultured human lymphocytes. Drug Chem. Toxicol. 0, 1–8. https://doi.org/10.1080/01480545.2017.1414834

Błędzka, D., Gromadzińska, J., Wąsowicz, W., 2014. Parabens . From environmental studies to human health. Environ. Int. J. 67, 27–42. https://doi.org/10.1016/j.envint.2014.02.007

Bo, L., Shengen, Z., Chang, C., 2016. Emerging Pollutants - Part II : Treatment. Water Environ. Res. 88, 1876–1904. https://doi.org/10.2175/106143016X14696400495857

Brunelli, E., Mauceri, A., Maisano, M., Bernabo, I., Giannetto, A., Elena De Domenicob, Barbara Corapi a, Sandro Tripepi a, n, S.F. b, 2011. Ultrastructural and immunohistochemical investigation on the gills of the teleost , Thalassoma pavo L ., exposed to cadmium. acta Histochem. 113, 201–213. https://doi.org/10.1016/j.acthis.2009.10.002

Burgeot, T., Bocquene, G., Galganil, F., 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. 131, 125–141.

Carmona, E., Andreu, V., Picó, Y., 2014. Science of the Total Environment Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin : From waste to drinking water. Sci. Total Environ. 484, 53–63. https://doi.org/10.1016/j.scitotenv.2014.02.085

Carrasco, K.R., Tilbury, K.L., Myers, M.S., 1990. Assessment of the Piscine Micronuc eus Test as an in situ Biological Indicator of Chemica Contaminant Effects. Fish. Aquat. Sci. 47, 21 23-2436. https://doi.org/doi.org/10.1139/f90-237

Choi, C.Y., An, K.W., An, M.I., 2008. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (Paralichthys olivaceus). Comp. Biochem. Physiol. - A Mol. Integr. Physiol.

149, 330–337. https://doi.org/10.1016/j.cbpa.2008.01.013

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–315. https://doi.org/10.1016/S0147-6513(03)00141-6

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194. https://doi.org/10.1016/S0378-4274(03)00068-7

Corredor-Santamaría, W.M.S.G., Velasco-Santamaría, Y.M., 2016. Using genotoxic and haematological biomarkers as an evidence of environmental contamination in the Ocoa River native fish ,. Springerplus. https://doi.org/10.1186/s40064-016-1753-0

Damasceno de Oliveira, L.L., Nunes, B., Antunes, S.C., Campitelli-Ramos, R., Rocha, O., 2018. Acute and Chronic Effects of Three Pharmaceutical Drugs on the Tropical Freshwater Cladoceran Ceriodaphnia silvestrii. Water. Air. Soil Pollut. 229. https://doi.org/10.1007/s11270-018-3765-6

Dambal, V.Y., Selvan, K.P., Lite, C., Barathi, S., Santosh, W., 2017. Developmental toxicity and induction of vitellogenin in embryo-larval stages of zebrafish (Danio rerio) exposed to methyl Paraben. Ecotoxicol. Environ. Saf. 141, 113–118. https://doi.org/10.1016/j.ecoenv.2017.02.048

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and Personal Care Products in the Environment : Agents of Subtle Change ? Environ. Health Perspect. 107, 907–938.

DeLorenzo, M.E., Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species Dunaliella tertiolecta. Arch. Environ. Contam. Toxicol. 54, 203–210. https://doi.org/10.1007/s00244-007-9032-2

Dobbins, L.L., Usenko, S., Brain, R.A., Brooks, B.W., 2009. Probabilistic ecological hazard

assessment of parabens using Daphnia magna and Pimephales promelas. Environ. Toxicol. Chem. 28, 2744–2753. https://doi.org/10.1897/08-523.1

Du, J., Mei, C.F., Ying, G.G., Xu, M.Y., 2016. Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea Daphnia magna. Bull. Environ. Contam. Toxicol. 97, 84–90. https://doi.org/10.1007/s00128-016-1806-7

Eggens, M.L., Galgani, F., 1992. Ethoxyresorufin-O-deethylase (EROD) activity in flatfish: Fast determination with a fluorescence plate-reader. Mar. Environ. Res. 33, 213–221. https://doi.org/10.1016/0141-1136(92)90149-G

Epel, D., Luckenbach, T., Steveson, C.N., MacManus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: Newly Appreciated Roles in Protection against Pollutants. Environ. Sci. e Technol. 3914–3920. https://doi.org/doi/abs/10.1021/es087187v

Escher, B.I., Hermens, J.L.M., 2002. Critical Review Modes of Action in Ecotoxicology : Their Role in Body Burdens , Species Sensitivity , QSARs , and Mixture Effects. Environ. Sci. e Technol. 36, 4201–4217. https://doi.org/doi.org/10.1021/es015848h

Freire, M.M., Santos, V.G., Ginuino, I.S.F., Linde, A.R., 2008. Biomarcadores na avaliação da saúde ambiental dos ecossistemas aquáticos. oecologia Bras. 12, 347–354.

Freiry, R., Stelzer, J.A.A., Maltchik, L., Arenzon, A., 2014. Sensitivity of Danio rerio (Teleostei, Cyprinidae) during Two Stages of Development Based on Acute Toxicity Tests. Bull. Environ. Contam. Toxicol. 93, 442–445. https://doi.org/10.1007/s00128-014-1367-6

Ghiselli, G., 2006. Avaliação das águas destinadas ao abastecimento público na região de Campinas: ocorrência e determinação dos interferentes endócrinos (IE) e produtos farmacêuticos e de higiene pessoal (PFHP). Unicamp 181p.

Gómez-Oliván, L.M., Neri-Cruz, N., Galar-Martínez, M., Islas-Flores, H., García-Medina, S., 2014. Binary mixtures of diclofenac with paracetamol, ibuprofen, naproxen, and

acetylsalicylic acid and these pharmaceuticals in isolated form induce oxidative stress on Hyalella azteca. Environ. Monit. Assess. 186, 7259–7271. https://doi.org/10.1007/s10661-014-3925-0

Gryta, A., Fr, M., Oszust, K., 2014. The Application of the Biolog EcoPlate Approach in Ecotoxicological Evaluation of Dairy Sewage Sludge. Appl. Biochem. Biotechnol. 174, 1434–1443. https://doi.org/10.1007/s12010-014-1131-8

Guiloski, I.C., Luiz, J., Ribas, C., Pereira, S., Paula, A., Neves, P., Cristina, H., Assis, S. De, 2015. Ecotoxicology and Environmental Safety Effects of trophic exposure to dexamethasone and diclofenac in freshwater fi sh. Ecotoxicol. Environ. Saf. 114, 204–211. https://doi.org/10.1016/j.ecoenv.2014.11.020

Guiloski, I.C., Piancinib, L.D.S., Ana Carolina Dagostimb, Sabrina Loise de Morais Caladoc, Luis Fernando Fávarod, S.L.B., Marta Margarete Cestarib, Cláudio da Cunhaa, H.C.S. de A., 2017. Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish Rhamdia quelen. Ecotoxicol. Environ. Saf. 139, 291–300. https://doi.org/10.1016/j.ecoenv.2017.01.053

Haman, C., Dauchy, X., Rosin, C., Munoz, J.F., 2015. Occurrence, fate and behavior of parabens in aquatic environments: A review. Water Res. 68, 1–11. https://doi.org/10.1016/j.watres.2014.09.030

Heath, A.G., 1995. Water pollution and Fish Physiology., 1st ed. crcpress, Florida.

Hinton, D.E., Segner, H., Braunbeck, T., 2017. Toxic responses of the liver, in: Target Organ Toxicity in Marine and Freshwater Teleosts. p. 45.

Hoeger, B., Bernd, K., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f . fario). Aquat. Toxicol. 75, 53–64. https://doi.org/10.1016/j.aquatox.2005.07.006

Hopkins, Z.R., Blaney, L., 2016. An aggregate analysis of personal care products in the

environment: Identifying the distribution of environmentally-relevant concentrations. Environ. Int. 92–93, 301–316. https://doi.org/10.1016/j.envint.2016.04.026

Insam, H., 1997. A new set of substrates proposed for community characterization in environmental samples. Microbial communities: functional versus structural approaches 259–260.

Jonkers, N., Sousa, A., Galante-oliveira, S., 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro , Portugal. Environ. Sci. Pollut. Res. 17, 834–843. https://doi.org/10.1007/s11356-009-0275-5

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

Kroon, F., Streten, C., Harries, S., 2017. A protocol for identifying suitable biomarkers to assess fish health : A systematic review. PLoS One 12, 1–43. https://doi.org/10.1371/journal.pone.0174762

Kurelec, B., 1992. The Multixenobiotic Resistance Mechanism in Aquatic Organisms. Crit. Rev. Toxicol. 22, 23–43. https://doi.org/doi/abs/10.3109/10408449209145320

Láng, J., Kohidai, L., 2012. Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate Tetrahymena. Chemosphere 89, 592–601. https://doi.org/10.1016/j.chemosphere.2012.05.058

Lee, J., Ji, K., Lim, Y., Kim, P., Choi, K., 2011. Ecotoxicology and Environmental Safety Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. Ecotoxicol. Environ. Saf. 74, 1216–1225. https://doi.org/10.1016/j.ecoenv.2011.03.014

Lima, C.S., C, E., 2018. Ecotoxicology and Environmental Safety Removal of emerging contaminants from the environment by adsorption. Ecotoxicol. Environ. Saf. 150, 1–17. https://doi.org/10.1016/j.ecoenv.2017.12.026

Lonappan, L., Brar, S.K., Das, R.K., Verma, M., Surampalli, R.Y., 2016. Diclofenac and its transformation products: Environmental occurrence and toxicity - A review. Environ. Int. 96, 127–138. https://doi.org/10.1016/j.envint.2016.09.014

McKim, J.M., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. J. Fish. Res. Board Canada 34, 1148–1154. https://doi.org/10.1139/f77-172#.XjS4RTJKjIU

Michael, I., Hapeshi, E., Michael, C., Varela, A.R., Kyriakou, S., Manaia, C.M., Fatta-kassinos, D., 2012. Solar photo-Fenton process on the abatement of antibiotics at a pilot scale : Degradation kinetics , ecotoxicity and phytotoxicity assessment and removal of antibiotic resistant enterococci. Water Res. 46, 5621–5634. https://doi.org/10.1016/j.watres.2012.07.049

Moermond, C.T.A., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: Criteria for reporting and evaluating ecotoxicity data. Environ. Toxicol. Chem. 35, 1297–1309. https://doi.org/10.1002/etc.3259

Nakada, N., Shinohara, H., Murata, A., Kiri, K., Managaki, S., Sato, N., Takada, H., 2007. Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. Water Res. 41, 4373–4382. https://doi.org/10.1016/j.watres.2007.06.038

Nieto, A., Borrull, F., Marcé, R.M., Pocurull, E., 2009. Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1216, 5619–5625. https://doi.org/10.1016/j.chroma.2009.05.061

Oliveira, L.L.D. de, 2014. Biomarcadores enzimáticos e testes ecotoxicológicos na avaliação da toxicidade de fármacos em invertebrados aquáticos. Universidade de São Paulo, São

Carlos. https://doi.org/10.11606/T.18.2014.tde-09022015-110612

Peng, X., Ou, W., Wang, C., Wang, Z., Huang, Q., Jin, J., Tan, J., 2014. Science of the Total Environment Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal land fi lls in China. Sci. Total Environ. 490, 889–898. https://doi.org/10.1016/j.scitotenv.2014.05.068

Pérez, S., Rial, D., Beiras, R., 2015. Acute toxicity of selected organic pollutants to saltwater (mysid Siriella armata) and freshwater (cladoceran Daphnia magna) ecotoxicological models. Ecotoxicology 24, 1229–1238. https://doi.org/10.1007/s10646-015-1489-6

Praskova, E., Plhalova, L., Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Hostovsky, M., Skoric, M., Maršálek, P., Voslarova, E., Svobodova, Z., 2014. Effects of Subchronic Exposure of Diclofenac on Growth , Histopathological Changes , and Oxidative Stress in Zebrafish (Danio rerio). Sci. World J. 2014, 1–5. https://doi.org/https://doi.org/10.1155/2014/645737

Praskova, E., Voslarova, E., Siroka, Z., Plhalova, L., Macova, S., Marsalek, P., Pistekova, V., Svobodova, Z., 2011. Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (Danio rerio). Pol. J. Vet. Sci. 14, 545–549. https://doi.org/10.2478/v10181-011-0081-0

Puerta, Y.T., Guimarães, P.S., Martinsa, S.E.M., Gaspar, C. de M., 2020. Ecotoxicology and Environmental Safety Toxicity of methylparaben to green microalgae species and derivation of a predicted no effect concentration (PNEC) in freshwater ecosystems. Ecotoxicol. Environ. Saf. 1–8. https://doi.org/10.1016/j.ecoenv.2019.109916

Quinn, B., Schmidt, W., Rourke, K.O., Hernan, R., 2011. Chemosphere Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (Dreissena polymorpha) and their comparison with standardised toxicity tests. Chemosphere 84, 657–663. https://doi.org/10.1016/j.chemosphere.2011.03.033

Ramaswamya, B.R., Kima, J.-W., Isobea, T., Changa, K.-H., Amanoe, A., W., T.M., Fernando P. Siringanf, S.T., 2011. Determination of preservative and antimicrobial compounds in fish from Manila Bay , Philippines using ultra high performance liquid chromatography tandem mass spectrometry , and assessment of human dietary exposure. J. Hazard. Mater. 192, 1739–1745. https://doi.org/10.1016/j.jhazmat.2011.07.006

Ribeiro, S., Torres, T., Martins, R., Santos, M.M., 2015. Toxicity screening of Diclofenac, Propranolol, Sertraline and Simvastatin using Danio rerio and Paracentrotus lividus embryo bioassays. Ecotoxicol. Environ. Saf. 114, 67–74. https://doi.org/10.1016/j.ecoenv.2015.01.008

Roberts, J., Kumar, A., Du, J., Hepplewhite, C., Ellis, D.J., Christy, A.G., Beavis, S.G., 2016. Pharmaceuticals and personal care products (PPCPs) in Australia's largest inland sewage treatment plant, and its contribution to a major Australian river during high and low flow. Sci. Total Environ. 541, 1625–1637. https://doi.org/10.1016/j.scitotenv.2015.03.145

Samanta, P., Im, H., Na, J., Jung, J., 2018. Ecological risk assessment of a contaminated stream using multi-level integrated biomarker response in Carassius auratus *. Environ. Pollut. 233, 429–438. https://doi.org/10.1016/j.envpol.2017.10.061

Sandermann, H., 2008. Chemosphere Ecotoxicology of narcosis : Stereoselectivity and potential target sites. Chemosphere 72, 1256–1259. https://doi.org/10.1016/j.chemosphere.2008.05.002

Scheurell, M., Franke, S., Shah, R.M., Hühnerfuss, H., 2009. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. Chemosphere 77, 870–876. https://doi.org/10.1016/j.chemosphere.2009.07.066

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B., 2011. Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (Mytilus spp.) and their comparison

with standardized toxicity tests. Mar. Pollut. Bull. 62, 1389–1395. https://doi.org/10.1016/j.marpolbul.2011.04.043

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Aquat. Toxicol. 68, 141–150. https://doi.org/10.1016/j.aquatox.2004.03.014

Silva, D.C., Serrano, L., Oliveira, T.M.A., Mansano, A.S., Almeida, E.A., Vieira, E.M., 2018. Effects of parabens on antioxidant system and oxidative damages in Nile tilapia (Oreochromis niloticus). Ecotoxicol. Environ. Saf. 162, 85–91. https://doi.org/10.1016/j.ecoenv.2018.06.076

Silveira, M.A.K., Caldas, S.S., Guilherme, J.R., Costa, F.P., Guimarães, B.D.S., Cerqueira, M.B.R., Soares, B.M., Primel, E.G., 2013. Quantification of pharmaceuticals and personal care product residues in surface and drinking water samples by SPE and LC-ESI-MS/MS. J. Braz. Chem. Soc. 24, 1385–1395. https://doi.org/10.5935/0103-5053.20130176

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J., Svobodova, Z., 2013. The effects of diclofenac on early life stages of common carp (Cyprinus carpio). Environ. Toxicol. Pharmacol. 35, 454–460. https://doi.org/10.1016/j.etap.2012.09.011

Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Science of the Total Environment Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21. https://doi.org/10.1016/j.scitotenv.2011.11.072

Sturm, A., Segner, H., 2005. P-glycoproteins and xenobiotic efflux transport in fish. Biochem. Mol. Biol. Fishes 6, 495–533. https://doi.org/doi/abs/10.3109/10408449209145320

Terasaki, M., Makino, M., Tatarazako, N., 2009. Acute toxicity of parabens and their chlorinated by-products with Daphnia magna and Vibrio fischeri bioassays. J. Appl.

Toxicol. 29, 242–247. https://doi.org/10.1002/jat.1402

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H., Schwaiger, J., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II . Cytological effects in liver , kidney , gills and intestine of rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 68, 151–166. https://doi.org/10.1016/j.aquatox.2004.03.015

Trombini, C., Hampel, M., Blasco, J., 2016. Evaluation of acute effects of four pharmaceuticals and their mixtures on the copepod Tisbe battagliai. Chemosphere 155, 319–328. https://doi.org/10.1016/j.chemosphere.2016.04.058

Ueda, T., Hayashi, M., Ohtsuka, Y., Nakamura, T., Kobayashi, J., Sofuni, T., 1992. A Preliminary study of the micronucleus test by acridine orange fluorescent staining compared with chromosomal aberration test using fish erythropoietic and embryonic cells. wal Sci. Technol. Res. 25, 235–240. https://doi.org/doi.org/10.2166/wst.1992.0297

Van Wezel, A.P., Opperhuizenb, A., 1995. Narcosis Due To Environmental Pollutants in Aquatic Organisms : Residue-Based Toxicity , Mechanisms , and Membrane Burdens. Crit. Rev. Toxicol. 25, 255–279. https://doi.org/doi.org/10.3109/10408449509089890

Verlicchi, P., Zambello, E., 2015. Science of the Total Environment Pharmaceuticals and personal care products in untreated and treated sewage sludge : Occurrence and environmental risk in the case of application on soil — A critical review. Sci. Total Environ. 538, 750–767. https://doi.org/10.1016/j.scitotenv.2015.08.108

Wu, Y., Sun, Q., Wang, Y., Deng, C., Yu, C., 2017. Comparative studies of aerobic and anaerobic biodegradation of methylparaben and propylparaben in activated sludge. Ecotoxicol. Environ. Saf. 138, 25–31. https://doi.org/10.1016/j.ecoenv.2016.12.017

Yamamoto, H., Tamura, I., Hirata, Y., Kato, J., Kagota, K., Katsuki, S., Yamamoto, A., Kagami, Y., Tatarazako, N., 2011. Aquatic toxicity and ecological risk assessment of seven parabens: Individual and additive approach. Sci. Total Environ. 410–411, 102–

111. https://doi.org/10.1016/j.scitotenv.2011.09.040

Yu, Y., Wu, L., Chang, A.C., 2013. Science of the Total Environment Seasonal variation of endocrine disrupting compounds , pharmaceuticals and personal care products in wastewater treatment plants. Sci. Total Environ. 442, 310–316. https://doi.org/10.1016/j.scitotenv.2012.10.001

Ziylan, A., Ince, N.H., 2011. The occurrence and fate of anti-inflammatory and analgesic pharmaceuticals in sewage and fresh water: Treatability by conventional and non-conventional processes. J. Hazard. Mater. 187, 24–36. https://doi.org/10.1016/j.jhazmat.2011.01.057

CAPÍTULO 2

Effects of diclofenac assessed by toxicity tests and biomarkers in adults and larvae of *Danio rerio*

Manuscrito será submetido para para a revista Environmental Toxicology and Pharmacology.

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Abstract

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (ANEs) widely used for the treatment of pain and inflammation in humans and animals. Inappropriate use, incorrect disposal, the inefficiency of wastewater treatment plants (WWTPs) in removing this compound and lack of environmental regulation contribute to DCF presence in water bodies, affecting aquatic biota. The objective of this work was to evaluate the effects of the diclofenac through toxicity tests (LC50) and biomarkers in larvae and adults of *Danio rerio*. A set of biochemical, genotoxic and physiological biomarkers were evaluated in blood, liver, and gills at an environmental concentration of 2µg/L of DCF and the non-effect concentration (NOEC) based on the mortality of adults and larva which was 3mg/L of DCF. The LC50 values were 5.49 mg/L for adults and 5.22 mg/L for the larvae. At NOEC it was observed changes in activities of glutathione-S-transferase (GST) enzyme and ATP Binding Cassete (ABC) transporters in gills of adult fish and also it was seen an increase in lipoperoxidation (LPO) in gills of adults and larvae, without statistical evidence of micronuclei (MNs) and other erythrocytic anomalies (EAs). Gills seem to be a target organ of DCF toxicity which causes oxidative stress. Also, toxicity tests and biomarkers are tools that complement each other to provide predictive effects of DCF to aquatic ecosystems.

Keywords: PPCP, fish; acute test; biomarkers; anti-inflammatory.

1. Introduction

Diclofenac (DCF) is a nonsteroidal anti-inflammatory drug used to treat pain and inflammation in humans and animals (Gan, 2010). DCF can be purchased in drugstores without the needfor prescription and this results in high consumption of this drug. The annual consumption ranges from 195 to 940 mg per inhabitant, based on data from different countries (Sakshaug, 2012). Therefore, DCF is commonly found in domestic wastewater (Américo-Pinheiro et al., 2017; Ghiselli, 2006; Lee et al., 2011; Lonappan et al., 2016; Schmidt et al., 2011). However, the lack of sanitation, mainly in middle-income and low-income countries, and the inefficiency of Wastewater Treatment Plants (WWTPs) to remove Pharmaceuticals and Personal Care Products (PPCPs) from the sewage (Carmona et al., 2014; Ghiselli, 2006; Michael et al., 2012; Verlicchi and Zambello, 2015) led to contamination of natural aquatic environments with DCF (Lonappan et al., 2016). DCF has a high solubility in water (2.37 mg/L at 25°C) but it has a short half-life, about 3.3 h (Ziylan and Ince, 2011).

Despite that, it has often been detected in domestic wastewater and water bodies around the world due to its continuous input. Carmona et al. (2014) reported DFC concentrations in the Turia River in Spain at 49 ng/L and they also reported DCF in mineral water at 25 ng/L; but the highest concentration reported was 4900 ng/L in rivers of Pakistan (Scheurell et al., 2009).

The literature has described quite varied effects of DCF such as cellular changes in the liver, kidneys, and gills of fish as well as histological anomalies and necrosis (Hoeger et al., 2005; Schwaiger et al., 2004; Triebskorn et al., 2004). Also and anomalies development of *Danio rerio* embryos were reported by Ribeiro et al. (2015). Despite these evidence, mechanisms of DCF toxicity, risk assessment, and its regulation are not well established. In 2013, DCF was included in the list of priority substances in a European Water Quality

Directive (EU Water Framework Directive, 2013) with a maximum permitted concentrations of 0.1 µg/L DCF in freshwater and 0.01 µg/L DCF in seawater. In fact, few regulations provide legislation and/or guidelines to establish limits of DCF concentration in aquatic environments, but they are not significant on a global scale.

Considering the information exposed above, this study assessed the toxicity of DCF and its sublethal effects through biomarkers using larvae and adults of *D. rerio* as biological models. Biomarkers are sensitive tools used to anticipate adverse effects that precede mortality, thus, they provide a prospective assessment about the health of organisms living in polluted ecosystems (Freire et al., 2008).

Biomarkers investigated in the present work were: activity of 7-ethoxyresorufin-O-desethylase (EROD) that is a way to measure CYP1A activity, activity of glutathione S-transferase (GST) and activity of ABC transporters as biomarkers of exposure; and lipoperoxidation (LPO) and analysis of micronuclei (MNs) and other erythrocyte nuclear abnormalities (ENAs) as biomarkers of damage (oxidative stress and genotoxicity, respectively). It is worth saying that activities of EROD, GST and ABC transporters represent phase 1, phase 2, and phase 3 of the biotransformation process.

2. Materials and methods

2.1 Zebrafish acclimation and larvae obtention

Adults of *D. rerio* purchased commercially were acclimated for 15 days in a cultivation system at the Institute of Biological Sciences of the Federal University of Rio Grande in 15 liter aquariums (1g fish/liter of water) filled with dechlorinated tap water, constantly aerated at a temperature of 27ºC and photoperiod of 12hD:12hL. During acclimation, fish were fed twice a day with commercial food for fish (Tetra).

Larvae of *D. rerio* were obtained at the same location where adults were acclimated. For that, couples were separated and placed in breeding aquariums overnight. After spawning, the eggs were collected and put in beakers filled with 250 mL of E3 medium (suitable for egg incubation) and kept at 27ºC for hatch. Larvae up to 24 h post-hatching (hph) were used for experiments.

Physic-chemical parameters of water were measured in acclimation and over the experiments (see sections 2.2 and 2.3) and they are described in Table 1.

Table 1. Physic-chemical parameters of water measured during acclimation and experiments. Values are presented as mean ± standard error (SE).

Parameter	Measurement
Temperature	25.9 ± 0.3 ºC
pH	7.3 ± 0.3
Dissolved oxygen	6.07 ± 0.1 mg/L
Conductivity	200.2 ± 2 $\mu S \cdot cm^{-1}$
Nitrite	0.5 ± 0.01 ppm
Ammonia	0.5 ± 0.01 ppm

2.2 Toxicity tests

Toxicity tests performed with adults (acute) and larvae (chronic) of zebrafish followed the guidelines ABNT NBR 15499 (ABNT, 2009) and ABNT NBR 15088 (ABNT, 2017), respectively. For tests with adults of *D. rerio*, males and females (n = 10, 0.32 ± 0.2 g of weight and 2.8 ± 0.02 cm in length) were randomly exposed to concentrations ranging from 2 to 10 mg/L of DCF plus a control group (without DCF in water). The duration of the test was 96 h, but the media was totally renewed after 48 h of exposure. Water was constantly aerated and fish were not feeding over the test. Larvae (24 hph) were exposed (n = 40 divided into 4 replicates) to the same concentrations used in the tests with adults, but the exposure lasted 168

h, with the total renewal of the media every 48 h. For both test lethality was recorded and used to estimate LC_{50}.

An aliquot of water was sampled at the beginning of the test from the control group and exposure concentrations of 2 and 10 mg/L DCF from both tests (with adults and larvae) for quantification of the contaminant. DCF sodium salt was purchased from manipulation pharmacy (CAS 15307-79-6, 99% purity). Physic-chemical parameters of water are summarized in Table 1, above.

2.3 Sublethal exposures for biomarkers assay

For the sublethal exposure, adult males of *D.rerio* (n= 36, 0.35g ± 0.01 g of weight and 2.35 cm ± 0.03 in length) were exposed in triplicate to the Non-Effect Observed Concentration (NOEC) of 3 mg/L DCF, estimated in the toxicity test (section 2.2), and to the environmental relevant concentration of 2 μg/L (Haman et al., 2015). A control group containing water without DCF was kept over the test. The test lasted 96 h, with a total renewal of water at 48 h. Animals did not feed over the experiment. Larvae (n = 40 divided in 4 replicates) were also exposed (168 h) to 3 mg/L and 2 μg/L of DCF. The concentration of 3 mg/L also represents larvae NOEC. An aliquot of water was sampled at the beginning of the test from all treatments for DCF measurement.

After exposure, adults (n = 6 per treatment) were anesthetized with tricaine (MS-222) (200 mg/L) and blood was punctured from the caudal vein using an insulin needle fixed to a syringe. Blood smears collected from each fish were prepared on clean slides and then fixed with methanol (99.8%) for 10 min. The slides were used for micronucleus (MNs) and erythrocytic anomalies (ENAs).

Then, punctured fish and the other fish submitted to the experimentation were euthanized with tricaine (MS-222) (500 mg/L) and their liver and gills were dissected. Tissues

used for quantification of EROD activity, GST activity and LPO were stored at –80ºC for further analysis, but tissues used for ABC transporters activity measurement were immediately used in the assay. On the other hand, larvae submitted do exposures for 168 h were directly immersed in tricaine (MS-222) solution (500 mg/L) for euthanasia and stored at –80ºC for later LPO analysis.

2.5 Genotoxic analysis

MNs and ENAs were performed according to the technique described by Ueda et al. (1992) with modifications. The slides with fixed smears were stained with acridine orange (0.003%) dissolved in Sorenson buffer pH 6.8, for 2 to 3 min. Then, they were examined under a fluorescence microscope (Opticam O500R FLUO) at 1000x magnification. It was counted 2000 cells for each fish and they were examined for the presence of MNs and ENAs (Carrasco et al., 1990). Data were expressed as means ± standard error (SE).

2.6 Biochemical Analyzes

Gills and liver dissected from the adult fish (6 pools of 3 fish organs per treatment) were thawed on ice, homogenized in a phosphate buffer (0.1 M, pH 7.0) and centrifuged (20000 g, 20 min, 4°C). EROD and GST were analyzed in supernatant according to Eggens and Galgani (1992) and Keen et al. (1976), respectively. EROD activity was determined by the conversion of 7-ethoxyresorufin to resorufin and expressed as pmol resorufin/mg protein/min, eleven readings were done for 10 min. The GST activity was analyzed by the monitoring of the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) and it was expressed by nmol of GSH/mg protein/min, with readings over 270 s.

The activity of ABC transporters was analyzed in gills and livers (n = 6 per treatment) based on the method of Kurelec (1992). After dissection, the tissues were weighted and immediately incubated in Rhodamine B (10 µM) for 1 h in the dark at 24ºC. After that, the tissues were washed twice with Phosphate-Buffered Saline (PBS) to remove the unabsorbed fluorescent. Subsequently, 300 µl of distilled water was added to each sample and the tissues were homogenized and centrifuged at 7500 g for 5 min. Finally, fluorescence was analyzed in 100 µL of the supernatant at the wavelength of 540/590 nm excitation/emission using a fluorimeter (F5 Multi-Mode Microplate Reader – Molecular Devices). Results were expressed as a percentage of ABC protein activity.

LPO was determined using a TBARS assay kit (Cayman chemical, 10009055) in gills and liver of adult fish (6 pools of 2 fish organs per treatment) and also in the whole larvae (4 pools of 10 larvae). The method is based on the reaction of malondialdehyde (MDA) with acid thiobarbituric (TBA) under conditions of high temperature and acidity, which generates a fluorochrome detected by a fluorimeter (F5 Multi-Mode Microplate Reader – Molecular Devices) at 553 nm of emission and 515 nm of excitation. This assay measured MDA, which is one of the final products of LPO. Results were expressed as µM MDA/mg protein.
As noted, all results described in this section were standardized by protein content in supernatant which was quantified by the biuret method (commercial kit Doles Reagentes Ltda., Brazil).

2.7 DCF quantification

Samples of water from acute fish toxicity tests and sublethal experiments were used to determine DCF. Samples were acidified with HCL to achieve the pH 3.0, then they were filtered (250 mL) using a GF / F filter (Glass Microfiber Filters, 47 mm in diameter, Whatman), to follow the solid phase extraction (EFS) processing. Conditioning was carried

out by passing 5 ml of ethyl acetate by gravity, followed by 5 ml of methanol and 5 ml of ultrapure water (pH 3.0 - HCl). The 250 mL of the sample was passed through the cartridge with the aid of a vacuum pump with a pressure of -300 mmHg.

After passing the entire sample through the cartridge, it was dried under vacuum for 30 min. Dried samples were eluted with 2 aliquots of 4 ml of ethyl acetate followed by 1 aliquot of 4 ml of dichloromethane. All 12 ml were collected in the same graduated glass tube and subjected to evaporation under nitrogen flow at a volume of 1 mL for further analysis by gas chromatography (Shimadzu CG-2010) coupled to mass spectrometry detector (Shimadzu CG-EM-QP 2020) and a capillary column Shimadzu SH-RTx-5MS (30m, ID 0.25 mm, 0, 25 μm). Concentration ranged in ηg/mL: 500-1000-2500-5000-7500-10000; LOQeq: 500 ηg/mL; LOQmet: 5 ηg/mL (considering the dilution factor). The standard curve was performed using diclofenac sodium salt (Sigma-Aldrich®, Brazil).

2.8 Statistical analysis

Toxicity data expressed as LC_{50} were calculated using *Trimmed Spearman Karber* software. The NOEC value used for the biomarker assays was obtained from the highest concentration that did not affect the survival of the exposed organisms. Data from biomarker analysis were expressed as mean ± SE. Differences between treatments were verified by one-way analysis of variance (ANOVA) followed by the *a posteriori* Tukey test. Residual analysis was performed to validate the assumptions of normality (Shapiro-Wilk test), homoscedasticity (Levene test) and independence (autocorrelation function (acf)). All analyses were performed using the R- Studio.

A Species Sensitivity Distribution (SSD) curve was also obtained using our data of toxicity and other available in the literature. For the revision we used the Web of Science platform and keywords were: "diclofenac toxicity", "diclofenac LC50" and "diclofenac

EC50". Only files containing the following criteria were used: tests performed under laboratory conditions; biological model exposed to freshwater; exposure to a single compound; endpoint (s) and exposure time indicated in the text. All ecotoxicological studies were assessed according to their reliability using the SciRAP website (http://www.scirap.org/), which is a tool for assessing the reliability and relevance of toxicity data, being useful for the regulatory assessment of chemicals (ACES and IMM, 2016; Moermond et al., 2016). For DCF, a total of 95 ecotoxicity test results were evaluated and only studies with reliability higher than 55% were used for the preparation of the SSD.

3. Results

Mean values of DCF concentration were < LOD in media from control groups, regardless of the test (toxicity or sublethal exposure). For the toxicity tests with adults and larvae, it was determined 1.18 mg/L of DCF for the concentration of 2 mg/L and 6.54 mg/L of DCF for the concentration of 10 mg/L. For sublethal exposures with adults and larva, it was measured 1.34 µg/L for the nominal concentrations of 2µg/L and 3.3 mg/L for the NOEC concentration of 3 mg/L of DCF. The LC50 of DCF estimated for adults and larvae were 5.49 mg/L (4.76 - 6.35 mg/L) and 5.22 mg/L (4.79 - 5.69 mg /L), respectively. Based on the ecotoxicity assessment we performed for DCF, added to the literature data considered "reliable", we developed a sensitivity curve (SSD) which is shown in Figure 1.

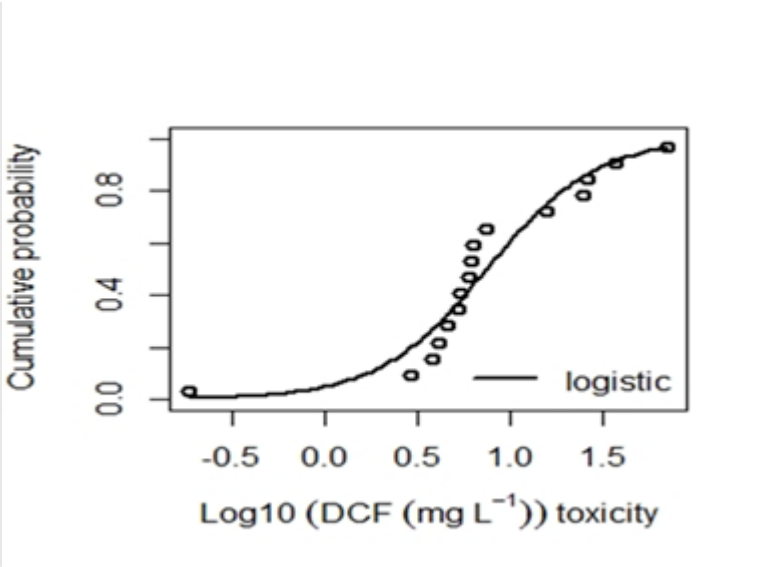

Figure 1. Species sensitivity distribution (SSD) for DCF showing the species sensitivity and survival probability (represented by the circles) based on the IC_{50}, EC_{50} and CL_{50} values in Log. The values are shown in ascending order, indicating as the most sensitive species with the less probability of survival: *Dunaliella tertiolecta* (DeLorenzo and Fleming, 2008), followed by *Siriella armata* (Pérez et al., 2015), *Vibrio fischeri* (Adame and Rocha, 2014), *Dugesia trigrida* (Oliveira, 2014), *Hyalella azteca* (Gómez-Oliván et al., 2014), *D .rerio* larvae (present study), *D. rerio* adult (present study), *D. rerio* embryo (Praskova et al., 2011), *Daphnia magna* (Du et al., 2016), *Atyaephyra desmarestii* (Nieto et al., 2009), *Lemna minor* (Cleuvers, 2003), *Tisbe battagliai* (Trombini et al., 2016), *Clarias gariepinus* (Ajima et al., 2015), *Tetrahymena pyriformis* (Láng and Kohidai, 2012), *Ceriodaphnia silvestrii* (Damasceno de Oliveira et al., 2018), *Desmodesmus subspicatus* (Cleuvers, 2004). The mathematical model used to calculate the curve was the logistic.

Regarding the biomarkers, Table 3 shows that there was no significant augment of MNs and ENAs in the erythrocyte of *D. rerio* after 96 h of exposure to 2 µg/L and 3 mg/L of DCF.

Table 2. Results of genotoxicity in erythrocyte of *D. rerio* exposed (96 h) to the of 2 µg/L DCF (environmental concentration) and 3 mg/L (NOEC). ENAs are represented by the segmented, lobulated, binucleated and kidney. A total of 2000 cells were accounted for from each fish (n= 6). Data are expressed as means ± SE.

Abnormality	Control	2µg/L DCF	3mg/L DCF
Segmented	0	0	0
Lobulated	2	1	3
Kidney	2	3	3
Binucleated	0	0	0
Micronucleus (MNs)	0	0	0

EROD activity in liver and gills are shown in Figure 2A and Figure 2B, respectively and it was non-affected by DCF at the nominal concentrations of 2 µg/L and 3 mg/L. GST activity was not altered by DCF in the liver but in gills, it was significantly higher at 3 mg/L of DCF (Figure 3A and Figure 3 B, respectively). On the other hand, the ABC transporters activity augmented in both concentrations of DCF in gills and again it was not observed any effect in the liver (Figure 4). There was an increase in LPO in gills of fish exposed to 3 mg/L of DCF compared to the other treatments, but it was statistically equal in the liver of control and exposed fish (Figure 5). For whole-body larvae, there was also a significant increase in LPO at the highest concentration tested (Figure 6).

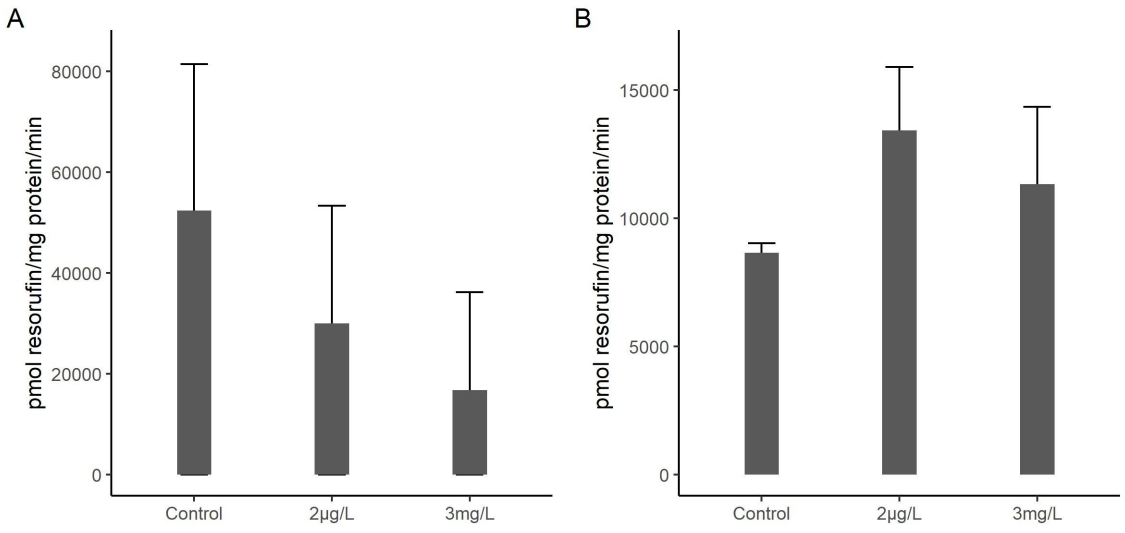

Figure 2. Ethoxyresorufin-O-deethylase (EROD) activity in the liver (A) and gills (B) of adult zebrafish males under control condition or exposed to DCF at 2 µg/L (environmental concentration) and 3 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE

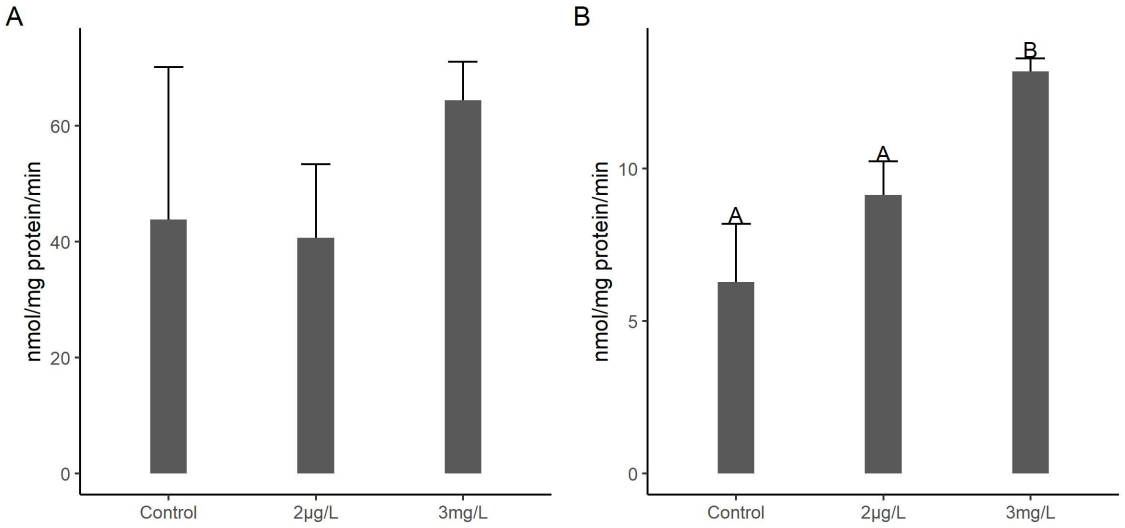

Figure 3. Glutathione S-transferase (GST) activity in the liver (A) and gills (B) of adult zebrafish males under control condition or exposed to DCF at 2 µg/L (environmental concentration) and 3 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE. Different letters indicate statistical differences between treatments ($p<0.05$)

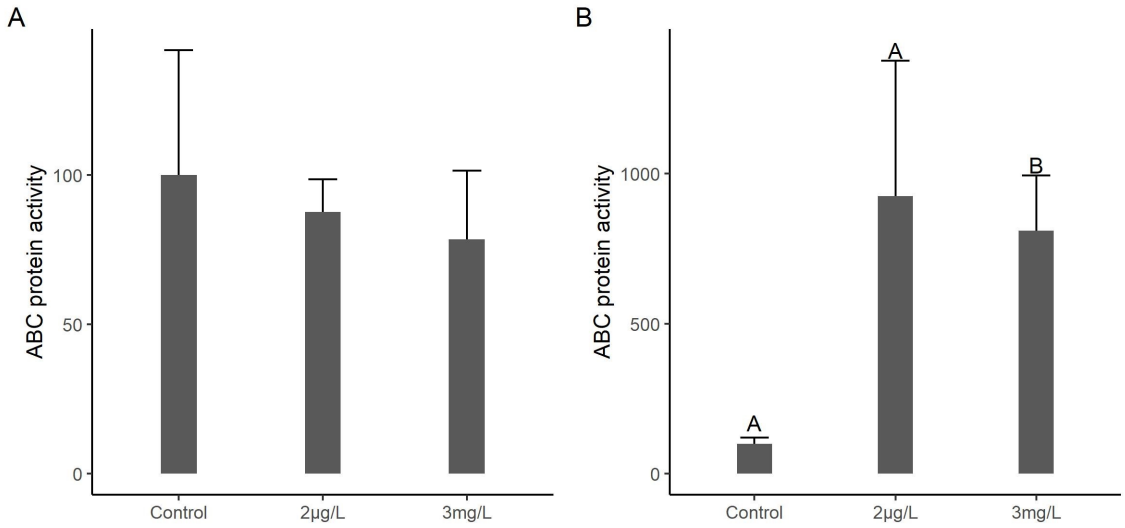

Figure 4. ABC protein activity percentage in the liver (A) and gills (B) of adults zebrafish males under control condition or exposed to DCF at 2 µg/L (environmental concentration) and 3 mg/L (NOEC) for 96 h. Results were estimated based in the control group which was

considered as 100% of activity. Data are expressed as mean ± SE. Different letters indicate statistical differences between treatments ($p<0.05$)

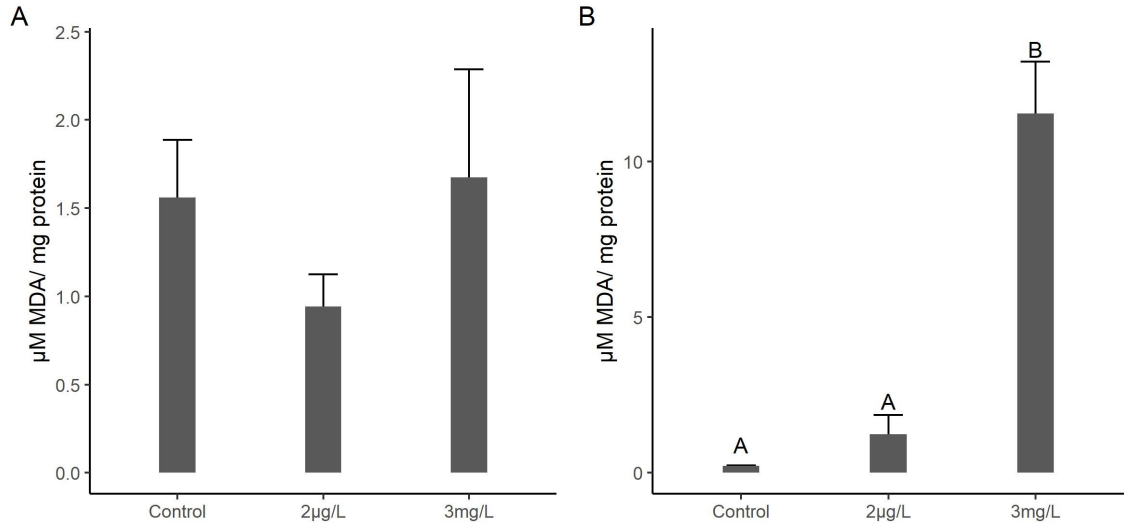

Figure 5. Lipid peroxidation (LPO), expressed as MDA formation in liver (A) and gills (B) of adults zebrafish males under control condition or exposed to DCF at 2 µg/L (environmental concentration) and 3 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE. Different letters indicate statistical differences between treatments ($p<0.05$)

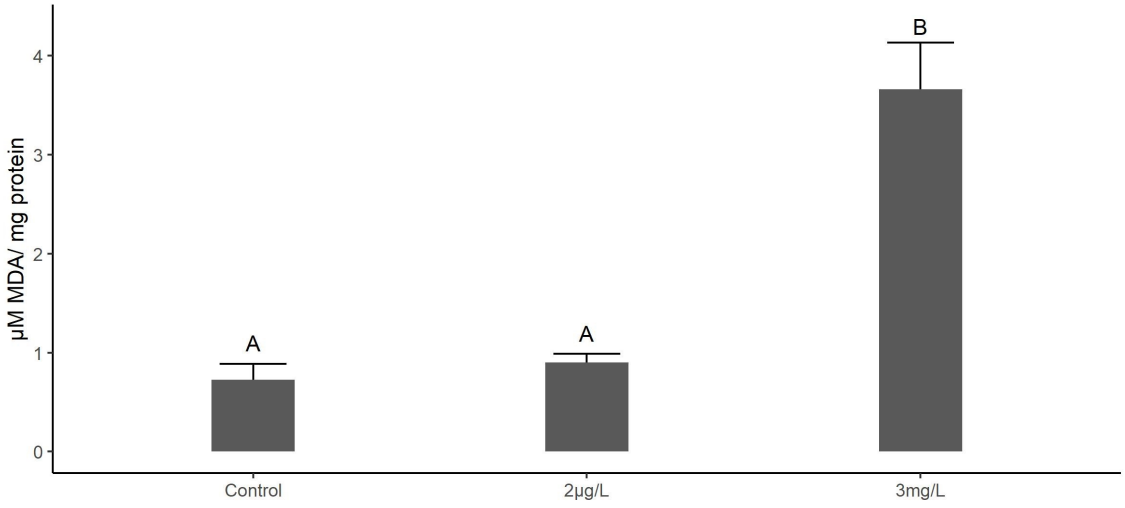

Figure 6. Lipid peroxidation (LPO), expressed as the formation of MDA of larvae zebrafish under control condition or exposed to DCF at 2 µg/L (environmental concentration) and 3 mg/L (NOEC) for 96 h. Data are expressed as mean ± SE. Different letters indicate statistical differences between treatments ($p<0.05$)

4. Discussion

Regarding LC_{50}, the values estimated for adults and larvae of *D. rerio* suggest that both life stages have similar sensitivity to DCF.

According to the SSD, the microalgae *D. subspicatus* (Cleuvers, 2004) is the organism more tolerant to DCF (IC_{50} of 71.9 mg/L of DCF); while the microalgae *D. tertiolecta* is the most sensitive (IC_{50} of 0.185 mg/L of DCF); however, algae were generally more tolerant to DCF, which may be related to the presence of cell wall that can work as a barrier avoiding incorporation of contaminants by microalgae and also to the DCF mechanism of action which targets biochemical processes in animals. Fish are the most sensitive organism to DCF, demonstrated by the greater sensitivity of *D. rerio*. Over organisms represented in the SSD curve, fish are the closest to humans on the evolutionary scale, so they may have similar biochemical and physiological processes.

This means that fish can, therefore, be more sensitive to DCF than other organisms. It is worth mentioning that *D. rerio* has been used as a biological model even for initial studies of drugs developed for humans. Although other studies have already built sensitivity curves, none of them followed SciRAP as the criterion of choice for the data. This tool increases the reliability of the data, generating more consistent results for the construction of risk analyzes and PNEC, reflecting the sensitivity of ecosystems.

The “toxicity tests were based on mortality which is a parameter of low sensitiveness, and is not exactly a good way to ensure the quality of the environment. Despite that, this kind of test is often used to determine Water Quality Criteria (WQC) for chemical compounds. However, to increase the sensitivity of our results, we used biomarkers associated with cell damage and its detoxification processes, which comprise biotransformation reactions.

The liver is the main organ participating in the detoxification of DCF (Guiloski et al., 2017, 2015; Stepanova et al., 2013), however, in this study liver did not respond to DCF as gills did. For humans it is clear that DCF is metabolized in the liver to 4-hydroxydiclofenac (metabolite principal) by a cytochrome P450 isoenzyme and other hydroxylated compounds; after glucuronization and sulfation, metabolites are excreted in the urine (65%) and bile (35%) (information present in drug leaflet of DCF). However, EROD measure in gills and liver was not considered as a good biomarker of DCF exposure since it did not respond in both tissues after fish exposure to DCF. Similarly, Guiloski et al. (2017; 2015) did not observe an increase or inhibition of EROD in the fish *Hoplias malabaricus* and *Rhamdia quelen*, both exposed to an environmental concentration of DCF (2µg/L).

On the other hand, the increase in GST activity in gills after exposure of fish to DCF at 3 mg/L is in agreement with data reported by Stepanova et al., (2013) who detected an augmented in GST in *Cyprinus carpio* larvae following exposure to 3 mg/L of DCF. Another study also observed an induction of GST activity, but at this time in the liver of the fish *Rhamdia quelen* chronically exposed to low concentrations (2µg/L) of DCF (Guiloski et al., 2017).

These results could indicate that DCF activates phase 2 of the biotransformation process in conjugation with GSH. Results presented in this study also show that cells activate their ABC transporters to eliminate DCF since the ABC activity augmented in gills after exposure of fish to DCF. In fact, cells use phase 3 detoxification even when DCF is at

environmental concentration. Although we have not evaluated the activity of specific groups of ABC transporters, we may suggest that the forms ABCCs and ABCG2 are the main involved in DCF cell extrusion since they eliminate GSH conjugates (Epel et al., 2008; Sturm e Segner, 2005).

Despite the augment in activities of GST enzyme and ABC transporters towards detoxification, LPO increased at the higher concentration of DCF (3 mg/L) in gills of adult fish and larvae of *D. rerio*. This means that DCF can cause oxidative stress. Moreover, larvae and adults appear to be similarly sensitive to DCF. However, contrary to our study, Stepanova et al. (2013) and Praskova et al. (2014) testing DCF at 3 mg/L and 5 mg/L observed a decrease in LPO in *D. rerio* larvae when compared to the control group. The authors suggested that exposure to NSAIDs may result in a protective action against oxidative stress.

Although DCF caused an increase in LPO, we did not evidence an effect of this drug on the incidence of MNs and ENAs. Thus, our results indicate that DCF is not genotoxic at 2 µg/L and 3 mg/L. DCF is rapidly metabolized and for this reason, it does not accumulate; therefore, its potential negative effects can be reduced due to its short biological half-life (2h) in the blood (information present in drug leaflet). Another point is that an increase in cellular defenses such as an increase in GST can be protective against DNA damage. In this sense, Quinn et al. (2011) reported the importance of GST activity in reducing DNA damage in fish, indicating a protective role of GST activity.

Finally, gills seem to be a target of DCF action in fish, perhaps because the interface between fish and water, being the first organ to be in contact with the contaminant and, thus, the first to answer . Another point is that the DCF incorporated by the gills would affect mainly this organ because it is rapidly degraded and does not effectively reach the blood and other organs.

5. Conclusion

Larvae and adults of *D. rerio* have similar sensitivity to DCF, and SSD shows that fish are very sensitive to this compound compared to other groups. DCF can cause LPO as a meaning of oxidative stress, at a sublethal concentration (3 mg/L), for both life stages of *D. rerio* tested here, even with an increase in the activities of GST enzyme and ABC transporters in adults. However, the drug did not cause negative effects on fish at the environmental concentration of 2 μg/L. In fact, this environmental concentration was able to trigger ABC activity as a response to fish contamination with DCF.

6. Acknolegements

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7. References

[ACES] - Department of Environmental Science and Analytical Chemistry, [IMM] Institute of Environmental Medicine, 2016. Science in Risk Assessment and Policy [WWW Document]. Eval. ecotoxicity Stud. URL http://www.scirap.org/Page/Index/44d2dba9-490a-4c66-9b9c-98e32bfc026a/evaluate-reliability-relevance (acessado20.02.20).

[ABNT] - Brazilian Association of Technical Standards. (2007). Aquatic ecotoxicology - Short-term chronic toxicity - test method with fish. ABNT-NBR 15499 (portuguese).

[ABNT] - Brazilian Association of Technical Standards. (2016). Aquatic ecotoxicology - Acute toxicity Test method with fish (Cyprinidae) ABNT NBR 15088, third edition, 13.12.2016.

Adame, A., Rocha, F.R.P., 2014. A multi-pumping flow system for acute toxicity bioassay using the Vibrio fischeri bacteria. Anal. Methods 6, 7367–7373. https://doi.org/10.1039/C4AY00789A

Ajima, M.N.O., Ogo, O.A., Audu, B.S., Ugwoegbu, K.C., 2015. Chronic diclofenac (DCF) exposure alters both enzymatic and haematological profile of African catfish, Clarias gariepinus. Drug Chem. Toxicol. 38, 383–390. https://doi.org/10.3109/01480545.2014.974108

Alnuqaydan, A.M., Sanderson, B.J., 2016. Toxicity and Genotoxicity of Beauty Products on Human Skin Cells In Vitro 6, 1–9. https://doi.org/10.4172/2161-0495.1000315

Alslev, B., Korsgaard, B., Bjerregaard, P., 2005. Estrogenicity of butylparaben in rainbow trout Oncorhynchus mykiss exposed via food and water. Aquat. Toxicol. 72, 295–304. https://doi.org/10.1016/j.aquatox.2005.01.005

Américo-Pinheiro, J.H.P., Isique, W.D., Nádia Hortense Torres, A.A.M., Carvalho, S.L. de, Filho, W.V.V., Ferreira, L.F.R., 2017. Ocorrência de diclofenaco e naproxeno em água superficial no município de Três Lagoas (MS) e a influência da temperatura da água na detecção desses anti - inflamatórios. Eng. sanitária e Ambient. 22, 429–435. https://doi.org/10.1590/S1413-41522017128719

Ameur, W. Ben, El Megdiche, Y., de Lapuente, J., Barhoumi, B., Trabelsi, S., Ennaceur, S., Camps, L., Serret, J., Ramos-López, D., Gonzalez-Linares, J., Touil, S., Driss, M.R., Borràs, M., 2015. Oxidative stress, genotoxicity and histopathology biomarker responses in Mugil cephalus and Dicentrarchus labrax gill exposed to persistent pollutants. A field study in the Bizerte Lagoon: Tunisia. Chemosphere 135, 67–74.

https://doi.org/10.1016/j.chemosphere.2015.02.050

Ates, P.S., Alturfan, A.A., Emekli-alturfan, E., 2018. Methylparaben induces malformations and alterations on apoptosis , oxidant – antioxidant status , ccnd1 and myca expressions in zebrafish embryos. J. Biochem. Mol. Toxicol. 1–6. https://doi.org/10.1002/jbt.22036

Azambuja, C.R., Mattiazzi, J., Riffel, A.P.K., Finamor, I.A., Garcia, L. de O., Heldwein, C.G., Heinzmann, B.M., Baldisserotto, B., Pavanato, M.A., Llesuy, S.F., 2011. Effect of the essential oil of Lippia alba on oxidative stress parameters in silver catfish (Rhamdia quelen) subjected to transport. Aquaculture 319, 156–161. https://doi.org/10.1016/j.aquaculture.2011.06.002

Barse, A. V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., Kumar, N., Raman, R.P., Jadhao, S.B., 2010. Vitellogenin induction and histo-metabolic changes following exposure of Cyprinus carpio to methyl paraben. Asian-Australasian J. Anim. Sci. 23, 1557–1565. https://doi.org/10.5713/ajas.2010.10118

Bayülken, D.G., Tüylü, B.A., Sinan, H., Sivas, H., 2017. Investigation of genotoxic effects of paraben in cultured human lymphocytes. Drug Chem. Toxicol. 0, 1–8. https://doi.org/10.1080/01480545.2017.1414834

Błędzka, D., Gromadzińska, J., Wąsowicz, W., 2014. Parabens . From environmental studies to human health. Environ. Int. J. 67, 27–42. https://doi.org/10.1016/j.envint.2014.02.007

Bo, L., Shengen, Z., Chang, C., 2016. Emerging Pollutants - Part II : Treatment. Water Environ. Res. 88, 1876–1904. https://doi.org/10.2175/106143016X14696400495857

Brunelli, E., Mauceri, A., Maisano, M., Bernabo, I., Giannetto, A., Elena De Domenicob, Barbara Corapi a, Sandro Tripepi a, n, S.F. b, 2011. Ultrastructural and immunohistochemical investigation on the gills of the teleost , Thalassoma pavo L ., exposed to cadmium. acta Histochem. 113, 201–213. https://doi.org/10.1016/j.acthis.2009.10.002

Burgeot, T., Bocquene, G., Galganil, F., 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. 131, 125–141.

Carmona, E., Andreu, V., Picó, Y., 2014. Science of the Total Environment Occurrence of acidic pharmaceuticals and personal care products in Turia River Basin : From waste to drinking water. Sci. Total Environ. 484, 53–63. https://doi.org/10.1016/j.scitotenv.2014.02.085

Carrasco, K.R., Tilbury, K.L., Myers, M.S., 1990. Assessment of the Piscine Micronuc eus Test as an in situ Biological Indicator of Chemica Contaminant Effects. Fish. Aquat. Sci. 47, 21 23-2436. https://doi.org/doi.org/10.1139/f90-237

Choi, C.Y., An, K.W., An, M.I., 2008. Molecular characterization and mRNA expression of glutathione peroxidase and glutathione S-transferase during osmotic stress in olive flounder (Paralichthys olivaceus). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 149, 330–337. https://doi.org/10.1016/j.cbpa.2008.01.013

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–315. https://doi.org/10.1016/S0147-6513(03)00141-6

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194. https://doi.org/10.1016/S0378-4274(03)00068-7

Corredor-Santamaría, W.M.S.G., Velasco-Santamaría, Y.M., 2016. Using genotoxic and haematological biomarkers as an evidence of environmental contamination in the Ocoa River native fish ,. Springerplus. https://doi.org/10.1186/s40064-016-1753-0

Damasceno de Oliveira, L.L., Nunes, B., Antunes, S.C., Campitelli-Ramos, R., Rocha, O., 2018. Acute and Chronic Effects of Three Pharmaceutical Drugs on the Tropical Freshwater Cladoceran Ceriodaphnia silvestrii. Water. Air. Soil Pollut. 229.

https://doi.org/10.1007/s11270-018-3765-6

Dambal, V.Y., Selvan, K.P., Lite, C., Barathi, S., Santosh, W., 2017. Developmental toxicity and induction of vitellogenin in embryo-larval stages of zebrafish (Danio rerio) exposed to methyl Paraben. Ecotoxicol. Environ. Saf. 141, 113–118. https://doi.org/10.1016/j.ecoenv.2017.02.048

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and Personal Care Products in the Environment : Agents of Subtle Change ? Environ. Health Perspect. 107, 907–938.

DeLorenzo, M.E., Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species Dunaliella tertiolecta. Arch. Environ. Contam. Toxicol. 54, 203–210. https://doi.org/10.1007/s00244-007-9032-2

Dobbins, L.L., Usenko, S., Brain, R.A., Brooks, B.W., 2009. Probabilistic ecological hazard assessment of parabens using Daphnia magna and Pimephales promelas. Environ. Toxicol. Chem. 28, 2744–2753. https://doi.org/10.1897/08-523.1

Du, J., Mei, C.F., Ying, G.G., Xu, M.Y., 2016. Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea Daphnia magna. Bull. Environ. Contam. Toxicol. 97, 84–90. https://doi.org/10.1007/s00128-016-1806-7

Eggens, M.L., Galgani, F., 1992. Ethoxyresorufin-O-deethylase (EROD) activity in flatfish: Fast determination with a fluorescence plate-reader. Mar. Environ. Res. 33, 213–221. https://doi.org/10.1016/0141-1136(92)90149-G

Epel, D., Luckenbach, T., Steveson, C.N., MacManus-Spencer, L.A., Hamdoun, A., Smital, T., 2008. Efflux transporters: Newly Appreciated Roles in Protection against Pollutants. Environ. Sci. e Technol. 3914–3920. https://doi.org/doi/abs/10.1021/es087187v

Escher, B.I., Hermens, J.L.M., 2002. Critical Review Modes of Action in Ecotoxicology : Their Role in Body Burdens , Species Sensitivity , QSARs , and Mixture Effects.

Environ. Sci. e Technol. 36, 4201–4217. https://doi.org/doi.org/10.1021/es015848h

Freire, M.M., Santos, V.G., Ginuino, I.S.F., Linde, A.R., 2008. Biomarcadores na avaliação da saúde ambiental dos ecossistemas aquáticos. oecologia Bras. 12, 347–354.

Freiry, R., Stelzer, J.A.A., Maltchik, L., Arenzon, A., 2014. Sensitivity of Danio rerio (Teleostei, Cyprinidae) during Two Stages of Development Based on Acute Toxicity Tests. Bull. Environ. Contam. Toxicol. 93, 442–445. https://doi.org/10.1007/s00128-014-1367-6

Ghiselli, G., 2006. Avaliação das águas destinadas ao abastecimento público na região de Campinas: ocorrência e determinação dos interferentes endócrinos (IE) e produtos farmacêuticos e de higiene pessoal (PFHP). Unicamp 181p.

Gómez-Oliván, L.M., Neri-Cruz, N., Galar-Martínez, M., Islas-Flores, H., García-Medina, S., 2014. Binary mixtures of diclofenac with paracetamol, ibuprofen, naproxen, and acetylsalicylic acid and these pharmaceuticals in isolated form induce oxidative stress on Hyalella azteca. Environ. Monit. Assess. 186, 7259–7271. https://doi.org/10.1007/s10661-014-3925-0

Gryta, A., Fr, M., Oszust, K., 2014. The Application of the Biolog EcoPlate Approach in Ecotoxicological Evaluation of Dairy Sewage Sludge. Appl. Biochem. Biotechnol. 174, 1434–1443. https://doi.org/10.1007/s12010-014-1131-8

Guiloski, I.C., Luiz, J., Ribas, C., Pereira, S., Paula, A., Neves, P., Cristina, H., Assis, S. De, 2015. Ecotoxicology and Environmental Safety Effects of trophic exposure to dexamethasone and diclofenac in freshwater fi sh. Ecotoxicol. Environ. Saf. 114, 204–211. https://doi.org/10.1016/j.ecoenv.2014.11.020

Guiloski, I.C., Piancinib, L.D.S., Ana Carolina Dagostimb, Sabrina Loise de Morais Caladoc, Luis Fernando Fávarod, S.L.B., Marta Margarete Cestarib, Cláudio da Cunhaa, H.C.S. de A., 2017. Effects of environmentally relevant concentrations of the anti-inflammatory

drug diclofenac in freshwater fish Rhamdia quelen. Ecotoxicol. Environ. Saf. 139, 291–300. https://doi.org/10.1016/j.ecoenv.2017.01.053

Haman, C., Dauchy, X., Rosin, C., Munoz, J.F., 2015. Occurrence, fate and behavior of parabens in aquatic environments: A review. Water Res. 68, 1–11. https://doi.org/10.1016/j.watres.2014.09.030

Heath, A.G., 1995. Water pollution and Fish Physiology., 1st ed. crcpress, Florida.

Hinton, D.E., Segner, H., Braunbeck, T., 2017. Toxic responses of the liver, in: Target Organ Toxicity in Marine and Freshwater Teleosts. p. 45.

Hoeger, B., Bernd, K., Dietrich, D.R., Hitzfeld, B., 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f . fario). Aquat. Toxicol. 75, 53–64. https://doi.org/10.1016/j.aquatox.2005.07.006

Hopkins, Z.R., Blaney, L., 2016. An aggregate analysis of personal care products in the environment: Identifying the distribution of environmentally-relevant concentrations. Environ. Int. 92–93, 301–316. https://doi.org/10.1016/j.envint.2016.04.026

Insam, H., 1997. A new set of substrates proposed for community characterization in environmental samples. Microbial communities: functional versus structural approaches 259–260.

Jonkers, N., Sousa, A., Galante-oliveira, S., 2010. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro , Portugal. Environ. Sci. Pollut. Res. 17, 834–843. https://doi.org/10.1007/s11356-009-0275-5

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

Kroon, F., Streten, C., Harries, S., 2017. A protocol for identifying suitable biomarkers to assess fish health : A systematic review. PLoS One 12, 1–43. https://doi.org/10.1371/journal.pone.0174762

Kurelec, B., 1992. The Multixenobiotic Resistance Mechanism in Aquatic Organisms. Crit. Rev. Toxicol. 22, 23–43. https://doi.org/doi/abs/10.3109/10408449209145320

Láng, J., Kohidai, L., 2012. Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate Tetrahymena. Chemosphere 89, 592–601. https://doi.org/10.1016/j.chemosphere.2012.05.058

Lee, J., Ji, K., Lim, Y., Kim, P., Choi, K., 2011. Ecotoxicology and Environmental Safety Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. Ecotoxicol. Environ. Saf. 74, 1216–1225. https://doi.org/10.1016/j.ecoenv.2011.03.014

Lima, C.S., C, E., 2018. Ecotoxicology and Environmental Safety Removal of emerging contaminants from the environment by adsorption. Ecotoxicol. Environ. Saf. 150, 1–17. https://doi.org/10.1016/j.ecoenv.2017.12.026

Lonappan, L., Brar, S.K., Das, R.K., Verma, M., Surampalli, R.Y., 2016. Diclofenac and its transformation products: Environmental occurrence and toxicity - A review. Environ. Int. 96, 127–138. https://doi.org/10.1016/j.envint.2016.09.014

McKim, J.M., 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-Term Toxicity. J. Fish. Res. Board Canada 34, 1148–1154. https://doi.org/10.1139/f77-172#.XjS4RTJKjIU

Michael, I., Hapeshi, E., Michael, C., Varela, A.R., Kyriakou, S., Manaia, C.M., Fatta-kassinos, D., 2012. Solar photo-Fenton process on the abatement of antibiotics at a pilot scale : Degradation kinetics , ecotoxicity and phytotoxicity assessment and removal of antibiotic resistant enterococci. Water Res. 46, 5621–5634. https://doi.org/10.1016/j.watres.2012.07.049

Moermond, C.T.A., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: Criteria for reporting and evaluating ecotoxicity data. Environ. Toxicol. Chem. 35, 1297–1309.

https://doi.org/10.1002/etc.3259

Nakada, N., Shinohara, H., Murata, A., Kiri, K., Managaki, S., Sato, N., Takada, H., 2007. Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. Water Res. 41, 4373–4382. https://doi.org/10.1016/j.watres.2007.06.038

Nieto, A., Borrull, F., Marcé, R.M., Pocurull, E., 2009. Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1216, 5619–5625. https://doi.org/10.1016/j.chroma.2009.05.061

Oliveira, L.L.D. de, 2014. Biomarcadores enzimáticos e testes ecotoxicológicos na avaliação da toxicidade de fármacos em invertebrados aquáticos. Universidade de São Paulo, São Carlos. https://doi.org/10.11606/T.18.2014.tde-09022015-110612

Peng, X., Ou, W., Wang, C., Wang, Z., Huang, Q., Jin, J., Tan, J., 2014. Science of the Total Environment Occurrence and ecological potential of pharmaceuticals and personal care products in groundwater and reservoirs in the vicinity of municipal land fi lls in China. Sci. Total Environ. 490, 889–898. https://doi.org/10.1016/j.scitotenv.2014.05.068

Pérez, S., Rial, D., Beiras, R., 2015. Acute toxicity of selected organic pollutants to saltwater (mysid Siriella armata) and freshwater (cladoceran Daphnia magna) ecotoxicological models. Ecotoxicology 24, 1229–1238. https://doi.org/10.1007/s10646-015-1489-6

Praskova, E., Plhalova, L., Chromcova, L., Stepanova, S., Bedanova, I., Blahova, J., Hostovsky, M., Skoric, M., Maršálek, P., Voslarova, E., Svobodova, Z., 2014. Effects of Subchronic Exposure of Diclofenac on Growth , Histopathological Changes , and Oxidative Stress in Zebrafish (Danio rerio). Sci. World J. 2014, 1–5. https://doi.org/https://doi.org/10.1155/2014/645737

Praskova, E., Voslarova, E., Siroka, Z., Plhalova, L., Macova, S., Marsalek, P., Pistekova, V., Svobodova, Z., 2011. Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (Danio rerio). Pol. J. Vet. Sci. 14, 545–549. https://doi.org/10.2478/v10181-011-0081-0

Puerta, Y.T., Guimarães, P.S., Martinsa, S.E.M., Gaspar, C. de M., 2020. Ecotoxicology and Environmental Safety Toxicity of methylparaben to green microalgae species and derivation of a predicted no effect concentration (PNEC) in freshwater ecosystems. Ecotoxicol. Environ. Saf. 1–8. https://doi.org/10.1016/j.ecoenv.2019.109916

Quinn, B., Schmidt, W., Rourke, K.O., Hernan, R., 2011. Chemosphere Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (Dreissena polymorpha) and their comparison with standardised toxicity tests. Chemosphere 84, 657–663. https://doi.org/10.1016/j.chemosphere.2011.03.033

Ramaswamya, B.R., Kima, J.-W., Isobea, T., Changa, K.-H., Amanoe, A., W., T.M., Fernando P. Siringanf, S.T., 2011. Determination of preservative and antimicrobial compounds in fish from Manila Bay , Philippines using ultra high performance liquid chromatography tandem mass spectrometry , and assessment of human dietary exposure. J. Hazard. Mater. 192, 1739–1745. https://doi.org/10.1016/j.jhazmat.2011.07.006

Ribeiro, S., Torres, T., Martins, R., Santos, M.M., 2015. Toxicity screening of Diclofenac, Propranolol, Sertraline and Simvastatin using Danio rerio and Paracentrotus lividus embryo bioassays. Ecotoxicol. Environ. Saf. 114, 67–74. https://doi.org/10.1016/j.ecoenv.2015.01.008

Roberts, J., Kumar, A., Du, J., Hepplewhite, C., Ellis, D.J., Christy, A.G., Beavis, S.G., 2016. Pharmaceuticals and personal care products (PPCPs) in Australia's largest inland sewage treatment plant, and its contribution to a major Australian river during high and low flow. Sci. Total Environ. 541, 1625–1637.

https://doi.org/10.1016/j.scitotenv.2015.03.145

Samanta, P., Im, H., Na, J., Jung, J., 2018. Ecological risk assessment of a contaminated stream using multi-level integrated biomarker response in Carassius auratus *. Environ. Pollut. 233, 429–438. https://doi.org/10.1016/j.envpol.2017.10.061

Sandermann, H., 2008. Chemosphere Ecotoxicology of narcosis : Stereoselectivity and potential target sites. Chemosphere 72, 1256–1259. https://doi.org/10.1016/j.chemosphere.2008.05.002

Scheurell, M., Franke, S., Shah, R.M., Hühnerfuss, H., 2009. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. Chemosphere 77, 870–876. https://doi.org/10.1016/j.chemosphere.2009.07.066

Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B., 2011. Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (Mytilus spp.) and their comparison with standardized toxicity tests. Mar. Pollut. Bull. 62, 1389–1395. https://doi.org/10.1016/j.marpolbul.2011.04.043

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R.D., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Aquat. Toxicol. 68, 141–150. https://doi.org/10.1016/j.aquatox.2004.03.014

Silva, D.C., Serrano, L., Oliveira, T.M.A., Mansano, A.S., Almeida, E.A., Vieira, E.M., 2018. Effects of parabens on antioxidant system and oxidative damages in Nile tilapia (Oreochromis niloticus). Ecotoxicol. Environ. Saf. 162, 85–91. https://doi.org/10.1016/j.ecoenv.2018.06.076

Silveira, M.A.K., Caldas, S.S., Guilherme, J.R., Costa, F.P., Guimarães, B.D.S., Cerqueira, M.B.R., Soares, B.M., Primel, E.G., 2013. Quantification of pharmaceuticals and personal care product residues in surface and drinking water samples by SPE and LC-ESI-MS/MS. J. Braz. Chem. Soc. 24, 1385–1395. https://doi.org/10.5935/0103-

5053.20130176

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J., Svobodova, Z., 2013. The effects of diclofenac on early life stages of common carp (Cyprinus carpio). Environ. Toxicol. Pharmacol. 35, 454–460. https://doi.org/10.1016/j.etap.2012.09.011

Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Science of the Total Environment Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21. https://doi.org/10.1016/j.scitotenv.2011.11.072

Sturm, A., Segner, H., 2005. P-glycoproteins and xenobiotic efflux transport in fish. Biochem. Mol. Biol. Fishes 6, 495–533. https://doi.org/doi/abs/10.3109/10408449209145320

Terasaki, M., Makino, M., Tatarazako, N., 2009. Acute toxicity of parabens and their chlorinated by-products with Daphnia magna and Vibrio fischeri bioassays. J. Appl. Toxicol. 29, 242–247. https://doi.org/10.1002/jat.1402

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H., Schwaiger, J., 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part II . Cytological effects in liver , kidney , gills and intestine of rainbow trout (Oncorhynchus mykiss). Aquat. Toxicol. 68, 151–166. https://doi.org/10.1016/j.aquatox.2004.03.015

Trombini, C., Hampel, M., Blasco, J., 2016. Evaluation of acute effects of four pharmaceuticals and their mixtures on the copepod Tisbe battagliai. Chemosphere 155, 319–328. https://doi.org/10.1016/j.chemosphere.2016.04.058

Ueda, T., Hayashi, M., Ohtsuka, Y., Nakamura, T., Kobayashi, J., Sofuni, T., 1992. A Preliminary study of the micronucleus test by acridine orange fluorescent staining compared with chromosomal aberration test using fish erythropoietic and embryonic cells. wal Sci. Technol. Res. 25, 235–240. https://doi.org/doi.org/10.2166/wst.1992.0297

Van Wezel, A.P., Opperhuizenb, A., 1995. Narcosis Due To Environmental Pollutants in

Aquatic Organisms : Residue-Based Toxicity , Mechanisms , and Membrane Burdens. Crit. Rev. Toxicol. 25, 255–279. https://doi.org/doi.org/10.3109/10408449509089890

Verlicchi, P., Zambello, E., 2015. Science of the Total Environment Pharmaceuticals and personal care products in untreated and treated sewage sludge : Occurrence and environmental risk in the case of application on soil — A critical review. Sci. Total Environ. 538, 750–767. https://doi.org/10.1016/j.scitotenv.2015.08.108

Wu, Y., Sun, Q., Wang, Y., Deng, C., Yu, C., 2017. Comparative studies of aerobic and anaerobic biodegradation of methylparaben and propylparaben in activated sludge. Ecotoxicol. Environ. Saf. 138, 25–31. https://doi.org/10.1016/j.ecoenv.2016.12.017

Yamamoto, H., Tamura, I., Hirata, Y., Kato, J., Kagota, K., Katsuki, S., Yamamoto, A., Kagami, Y., Tatarazako, N., 2011. Aquatic toxicity and ecological risk assessment of seven parabens: Individual and additive approach. Sci. Total Environ. 410–411, 102–111. https://doi.org/10.1016/j.scitotenv.2011.09.040

Yu, Y., Wu, L., Chang, A.C., 2013. Science of the Total Environment Seasonal variation of endocrine disrupting compounds , pharmaceuticals and personal care products in wastewater treatment plants. Sci. Total Environ. 442, 310–316. https://doi.org/10.1016/j.scitotenv.2012.10.001

Ziylan, A., Ince, N.H., 2011. The occurrence and fate of anti-inflammatory and analgesic pharmaceuticals in sewage and fresh water: Treatability by conventional and non-conventional processes. J. Hazard. Mater. 187, 24–36. https://doi.org/10.1016/j.jhazmat.2011.01.057

CONSIDERAÇÕES FINAIS E PESPECTIVAS

Resumidamente, os peixes demonstraram uma maior sensibilidade à exposição ao fármaco DCF independente do estágio de vida, apresentando valores de CL50 muito próximos (adultos e larvas), isso pode estar associado ao fato dos peixes terem processos bioquímicos e fisiológicos mais semelhantes aos humanos para quem o DCF foi desenvolvido (devido ao seu mecanismo de ação Cox 1 e 2). Por outro lado, os valores de CL50 tanto dos adultos quanto das larvas são superiores aos valores encontrados na literatura para peixes, indicando uma resistência a essa substância. Os adultos de *D*. *rerio* foram mais sensíveis ao conservante MeP, no entanto, a hipótese para explicar a toxicidade associada a atividade antimicrobiana deste composto foi refutada, indicando apenas que a microbiota em condições adversar pode conseguir se manter absorvendo mais fontes de energia. Mais estudos são necessários para entender porque as larvas de *D. rerio* são mais resistentes provavelmente fatores da biológia de cada uma das fases de vida estão relacionados a essa diferença.

Quanto às análises de biomarcadores, a concentração ambiental dos compostos testados não causou efeitos significativos para nenhuma das respostas analisadas, no entanto nossas exposições foram aguadas o que pode refletir um curto tempo para causar efeitos, porém os organismos estão expostos cronicamente a essas substâncias. A efeito não-observado (CENO), apesar de não gerar efeitos nos testes de toxicidade clássicos, que tem como *endpoint* a mortalidade, causaram variações nos biomarcadores, incluindo biomarcadores de dano. Neste sentido, podemos destacar a maior eficácia e sensibilidade dos biomarcadores em revelar os efeitos danosos destes compostos. Sendo assim ressaltamos o uso conjunto dos ensaios de toxicidade e biomarcadores para fornecer respostas mais completas da saúde dos organismos e consequentemente do ambiente. Estudos assim são importantes pois, fornece dados que podem ser usados em propostas de avaliação de risco e regulamentação como maneiras de proteger a biota e os ambientes aquáticos.

ANEXOS

COMISSÃO DE ÉTICA EM USO ANIMAL
Universidade Federal do Rio Grande
Pró-Reitoria de Pesquisa e Pós-Graduação - PROPESP
ceua@furg.br http://www.propesp.furg.br

CEUA

CERTIFICADO Nº P047/2017

Certificamos que o projeto intitulado "Monitoramento de novos contaminantes – ecotoxicologia", protocolo nº 23116.005140/2017-29, sob a responsabilidade de Camila de Martinez Gaspar Martins - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 26 de setembro de 2017 (Ata 009/2017).

A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq014/2017
COLABORADORES AUTORIZADOS A MANIPULAR OS ANIMAIS	Samantha Eslava Gonçalves Martins; Ana Laura Venquiaruti Escarrone; Cássia Rodrigues da Silveira
VIGÊNCIA DO PROJETO	31/05/2019
ESPÉCIE/ LINHAGEM / RAÇA	*Danio rerio* (zebrafish)
NÚMERO DE ANIMAIS	1350 Adultos e 2520 Larvas
PESO/ IDADE	0,25 à 1,2 g / Larvas e adultos
SEXO	Indiferente
ORIGEM	Luiz Carlos Alves Machado Junior ME, Rua Gen. Netto, 367 - Centro, Rio Grande, RS - CNPJ: 22.857.229/0001-80
ENVIO DO RELATÓRIO FINAL	Junho de 2019

Rio Grande, 26 de setembro de 2017.

Med. Vet. Márcio de Azevedo Figueiredo
Coordenador da CEUA-FURG