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EFEITOS DO DIURON E SEUS METABÓLITOS DCA E DCMPU SOBRE O ZEBRAFISH: TOXICIDADE, COMPORTAMENTO E PARÂMETROS BIOQUÍMICOS.

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Patologia.

Orientadora: Profa. Dra. Lilian Cristina Pereira

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: MARIA CAROLINA ANDRADE CRUZ E SANTOS-CRB

Sales, Bianca Camargo Penteado.
Danos induzidos pelo diuron e seus metabólitos DCA e DCPMU sobre o zebrafish : comportamento e parâmetros bioquímicos / Bianca Camargo Penteado Sales. - Botucatu, 2023

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: Lilian Cristina Pereira
Capes: 40105008

1. Metabólitos. 2. Toxicologia. 3. Diuron. 4. Marcadores bioquímicos. 5. Herbicidas. 6. Zebrafish.

Palavras-chave: DCA; DCPMU; Diuron; Parametros bioquímicos; Zebrafish.

DEDICATÓRIA

Ao meu filho Diego, que chegou em minha vida e me mostrou o real sentido da vida.
Te amo muito.

AGRADECIMENTOS ESPECIAIS

À Deus por me guiar, iluminar e me dar tranquilidade para seguir em frente com meus objetivos e não desanimar com as dificuldades.

À minha orientadora Dra. Lílian Cristina Pereira, sempre tão atenciosa incentivadora e impulsionadora. Agradeço pelos anos de convivência, orientação, dedicação, compreensão e por todo o conhecimento transmitido durante esse período.

Ao Dr. Amadeu Mortágua Velho por aceitar minha ida até Portugal, onde realizei parte de meu doutoramento. Sou grata por toda confiança depositada e pelo grande aprenizado que tive na Universidade de Aveiro.

À Dra. Marta Monteiro e a Dra. Carla Quintadeiro pelos ensinamentos, disponibilidade, por toda a ajuda e acima de tudo pela paciência comigo durante meu doutorado sanduíche.

AGRADECIMENTOS

Aos meus pais José Antônio e Sonia e minha irmã Beatriz, que são meu porto seguro. Obrigada pelo apoio, amor incondicional e presença constante em minha vida. Vocês são meus exemplos, o verdadeiro significado da palavra família.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado sanduíche (CAPES-PrInt - Processo nº 88887.569799/2020-00).

Aos todos os colegas do laboratório TOXICAM pela troca de conhecimentos e ajuda para o desenvolvimento deste trabalho.

Ao Ítalo, Paloma, Cris e Thania meus colegas de bancada e biotério, por toda disposição, trocas de conheciemento e apoio para conduzir esse estudo.

Á Iza, Marquinhos e minhas colegas do departamento de Patologia, que sempre compreenderam minhas ausências e faziam o dia-a-dia de trabalho mais leve e divertido.

Ao Paulo e Vânia pelas orientações e apoio essenciais para o desenvolvimento deste trabalho.

Ao meu namorado Lucas, que sempre acreditou no meu potencial e que para mim é um exemplo de dedicação e persistência.

Ás amigas de verdade Preta e Monique, por estarem sempre presente na minha vida e me auxiliarem com a formatação do trabalho.

"A verdadeira viagem de descobrimento não consiste em procurar novas paisagens, mas em ter novos olhos".

Marcel Proust

RESUMO

O aumento da demanda por alimentos intensificou os sistemas de produção em larga escala, tornando o uso dos praguicidas indispensável no meio rural. Entretanto, o elevado consumo desses químicos é motivo de preocupação, devido às contaminações e consequentes efeitos á saúde dos organismos expostos. Dentre os herbicidas, se destaca o diuron [3- (3,4-diclorofenil) -1,1- dimetilureia], por este ser amplamente usado na agricultura e por poder se degradar em solo e água, gerando metabólitos como DCA [3,4 dicloroanilina] e o DCPMU [3,4 diclorofenil-N-metilureia]. Existem estudos com roedores relatando o potencial nocivo dessas substâncias, mas são poucas pesquisas que mostraram sua toxicidade em organismos aquáticos. Nesse cenário, o teleósteo zebrafish (Danio rerio) é considerado um modelo eficaz para testes de toxicidade. A tese de doutorado objetivou avaliar em duas diferentes pesquisas os possíveis danos induzidos pelo diuron, DCA e DCPMU em adultos, embriões e larvas de zebrafish. Para o primeiro manuscrito utilizou-se diferentes concetrações do diuron 0.003, 0.012, 0.048, 0.189, 0.754, 3.000 mg/L, DCA 0.020, 0.112, 0.633, 0.047, 0.267, 1.500 mg/L e DCPMU 0.020, 0.051, 0.129, 0.326, 0.828, 2.1 mg/L. Foram monitorados biomarcadores com embriões quanto às atividades das enzimas acetilcolinesterase (AChE), catalase (CAT), glutationa-S-transferase (GST), lactato desidrogenase (LDH) e níveis de peroxidação lipídica (LPO). O comportamento locomotor foi avaliado na fase larval usando o Zebrabox® e os registros foram classificados pelo software ZebraLab® (Viewpoint, FR). Parâmetros adicionais como comprimento total e a frequência cardíaca larval foram mensurados usando o programa ImageJ e um estereomicroscópio. Para segundo manuscrito utilizou-se concetrações que variavam de 0.5, 1.0, 5.0, 10.0, 50.0 e 100.0 µM do diuron, DCA e DCPMU. Avaliou-se a toxicidade com adultos seguindo o protocolo o OECD 203 "Fish Acute Toxicity Test". Para o teste de toxicidade com embriões, utilizou-se o protocolo OCDE 236 "Fish Embryo Acute Toxicity'' (FET). Por fim, a atividade da enzima acetilcolinesterase (AChE) foi monitorados também utilizando embriões.
No primeiro manuscrito, as atividade das enzimas estudadas e o comportamento natatório, foram detectadas alterações significativas nas diferentes concentrações estudadas de diuron, DCA e DCPMU. Além disso, as larvas expostas ao DCA tiveram redução em seu comprimento, e o batimento cardíaco das larvas mostrou-se diminuído pela exposição aos três químicos. No segundo manuscrito não foi possível observar alterações histológicas evidentes no cérebro, baço e fígado dos zebrafish adultos expostos ao diuron, DCA e DCPMU por 96 horas. No estudo com embriões, efeitos no desenvolvimento (malformações), como aparecimento de edemas e escoliose em zebrafish ocasionados pela ação dos contamintes foram observados. A atividade de AChE foi alterada na maior concentração que houve sobrevivência (10.0 µM) nas análises com o metabólito DCA. Nossos resultados indicam que o herbicida diuron e seus metabólitos DCA e DCPMU são capazes de causar efeitos adversos para o zebrafish. Esses resultados podem ainda oferecer embasamento científico adicional, para a implementação de estratégias coerentes e eficazes de controle desses contaminantes no meio ambiente.

Palavras chaves: *Danio Rerio*; herbicidas; toxicidade; contaminantes; biomarcadores enzimáticos; toxicidade embrionária

ABSTRACT

The increase in demand for food has intensified large-scale production systems, making the use of pesticides essential in rural areas. However, the high consumption of these chemicals is a cause for concern, due to contamination and consequent health effects on exposed organisms. Among herbicides, diuron [3- (3,4-dichlorophenyl) -1,1- dimethylurea] stands out, as it is widely used in agriculture and can degrade in soil and water, generating metabolites such as DCA [3, 4 dichloroaniline] and DCPMU [3,4 dichlorophenyl-N-methylurea]. There are studies with rodents reporting the harmful potential of these substances, but little research has shown their toxicity in aquatic organisms. In this scenario, the teleost zebrafish (Danio rerio) is considered an effective model for toxicity testing. The doctoral thesis aimed to evaluate, in two different studies, the possible damage induced by diuron, DCA and DCPMU in zebrafish adults, embryos and larvae. For the first manuscript, different concentrations of diuron were used: 0.003, 0.012, 0.048, 0.189, 0.754, 3.000 mg/L, DCA 0.020, 0.112, 0.633, 0.047, 0.267, 1.500 mg/L and DCPMU 0.020, 0.051 , 0.129, 0.326 , 0.828, 2.1 mg/L. Biomarkers were monitored with embryos regarding the activities of the enzymes acetylcholinesterase (AChE), catalase (CAT), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and lipid peroxidation (LPO) levels. Locomotor behavior was evaluated in the larval stage using Zebrabox® and records were classified by ZebraLab® software (Viewpoint, FR). Additional parameters such as total length and larval heart rate were measured using the ImageJ program and a stereomicroscope. For the second manuscript, concentrations ranging from 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 µM of diuron, DCA and DCPMU were used. Toxicity was evaluated in adults following the OECD 203 “Fish Acute Toxicity Test” protocol. For the toxicity test with embryos, the OECD 236 “Fish Embryo Acute Toxicity” (FET) protocol was used. Finally, the activity of the enzyme acetylcholinesterase (AChE) was also monitored using embryos.In the first manuscript, the activity of the studied enzymes and the swimming behavior, significant changes were detected in the different studied concentrations of diuron, DCA and DCPMU. Furthermore, the larvae exposed to DCA had a reduction in their length, and the heart rate of the larvae was reduced by exposure to the three chemicals. In the second manuscript, it was not possible to observe evident histological changes in the brain, spleen and liver of adult zebrafish exposed to diuron, DCA and DCPMU for 96 hours. In the study with embryos, effects on development (malformations), such as the appearance of edema and scoliosis in zebrafish caused by the action of contaminants, were observed. AChE activity was altered at the highest concentration at which survival occurred (10.0 µM) in analyzes with the DCA metabolite. Our results indicate that the herbicide diuron and its metabolites DCA and DCPMU are capable of causing adverse effects on zebrafish. These results can also provide additional scientific support for the implementation of coherent and effective strategies to control these contaminants in the environment.

Key word: *Danio Rerio;* herbicides; toxicity; contaminants; enzyme biomarkers; embryo toxicity.

SUMÁRIO

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CAPITULO I. **REVISÃO DE LITERATURA**

1. Praguicidas: O que são e o uso no Brasil e no mundo

O termo praguicida é definido como qualquer substância ou mistura de compostos de origem química ou biológica que tem como função a prevenção, destruição ou contenção de organismos considerados pragas (USEPA, 2017; Pathak et al., 2022). Os praguicidas são classificados, de acordo com seu espectro de ação, como herbicidas (substâncias que eliminam "ervas daninhas" que infestam a cultura no campo), inseticidas (compostos que controlam insetos no meio agrícola ou urbano), ou outros, como fungicidas (produtos que eliminam os fungos), bactericidas (produtos que eliminam bactérias), rodenticidas (produtos que combatem roedores). Os praguicidas também podem ser classificados pela sua estrutura química, é o caso dos organofosforados, feniluréias, carbamatos e piretróides (Zhang et al., 2022; Pathak et al., 2022). Na agricultura, os praguicidas são usados para elevar a produção do plantio, impedindo a proliferação de pragas, visando aumento de lucro e melhoria da qualidade dos produtos (OPAS/OMS, 2020; Zhang et al., 2022).

É relatado, desde 500 a.C o uso agrícola dos praguicidas para evitar perdas em suas colheitas. Porém, em meados de 1936 os compostos sintéticos como o inseticida diclorodifeniltricloroetano, conhecido como ''DDT'' foram desenvolvidos e usados indiscriminadamente para combater insetos. Na década de 1960 descobriu-se que a substância provocava severos danos à saúde dos seres vivos e ao meio ambiente, por essa razão teve seu uso banido em diversos países na década de 1970 (Abubakar et al., 2020).

Hoje, os praguicidas estão entre os principais instrumentos do modelo de agricultura atual. Ao quantificar o consumo médio mundial desses químicos, o uso dos herbicidas se destacou, com um consumo médio de aproximadamente 48%, seguido dos inseticidas, representando 29,5% e finalmente os fungicidas com 18% (Sharma et al., 2019). O consumo global de pesticidas em 2019 foi de aproximadamente 4,19 milhões de toneladas, onde a China foi o maior país consumidor de pesticidas (1,76 milhões de toneladas métricas), seguida pelos Estados Unidos (408 mil toneladas),

Brasil (377 mil toneladas) e Argentina (204 mil toneladas) (Fernández, 2021). No Brasil, a agricultura tem sido a principal atividade econômica ao longo dos séculos, devido às suas condições climáticas favoráveis, assim, o país desempenha hoje um importante papel no mercado agrícola, sendo considerado um dos maiores exportadores de alimentos em escala global (Pignati et al., 2017). Além do mais, o país possui aproximadamente 9% do seu território de área plantada, chegando a 76 milhões de hectares. Já produtividade aumenta, em média, 8% década (CEPEA, 2019; IBGE 2020). Por essas circunstâncias, o mercado brasileiro de praguicidas é estimado em US $ 10,5 bilhões por ano, o que representa cerca 20% do mercado mundial (Pignati et al., 2017; Barizon, 2020). Outro ponto a ser destacado sobre produção agrícola, é que essa atividade corresponde, aproximadamente, a 5% dos US$ 1,8 trilhão produto interno bruto (PIB) brasileiro, com uma variedade de produtos das cadeias de grãos e oleaginosas, fibras e frutas (MME, 2020).

Segundo a publicação de 2016 da Organização das Nações Unidas (ONU) para a Alimentação e Agricultura, o Brasil teve um consumo de 4,31 quilos de praguicidas por hectare (Kg/ha) cultivado, ficando atrás de países como Países Baixos (9,38 kg/ha), Bélgica (6,89 Kg/ha), Itália (6,66 Kg/ha), Irlanda (5,78 Kg/ha), Portugal (5,63 Kg/ha) e Suíça (5,07 Kg/ha) (FAO, 2020). Ainda, sabe-se que a soja (área plantada de 32,2 Mha), milho (área plantada de 15,8 Mha) e cana-de-açúcar (área plantada de 10,1 Mha) são as culturas de maiores safras brasileiras (Pignati et al., 2017), consumindo grandes quantidades de praguicidas por hectare (Pignati et al., 2017; Camargo et al., 2021). Estudos recentes indicam que o uso de praguicidas aumentará ao longo dos anos, acompanhando o crescimento da população, que provavelmente atingirá nove bilhões de pessoas em 2050 (Verger, 2020). Apesar de cumprirem o papel de proteger as culturas agrícolas, existe uma crescente preocupação com relação às consequências do uso indiscriminado de grande parte desses compostos e os possíveis danos que possam promover nos ecossistemas e na saúde humana.

2. Impactos pela ação dos praguicidas

O desenvolvimento industrial e o crescimento populacional, incentivou grande produção de inúmeros novos compostos (Pereira et al., 2015; Bambino et al., 2017; Starling et al., 2019). Desde então, esses químicos, incluindo os praguicidas estão presentes no ambiente, elevando a necessidade de conhecimento sobre sua toxicidade, assim como os possíveis riscos a saúde humana e as consequências do acúmulo desses compostos no ecossistema (Poynton; Robinson, 2018; Richardson e Kimura, 2020; Asgari et al 2023).

Especificamente sobre os praguicidas, após a aplicação, solo é o primeiro compartimento onde se dispersam, podendo ser absorvidos pelas raízes das plantas, lixiviados para estratos mais profundos do solo ou sofrer escoamento (Pathak, 2023). Por subsequência, o ambiente aquático também é destino desses resíduos (Hedlund et al., 2019; Bonifacio e Hued, 2019). Como agravante, a maioria dos compostos ativos desses produtos são moléculas quimicamente estáveis e persistentes, dispersando-se gradativamente no meio ambiente e contaminado os recursos hídricos (Storck et al., 2017). Hoje, é possível identificar quais praguicidas estão mais presentes no meio ambiente e em matrizes aquáticas (Escher et al., 2019). Outro dado relevante sobre os praguicidas, é que esses estão dentre os principais contaminantes presentes em águas brasileiras superficiais, subterrâneas, em sedimentos e até em água potável (Montagner et al., 2019). Publicações recentes relatam ingredientes ativos de praguicidas em 25% das águas superficiais do estado de São Paulo (CETESB, 2019). Por esses fatores, inúmeros trabalhos científicos vem sendo desenvolvidos para se conhecer os impactos gerados ambientais por essas substâncias.

Apesar dos dados sobre a presença desses poluentes nas águas e seus possíveis efeitos, sobre o meio biológico, alguns pesquisadores afirmam que as concentrações de contaminantes presentes no ambiente, como os praguicidas, estão em níveis muito abaixo das doses usualmente aplicadas em experimentos e consumidas diariamente (UNEP, 2019; Gupta et al., 2020). Assim, abordagens modernas têm alta sensibilidade, quando comparado a testes de toxicidade tradicionais ou testes que usam contaminantes em concentrações elevadas (Andrade et al., 2016; Domingues et al. 2010; Oliveira et al., 2013). Estudos evidenciaram que as alterações em marcadores bioquímicos e de comportamento locomotor, ocorrem nas concentrações dos praguicidas abaixo aquelas capazes

de alterar o desenvolvimento embrionário do modelo zebrafish (*Danio rerio*), fornecendo informações adicionais sobre esses compostos (Domingues et al., 2010; Zhang et al., 2012; Oliveira et al., 2013). Andrade e seus colaboradores (2016) avaliaram, em embriões de zebrafish, baixas concentrações do fungicida carbendazim, consideradas ambientalmente relevantes (0.16 a 500 μg/L). Foram observados pontos letais e de desenvolvimento como eclosão, edemas e malformações, mas também foram avaliados efeitos a nível bioquímico e comportamental. Como resultado, os marcadores bioquímicos (acetilcolinesterase, glutationa s-transferase e lactato desidrogenase) estavam alterados. No mesmo estudo, os autores evidenciaram que alterações comportamentais foram observadas em concentrações quase 9.000 vezes abaixo daquelas capazes de alterar o desenvolvimento do organismo (Andrade et al., 2016).

Por fim, é de suma importância a realização de avaliações toxicológicos experimentais dos praguicidas, que permitam uma melhor compreensão de impactos gerados por estes contaminantes, incluindo os estudos que avaliam as concentrações reais encontradas no ecossistema. Também é importante a realização de estudos mecanísticos para a compreensão do modo de ação (MoA) e identificação de endpoints desses químicos, para a detecção de seus possíveis efeitos deletérios, dentro deste contexto realista (Bahe et al., 2006; Pereira et al., 2015).

3. O herbicida diuron e seus metabólitos DCPMU, DCPU e DCA

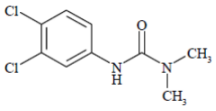
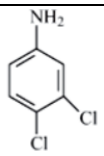
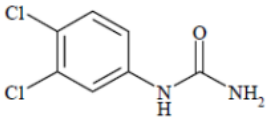
O diuron (3-(3,4-dichlorofenil)-1,1-dimetilureia) é um herbicida derivado da ureia, amplamente utilizado no meio agrícola que possui amplo espectro de ação em aplicações pré e pós-emergentes (Roque e Melo, 2000; USEPA, 2003; APVMA, 2005). É facilmente absorvido pelas raízes das plantas invasoras (ou ervas daninhas) e tem como função primordial bloquear a fotossíntese e inibir a produção de oxigênio (Iyer, 2002; APVMA, 2005; Ihlaseh et al., 2011). É muito utilizado no cultivo de cana-de-açúcar, mas também pode ser usado em culturas de frutas cítricas, banana e uva (Goldemberg, 2008; Tooge, 2019). Quanto às suas características, em sua forma pura, o diuron é um cristal, não iônico, com moderada solubilidade em água (42 mg/L a 20 °C) (Giacomazzi e Cochet, 2004).

Estima-se que 6,52 mil toneladas de diuron foram vendidas no Brasil, só no ano de 2021. De

acordo com o IBAMA (2023), o diuron é o 4º herbicida mais usado no país e o estado que faz mais uso desse produto é o de São Paulo, representando cerca de 3 mil toneladas das aquisições do herbicida. O diuron é altamente estável, persistente e sofre lenta degradação, sendo considerado um poluente biologicamente ativo no solo, em sedimentos e principalmente na água (Field et al., 2003; Giacomazzi e Cochet, 2004). Após as aplicações no solo, o herbicida sofre escoamento e lixiviação principalmente para os cursos de água e posteriormente cai nos rios e lagos. Em água, possui meia vida considerada longa, de aproximadamente 45 dias (Lamoree et al., 2002; Gooddy et al., 2002; Giacomazzi e Cochet, 2004; PUBCHEM, 2021a). Hoje, estima-se que suas concentrações em água variam entre 0,16 a 6742 ng/L na Europa, 3045 ng/L no Japão e entre 4 a 279 ng/L no Brasil (Konstantinou et al., 2004; Köck-Schulmeyer et al., 2013; Velki et al., 2017; Acayba et al., 2021).

No ambiente, o diuron também pode vir a se biotransformar, pela ação de fungos e bactérias ou pela hidrólise em água, em metabólitos conhecidos que exibem alta toxidade (Pätzold; Brümmer 2003; Sørensen et al., 2003; Giacomazzi e Cochet, 2004). O DCPMU (3- (3,4-Diclorofenil) -1- Metilureia) é formado após um primeiro passo de desmetilação, que na sequência se transforma em DCPU (1- (3,4 - Diclorofenil) ureia) e finalmente é hidrolisado em 3,4 - DCA (3,4 - Dicloroanilina) (Hodge et al., 1967). Para Thomas e seus colaboradores (2002), o diuron e o DCA são considerados substâncias bastante persistentes. Já os metabólitos DCPMU e DCPU tem menor persistência em água. Apesar de o metabólito DCPU ser um potencial contaminante ambiental, atualmente não vem sendo desenvolvidos estudos com essa molécula. Uma vez que, não é possível encontrá-la no mercado para análises, há uma limitação das possibilidades de estudos com esse composto.

Tabela 1) Nomenclatura e estrutura química do diuron, DCA e DCPMU.

Nome da substância	**Nomenclatura da substância**	**Estrutura química da substância**
Diuron	3-(3,4-diclorofenil)-1,1- dimetilureia	
DCA	3,4-dicloroanilina	
DCPMU	(3- (3,4-diclorofenil) -1-metilureia)	

Fonte: USEPA, 2003

4. A toxicidade do diuron e seus metabólitos DCA e DCPMU

De acordo com o Sistema Globalmente Harmonizado de classificação e rotulagem de produtos químicos (GHS, sigla em inglês), o diuron pertence à classe III de periculosidade, esse praguicida tem potencial carcinogênico, é tóxico se ingerido, e causa danos para órgãos-alvo específicos. Para a Comissão Europeia, o herbicida diuron é um composto perigoso (CETESB, 2019; Mohammed et al, 2020). No Brasil, o diuron é encaixado à categoria 3 e à faixa amarela, considerado substância moderadamente tóxica (Anvisa, 2017). Pela classificação do GHS, o metabólito do herbicida diuron (3,4- DCA, ou somente DCA) é tóxico por via oral, inalatória e pode causar de lesões oculares (PUBCHEM, 2022). De acordo com Dupraz et al (2016), o DCA é classificado como um produto químico altamente tóxico. Já os escassos dados sobre o DCPMU relatam que o metabólito é nocivo se ingerido e pode causar irritação ocular (PUBCHEM, 2021).

Para Asgari (2013) o diuron, tem ameaçado o ambiente e a dos organismos, incuindo o ser humano. Estudos experimentais com roedores mostraram que os principais alvos de toxicidade do

diuron são o sistema hematopoiético e a mucosa urotelial (USEPA, 1997; Iyer, 2002). A Australian Pesticides and Veterinary Medicines Authority (APVMA, 2011) relata que, em altas concentrações (2.500 ppm) o diuron induziu tumorização na bexiga e pelve renal de ratos. Frente a esses dados, a United States Environmental Protection Agency (USEPA, 1997) classificou a substância como um "provável cancerígeno para o homem". Além disso, foi observado que o diuron também causou danos ao sistema hematopoiético e anemia em ratos (USEPA, 2003). Na busca por conteúdo sobre potencial nocivo do diuron, é possível encontrar estudos que priorizam métodos alternativos à experimentação animal, que incluem, por exemplo, os organismos aquáticos (Mhadhbi e Beiras, 2012; Pereira, et al 2015; Velki et al., 2017; Felicio et al., 2018; Velki et al 2019). De acordo com Eissa (2010), os peixes são organismos sensíveis do ponto de vista toxicológico, apresentam desenvolvimento rápido e vivem em um ecossistema que é o principal destino dos praguicidas. Um ponto relevante nos estudos que favorecem toxicologia alternativa, é que grande parte dessas pesquisas avaliam os possíveis danos dos químicos, como o diuron concentrações ambientalmente relevantes (Pereira et al., 2015; Velki et al., 2017; Velki et al., 2019).

No estudo de Velki et al (2017), o herbicida diuron nas concetrações de 1, 2 e 3.8 mg/L se mostrou tóxico aos estágios iniciais da vida do modelo zebrafish. Mudanças nos desfechos comportamentais, como diminuição nos movimentos de rolamento espontâneos de embriões e timotaxia foram frequentemente observadas. Em outro estudo com embriões de zebrafish, realizado com concetrações ambientais de 8.6, 4.3, 2.15, 0.43 e 0.086 μM de diuron, foram detectadas alterações bioquímicas nas enzimas Catalase (CAT), glutationa S-Transferase (GST) e Acetilcolinesterase (AchE), apontando a importância de monitorar o herbicida em diferentes níveis de organização biológica (Velki et al., 2019). Em estudos para avaliar efeitos teratogênicos, o diuron induziu malformações no desenvolvimento e aumento significativo da mortalidade embrionária em peixes robalo (*Psetta maxima*), em adição os organismos que sobreviveram sofreram diminuição significativa no sucesso da incubação, malformações e edema pericárdico (Mhadhbi e Beiras, 2012). A exposição do peixe medaka javanês (Oryzias latipes) a concetrações subletais (1, 50, 100, 500 e 1.000 μg/L) de diuron, por 21 dias, induziu alterações histopatológicas nas gônadas (ovário e testículo), diminuição no estadiamento gonadal e maturidade das células germinativas na oogênese e

espermatogênese (Kamarudin et al., 2020). Foram também observadas, por Kamarudin et al. (2020), em embriões desse peixe deformidades de desenvolvimento para concetrações ambientalmente relevantes (0.60, 1.25, 2.50mg/L) de diuron. Outros endpoints como a diminuição da frequência cardíaca, eclodibilidade e taxa de sobrevivência foram reduzidas para a concentração mais altas (5 e 10 mg/L (Ibrahim et al., 2020). Kao et al. (2019) testaram, por 24 e 48 horas, diferentes concentrações de diuron (12,5µg/ml e 25 µg/ml) em células da linhagem de hepatoblastoma humano (HepG2) e em embriões do teleósteo zebrafish. Os autores observaram embriotoxicidade para zebrafish e geração de espécies reativas de oxigênio (EROS) para as células HepG2.

Outros estudos demostram que além do diuron, seus metábólitos, exercem efeitos tóxicos em organismos aquáticos (Guilhermino et al., 1998; Tixier et al., 2001; Osano et al., 2002). Em peixes da espécie Tilápia do Nilo *(Oreochromis niloticus*) a exposição ao diuron ou aos seus metabolitos DCA, DCPMU e DCPU, induziram diminuição dos níveis de testosterona e 11-ketotestosterona sérica, do índice gonadossomático, do diâmetro dos túbulos seminíferos e das porcentagens médias de células germinativas, caracterizando–os como anti-androgênicos para o sistema reprodutivo (Pereira et al., 2015). Parâmetros endócrinos e de biomarcadores enzimáticos CAT, GST e SOD (superóxido dismutase) foram estudados também *in vitro*, no fígado, cérebro e brânquias de tilápias do Nilo (*Oreochromis niloticus*) expostas ao diuron DCA, DCPMU e DCPU. O estudo detectou alterações nos parâmetros analisados, confirmando que podem ser considerados possíveis desreguladores endócrinos (Felicio, 2017). Ainda em peixes de diferentes espécies, o diuron e os seus metabólitos DCA, DCPU e DCPMU causaram alterações a nível morfológicas (Mhadhbi e Beiras, 2012), bioquímicas (Sanchez-Muros et al., 2013), e fisiológicas (Scheil et al., 2009).

Em um estudo avaliando o DCA, o composto mostrou-se tóxico para fêmeas do peixe menaka javanês expostas a 250µg/L de diuron. Foram observados índice gonadossomático significativamente baixo, a estrutura das células ovarianas foram interrompidas e houve redução significativa no desenvolvimento das gônadas femininas, mostrando que o DCA pode interferir na reprodução desses oganismos aquáticos, mor meio de efeitos sobre a fecundidade e alteração dos tecidos gonadais (Ibrahim et al., 2021). De acordo com Groshart e Okkerman (2000), o DCA é

reconhecido desregulador endócrino para a vida selvagem e à saúde humana. No entanto, dados sobre o potencial nocivo do DCA sobre os marcadores biológicos e comportamental, não são comuns, tornando esse tipo de avaliação a necessária. Já as informações sobre o DCPMU, que participa da formação do DCA são raros na literatura, embora alguns estudos apontam sua maior toxicidade em relação ao diuron (Tixier et al., 2000).

5. Zebrafish como método alternativo aplicado em ensaios toxicológicos

Os estudos toxicológicos in vivo são fundamentais para análise dos compostos químicos e para a avaliação dos efeitos adversos dos praguicidas (Koeter, 1993; Stokes, 2002; Meyer, 2003; Klaassen e Watkins, 2012). Apesar dos mamíferos serem os animais tradicionalmente utilizados em experimentações, eles possuem alto custo para manutenção e necessidade de grandes espaços para o seu cultivo (Zon e Perterso, 2005). Além do mais, o uso disseminado dos animais experimentais tradicionais na pesquisa tem sido causa de discussões, principalmente sob o caráter ético, em função do grande número de animais requerido e do sofrimento causado durante alguns tipos de experimento (Russel e Burch, 1992; Schechtman, 2002).

Na proposta de introduzir novos métodos de experimentação o programa denominado 3R’s (Reduction, Replacement, Refinement), objetiva reduzir o uso de animais de laboratório, substituir ensaios experimentais e refinar, minimizando o sofrimento dos modelos utilizados nas pesquisas (Russel e Burch, 1992; Schechtman, 2002). Nesse cenário, existe uma busca intensa por métodos alternativos a serem aplicados na área da toxicologia, para avaliação dos riscos potenciais de produtos químicos. Dentre a ampla gama de métodos alternativos que surgiram, o modelo experimental zebrafish, em substituição aos mamíferos, é um organismo experimental de baixo custo, com alta taxa de reprodução e amplamente utilizado em pesquisas ecotoxicológicas para avaliação de risco de contaminantes ambientais (Kimmel el al., 1955; Scholz et al., 2008; Cassar et al., 2019). Na fase embrionária é considerado método de substituição que se encaixa nos 3R’s, pois pela diretiva europeia 2010/63/EU, sobre a proteção de animais para fins científicos, os embriões da espécie se alimentam unicamente pelo conteúdo do saco vitelínico, sem necessidade de regulamentação em procedimentos experimentais, pois não tem a alimentação exógena durante esse

período (Scholz et al., 2008; Directive, 2010/63/EU; Teixidó et al., 2013).

O peixe zebrafish (Danio rerio), ou paulistinha, é um teleósteo de origem Asiática e tem aproximadamente quatro centímetros na fase adulta (Eaton e Farley, 1974; Scholz et al., 2008) (Figura 1). O peixe apresenta características fenotípicas diferentes em relação ao seu sexo. Geralmente os peixes machos têm uma barriga mais lisa, enquanto que as fêmeas mostram uma barriga arredondada e prateada (Nasiadka et al., 2012; Meyers, 2018). Esta espécie foi descrita pela primeira vez em meados de 1822 pelo britânico Francis Hamilton e se tornou modelo científico em 1983 pelo biologista George Streisinger (Spence et al., 2008). Atualmente, é um importante modelo vertebrado nas pesquisas científicas, sendo explorado em diversas áreas, mas com grande destaque na área da toxicologia, por possuir alta sensibilidade quando exposto a compostos químicos, devido à rápida absorção (Scholz et al., 2008; Carlsson et al., 2013). O peixe é um notável modelo para avaliação de neutotoxicidade, pois tem sistema nervoso central parecido ao humano, contendo os mesmos neurotransmissores, receptores e genes envolvidos nas alterações neuronais (Migliarini e Carnevali, 2009; Panula et al., 2010). Com as análises das funções motoras, sensoriais, autonômicas e cognitivas é possível também a compreender os efeitos dos compostos químicos no sistema neurológico, o que tem grande relevância, já que muitas das alterações comportamentais têm correlação com as dos humanos (Klaassen e Watkins, 2013).

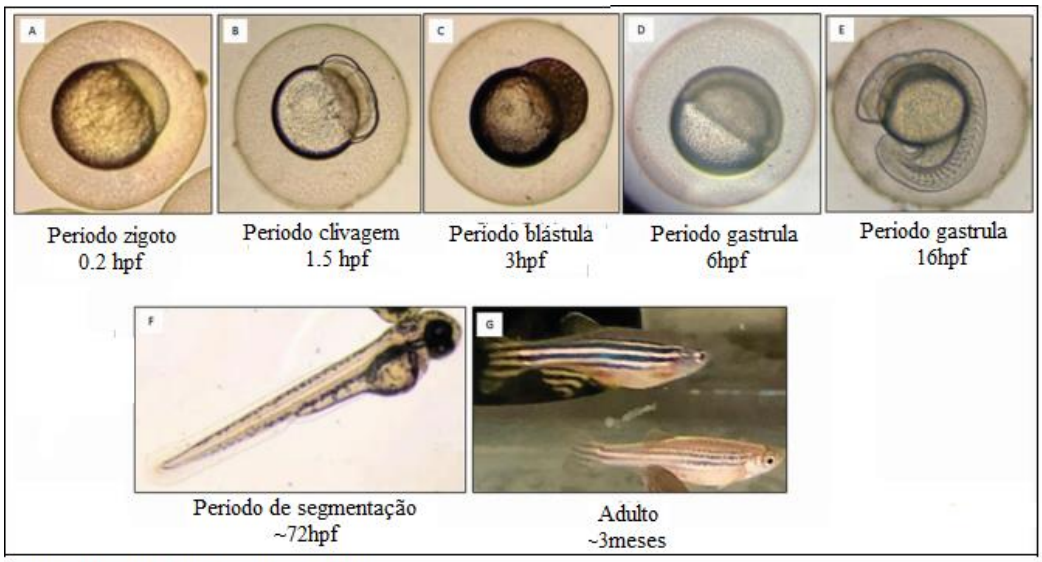
Sua importância como modelo para pesquisas também se dá principalmente por possuir grande parte do genoma sequenciado e pelas semelhanças com outros vertebrados, inclusive humanos, com cerca de 70% de genes semelhantes (Kimmel et al., 1995; Scholz et al., 2008; Carlsson et al., 2013; Teixidó et al., 2013) e cerca de 84% de ortologia com genes de doenças humanas (Howe et al, 1013). O peixe compartilha características anatômicas com os mamíferos (rins, cérebro, fígado, intestino, coração, coluna vertebral) que, embora tenham adaptações específicas à vida aquática, possui funções e fisiologia conservadas (Macrae e Peterson, 2015). As reações metabólicas em humanos acontecem semelhante no zebrafish, devido a genes ortólogos duplicados de um ancestral comum que continuam a ter uma função correspondente nas duas espécies, tornando o zebrafish um modelo na previsão do metabolismo de inúmeros compostos (De Souza et al., 2018). As vias (metabolismo e excreção) também funcionalmente semelhantes às dos

mamíferos. Já a bioconversão dos diferentes compostos, como os químicos, ocorrem principalmente no fígado em ambas as espécies (Macrae e Peterson, 2015; Hahn et al., 2015). Ainda dentro do contexto, o fígado é considerado biomarcador. O zebrafish possui processos moleculares e celulares, desenvolvimento hepático, maturação e função de enzimas metabólicas semelhantes quando comparados aos humanos, sendo reconhecido como organismo respeitável para análise hepática (Barros et al., 2008; Vliegenthart et al., 2014). Assim, por esse tecido, é possível visualizar os efeitos das exposições a xenobióticos, sendo considerado uma importante ferramenta na avaliação de alterações patológicas e caracterizado como indicador de toxicidade de compostos do ambiente aquático (Johnson et al. 1993; Van Der Oost et al. 1996; Bernet, et al. 1999, Boran, 2010).

Dentre outras características que enaltecem o zebrafish, é que o organismo tem rápido desenvolvimento e manipulação acessível, tornando-o um modelo fácil para avaliação experimental (Mcgrath, 2008). Seu ciclo de vida tem quatro estágios distintos (embrionária, larval, juvenil e adulta). Durante a fase embrionária, o organismo se desenvolve em um ovo translúcido que possibilita a avaliação do seu desenvolvimento. A fase larval ocorre após a eclosão do ovo, com aproximadamente 72 hpf, e as fases juvenil/adulta são atingidas com apenas três meses, etapa que a maturidade sexual é alcançada (Hill et al., 2005; Scholz, 2008). No desenvolvimento embrionário e larval, é possível se observar várias malformações resultadas da exposição a contaminantes (Hill et al., 2005). Além do mais, os estágios iniciais da vida (ovos, embriões e larvas) de peixes e invertebrados aquáticos demonstraram ser particularmente sensíveis a estressores ambientais (Hutchinson et al., 1998; Mohammed, 2013).

Por fim, métodos toxicológicos alternativos, como os que utilizam o zebrafish, têm grande potencial, uma vez que o organismo em questão compartilha inúmeras características genéticas, anatômicas e fisiológicas com os mamíferos, tornando possível a extrapolação dos resultados obtidos com este peixe para o ser humano (Ali et al., 2011; Garcia; Noves; Tanguay, 2016). E ainda, o organismo é um biomodelo promissor para análises comportamentais e ecotoxicológicas, principalmente para o biomonitoramento de ecossistemas aquáticos e contaminantes ambientais, tornando-o um importante aliado da ciência (Quiniou et al., 2007; Vethaak et al., 2015).

Figura 1) Esquema das fases de desenvolvimento do zebrafish (*Danio rerio*).



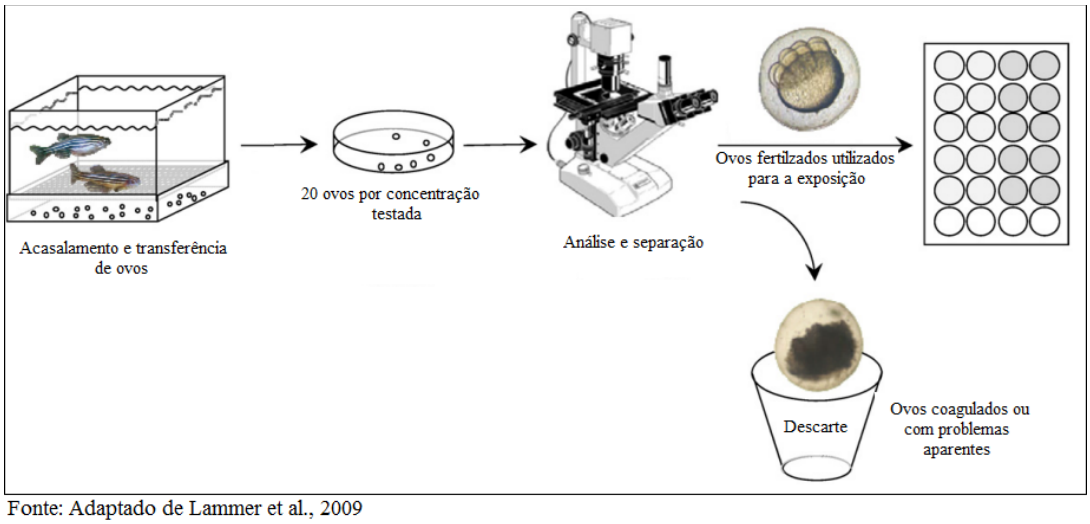
Fonte: Adaptado de Lima et al., 2020.
De A até E: embrião (Até 48 hpf), F: larval (48-72 hpf) e G: adultos (3 meses).

5.1 Teste de toxicidade aguda em embrião (teste FET)

O ensaio de toxicidade com os estágios embrio-larval de zebrafish (Fish Embryo Toxicity - FET) emergiu como análise promissora para investigação da toxicidade de compostos químicos (Braunbeck et al., 2005; Knöbel et al., 2012; Nagel, 2002). Estabelecido no ano de 2013 pela OECD, o teste FET objetiva acasalar adultos, obter ovos, expor os embriões e larvas de zebrafish recém-fertilizados ao composto-teste e analisar, a cada 24 horas, os parâmetros dos indicadores de toxicidade (Figura 2). De acordo com Kimmel et al., (1995) os indicadores de letalidade incluem a coagulação dos ovos fertilizados, falta de formação de somitos, falta de destacamento da cauda do saco vitelino e ausência de batimento cardíaco. Dentre os indicadores de subletalidade, destacam-se as malformações no saco vitelino, olhos e cabeça; embrião sem movimento, deformidades esqueléticas (cauda curvada, cauda encurtada, escoliose) e crescimento retardado (Carlsson et al., 2013). Inicialmente, o teste FET foi proposto como método toxicológico alternativo ao uso de peixes adultos, como o protocolo OECD 203 (2019), e apresentou grande potencial para sua aplicação em toxicologia regulatória. Desde sua implementação, tem se observado uma gradual adesão do teste FET dentro do contexto regulatório, resultado da associação entre um modelo robusto com um método alternativo eficiente Braunbeck et al., 2015). Em geral, os embriões de zebrafish são capazes

de metabolizar a maioria das substâncias da mesma maneira que os peixes adultos (Braunbek et al., 2014). Além do mais, a maioria dos ensaios FET ocorrem durante o período de 96 – 120 hpf, se encaixando no conceito de bem-estar animal (refinamento) e substituição dos modelos tradicionais de experimentação. Tais conceitos estão encaixados dentro dos 3Rs, outro motivo de serem também tão utilizados nos experimentos atuais (Astrogildo et al., 2018). É importante notar que, fatores como características físico-químicas e peso molecular das substâncias a serem testadas devem ser consideradas, uma vez que o córion do embrião de zebrafish possui poros com aproximadamente 0,5 µm de diâmetro, podendo limitar o alcance do composto em estudo ao organismo (Cheng et al., 2007). Contudo, apesar dessa restrição, em geral, a utilização de do FET teste para análises de substâncias químicas na toxicologia mostra-se uma tecnologia eficaz e de alto rendimento.

Figura 2) Esquema do procedimento do FET teste.



Fonte: Adaptado de Lammer et al., 2009

5.2 Teste de toxicidade aguda em peixes adultos

Para poder estimar os efeitos deletérios de compostos químicos ao meio ambiente e para os diferentes organismos, é necessário obter respostas rápidas (Barbieri et al., 1998; Barbieri et al., 2007; Phan et al., 2023). Os testes de toxicidade aguda são considerados métodos para determinar qual a concentração de uma substância produz efeito tóxico a um organismo por um curto tempo de exposição (Pankratz, 2011). São definidos como efeitos, em geral, severos e rápidos, sofridos pelos

organismos expostos ao agente químico, em um curto período de tempo (toxicidade aguda) (Aragão e Araújo, 2006).

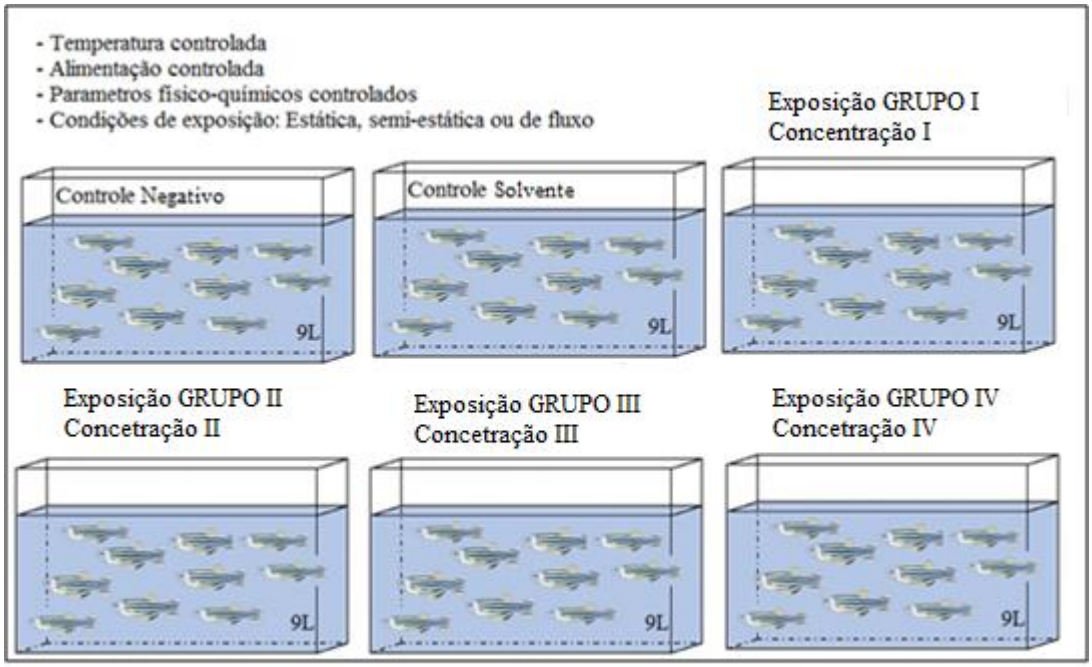
No Brasil, os testes toxicológicos também utilizam peixes como organismo teste. Esses testes são padronizados de acordo com a ABNT (Associação Brasileira de Normas Técnicas), que estabelece os possíveis modelos experimentais para avaliação de contaminantes, dentre os quais se inclui o zebrafish. A norma ABNT-NBR 15088 é a responsável pela normatização dos ensaios de toxicidade aguda (ABNT, 2011). Nesse contexto, as análises de toxicidade com peixes adultos são fundamentais, pois avaliam os possíveis efeitos tóxicos das substâncias químicas sobre o ecossistema aquático, uma vez que o ambiente é uma das principais rotas dos contaminantes (Rand et al., 1995, Kendaall et al., 2021). Outro ponto importante para utilização de peixes nos ensaios de toxicidade é que, os organismos são os principais representantes dos consumidores secundários na cadeia alimentar aquática (Costa et al., 2008). Além do mais, os peixes são considerados bioindicadores da qualidade da água. Uma vez expostos, podem ter alterações na taxa crescimento, maturação sexual e os danos em tecidos, que auxiliam na predição dos efeitos dos poluentes (Martinez e Souza, 2014). Por fim, a utilização de peixes para avaliação dos possíveis efeitos tóxicos de substâncias presentes no ecossistema aquático é de suma importância para conhecer o potencial nocivo de químicos, amparando a tomada de decisões, pelo do conhecimento das consequências do composto para o ambiente, e à saúde do homem.

O guideline de 2019, OECD TG 203 (Fish, Acute Toxicity Testing), é destaque para análise com peixes expostos a um curto período de tempo. Tem como interesse o bem-estar animal e a utilização eficiente de recursos. O princípio do teste se baseia em expor os peixes ao produto químico, durante um período agudo de até 96 horas, sob qualquer condição estática, semi-estáticas ou de fluxo (Figura 3). Durante o período de exposição à substância teste, são registradas a mortalidade e as anormalidades visíveis, relacionados à aparência e ao comportamento do organismo testado. O princípio do teste considera outros pontos relevantes, que devem ser seguidos durante a experimentação, com a presença de grupos controles, onde a mortalidade deve não exceder 10%, utilizar sempre recipiente de vidro, aço inoxidável ou outros recipientes quimicamente inertes e realizar sempre o controle dos parâmetros físico-químicos da água onde os animais estarão expostos

(OECD, 2019).

Apesar da importância destacada na utilização de adultos para avaliação toxicológica, alguns autores relatam que embriões e larvas são mais sensíveis que os animais adultos, o que pode ocasionar sensibilidades diferenciadas entre as fases descritas no método mencionado. Segundo Xiu e colaboradores (1991), os embriões e larvas de zebrafish são geralmente mais sensíveis a diversos poluentes do que os adultos. Em função desta sensibilidade diferenciada, o Órgão Ambiental Norte Americano, USEPA (USEPA, 2002) orienta que, os ensaios de toxicidade sejam também conduzidos com organismos diferentes fases vida.

Figura 3) Esquema da exposição do teste de toxicidade aguda com peixes adultos, contendo controles negativo, controle solvente e grupos de exposição com diferentes concetração dos contaminates.



Fonte: Adaptado de Ribeiro et al., 2022

5.3 Biomarcadores bioquímicos em Zebrafish

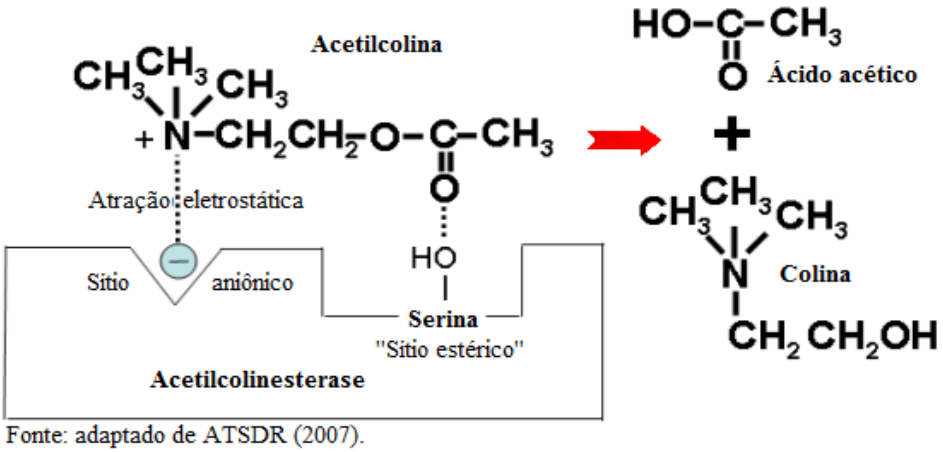
Biomarcadores são, por definição, alterações do organismo exposto a um produto (xenobiótico) em um nível sub-individual, medido por sinais bioquímicos, moleculares, genéticos, imunológicos e/ ou fisiológicos (VanGestel e VanBrummelen, 1996; Regoli et al., 2002). As alterações desses componentes indicam um comprometimento do estado normal do organismo, mostrando que, o contaminante em questão foi absorvido e distribuído sistemicamente, provocando toxicidade para o indivíduo. (Van Der Oost et al., 2003). Essas informações são importantes, pois auxiliam na compreensão dos efeitos e modo de ação de diferentes químicos e na prevenção de seus possíveis perigos (Domingues et al., 2019). Além do mais, análises de biomarcadores também podem auxiliar na detecção precoce da existência de substâncias tóxicas no ambiente, o que contribui para identificar espécies ou populações em risco, avaliar a contaminação e determinar o grau de severidade dos efeitos causados pelos xenobióticos (Stegeman et al., 1992; De Caprio, 1997; Van Der Oost et al., 2003).

Diferentes tipos de biomarcadores podem ser analisados em organismos expostos a agentes estressores presentes no meio ambiente, para avaliar impactos de compostos químicos (Van der Oost et al., 2003). As alterações das atividades enzimáticas, da concentração de compostos não enzimáticos e antioxidantes, e da toxicidade de oxidante mediada por oxidação/carbonilação proteica, peroxidação lipídica e danos ao DNA, são exemplos de biomarcadores (Printes, 2004). Geralmente, os biomarcadores mais sensíveis são aqueles que indicam alterações nos níveis e nas atividades de enzimas de biotransformação, sendo que, em peixes, a atividade destas enzimas pode ser induzida ou inibida por xenobióticos (Bucheli e Fent, 2018). Dentre os biomarcadores escolhidos para essa análise destacam-se a Acetilcolinesterase (AChE), uma importante enzima na manutenção da função nervosa (Olsen et al., 2001); Catalase (CAT), enzima que promove a detoxificação das células; Glutationa S-transferase (GST), uma família de enzimas com um papel fundamental na biotransformação de xenobióticos (Hyne e Maher, 2003); Lactato desidrogenase (LDH), envolvida no fisiologia muscular (Diamantino et al., 2001) e a peroxidação lipídica LPO, processo relacionado aos radicais livres nas membranas celulares (Esterbauer et al., 2001).

1) Acetilcolinesterase (AChE), é uma enzima envolvida no sistema nervoso central, em

funções neurais e musculares (Araújor et al., 2016). A AChE participa na hidrólise de acetilcolina (ACh) (Figura 4), cuja função primordial é transmitindo sinapses neuronais (Purves et al., 2008; Fischer et al 2015). Essa enzima é de suma importância, pois sua inibição pode acarretar no acúmulo do neurotransmissor ACh na fenda sináptica, levando a hiperestimulação dos receptores nos neurônios, portanto, à ativação da transdução de sinal constante pelas sinapses colinérgicas (Fukuto, 1990; Fulton et al., 2001; Russom et al., 2014). A mudança na atividade de AChE, também pode causar alterações no comportamento e na atividade neuromuscular, sendo considerada como um importante biomarcador de neurotoxicidade (Gagné, 2014). Determinados contaminantes podem causar a alteração/inibição de AChE, que causa a hiperestimulação de receptores de acetilcolina, ocasionando alterações fisiológicas e neurológicas (Haverrot et al., 2015). Normalmente, em ecotoxicologia a quantificação da atividade da AChE é utilizada para avaliar organofosforados e inseticidas (Jadhav e Rajini 1994; Drobne et al., 2018), herbicidas (Moraes et al., 2009), antibióticos (Tu et al., 2009) e metais (Elumalai et al., 2002; Calisi et al., 2009).

Figura 4) Esquema da quebra enzimática da acetilcolina

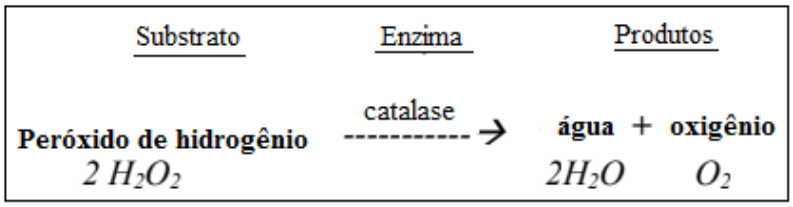


Fonte: adaptado de ATSDR (2007).

2) As Catalases (CAT) são enzimas que participam dos sistemas de defesa, principalmente contra as moléculas geradas durante o estresse oxidativo, conhecidas como espécies reativas de oxigênio (ERO), catalisando o peróxido de hidrogênio (composto tóxico), em água e oxigênio (compostos não nocivos) (Scibior e Czeczot, 2016) (Figura 5). A principal atividade da CAT

está associada aos peroxissomos (presentes principalmente no fígado) ou microcorpos que atuam no metabolismo dos ácidos graxos (Huggett et al., 1992). CAT está conectada a atividade da glutationa peroxidase (GPx) para combater a exposição ao estresse oxidativo causada por contaminantes ambientais (Diesseroth e Dounce 1970). A CAT desempenha um papel importante no processo de detoxificação de xenobióticos em peixes (Oliveira et al., 2009). É comum observar aumento de CAT em modelos experimentais expostos a diferentes químicos potencialmente tóxicos (Oliveira et al., 2009).

Figura 5) Esquema da da catalização da enzima catalase (CAT).

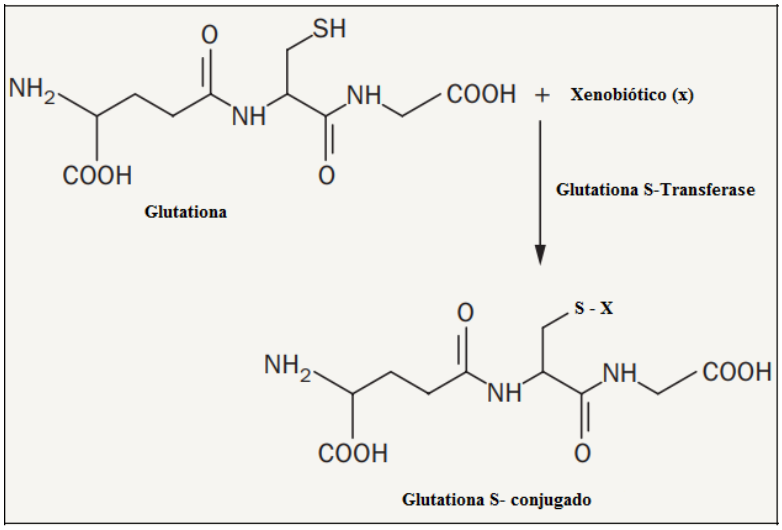


Fonte: https://bio.libretexts.org/

3) A Glutationa S-transferase (GST), é considerada a principal enzima de fase II. Resumidamente os sitemas de detoxificação de xenobióticos convertem compostos lipofílicos em produtos mais facilmente excretados, por meio da ação de uma variedade de proteínas e enzimas. Na fase I, reações de oxidação, redução e hidrólise resultam em metabólitos mais polares do que as substâncias originais. Essas mudanças aumentam a polaridade dos xenobióticos, criando sítios moleculares que facilitam sua conjugação durante a fase II. Na fase II, os metabólitos da primeira fase e os xenobióticos polares ligam-se a substratos endógenos, produzindo conjugados mais solúveis em água, os quais podem ser facilmente excretados da célula (Van Der Oost et al., 2003). Os xenobióticos são conjugados com diversas substâncias e, dentre as reações importantes da fase II, tem-se a conjugação da glutationa (GSH) aos compostos químicos (xenobióticos). A conjugação de agentes tóxicos com o tripeptídeo GSH é catalisada, na sua fase inicial, pela GST, que atua na fase II da biotransformação, prevenindo danos à membrana celular e a outras macromoléculas (Figura 6) (Hayes et al, 1995; Dybing et al., 2002; Van Der Oost et al., 2003; Malmezat et al., 2000).

Para Gagné (2014), a conjugação de xenobióticos polares à glutationa (GSH) representa uma importante via da fase II de invertebrados e peixes. Esta reação ocorre pela da ligação de compostos eletrofílicos ao grupo nucleofílico tiol do aminoácido cisteína da GSH, sendo catalisada pela glutationa S-transferase (GST) (Torres et al., 2004; Nascimento, 2008). Assim, as GST estão envolvidas na biotransformação de poluentes, sendo utilizada como biomarcador ambiental (Monteiro et al., 2006). Por fim, o aumento de GST no organismo pode estar relacionado à ação de compostos nocivos no organismo (Schelin et al., 1983).

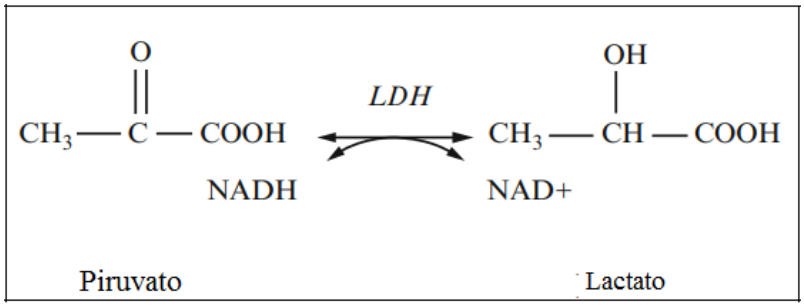
Figura 6) Esquema da Ação das glutationas S-transferases.



Fonte: Adaptado de Simic et al. (2009).

4) Lactato desidrogenase (LDH), faz parte do metabolismo celular, que participa do processo de transformação da glicose em energia nas células. Está enzima está no processo de conversão de piruvato em lactato, pela via anaeróbia nos processos de produção de energia celular, e, simultaneamente, converte o NADH em NAD+ (Neves, 2012; Silva, 2015) (Figura 7). Tem grande relevância em condições de estresse induzidos por contaminantes ambientais, quando a energia pode ser necessária em um curto período de tempo (De Coen et al., 2001; Monteiro et al., 2016). As mudanças na atividade de LDH são usadas como um indicador de exposição a produtos químicos (Gupta et al.,1991). Geralmente, aumentos de atividade de LDH são comumente relatadas em invertebrados expostos a xenobióticos (Ribeiro 1999; Diamantino et al., 2001).

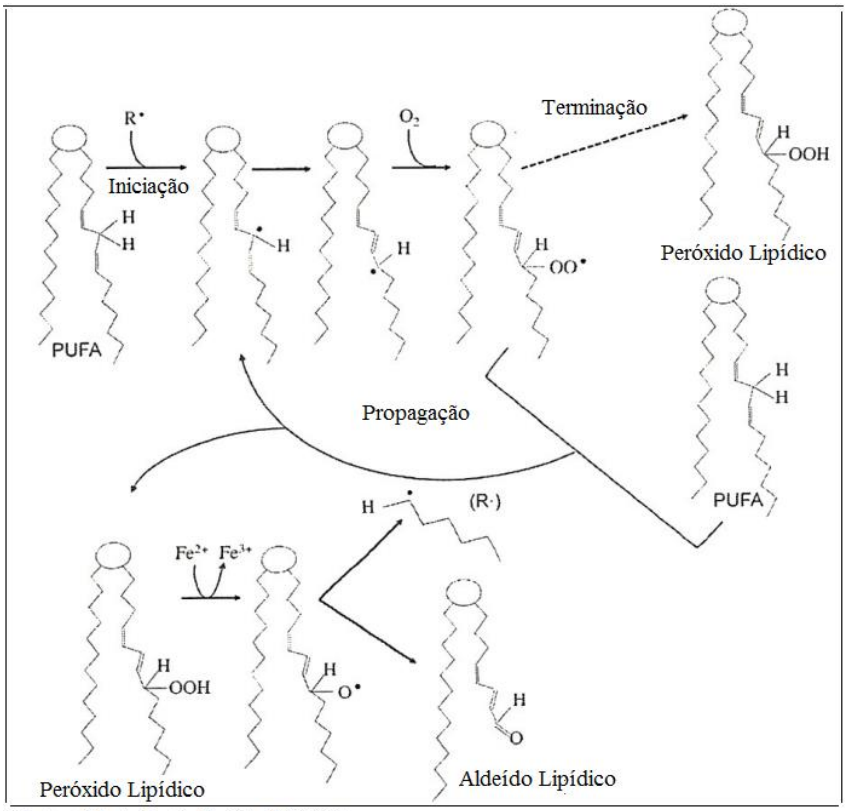
Figura 7) Esquema de catalização do lactato desidrogenase (LDH)



Fonte: Adaptado de Maes et al. (2014).

5) Peroxidação lipídica (LPO), é a degradação oxidativa dos lípidos. O excesso de ERO não removido pelo sistema antioxidante pode causar danos aos lipídeos pela sua oxidação por via enzimática ou por interação com radicais livres (Smart e Hodgson, 2008; Gagné, 2014). Quando as ERO apresentam reatividade suficiente para extrair um átomo de hidrogênio de um lipídeo intacto, inicia-se a lipoperoxidação ou peroxidação lipídica, que levam a degradação das membranas (Filipak, 2014). Este processo é iniciado por um mecanismo de reação em cadeia de um radical livre (R), que remove um hidrogênio de um ácido graxo poli-insaturado (PUFA). A fase de terminação é quando o radical lipídico formado pode reagir com oxigênio (O2), produzindo um radical de peróxido lipídico. Caso o PUFA contenha mais de três insaturações, o radical gerado reage com outro radical de peróxido lipídico, formando um peróxido cíclico. Este radical pode ainda reagir com outro ácido graxo, produzindo outro radical lipídico e um peróxido lipídico, fase denominada de propagação (Smart e Hodgson, 2008; Filipak, 2014) (Figura 8). Estudos demonstraram aumento da LPO em tecidos como consequência de toxicidade de compostos (Franco-Bernardes et al., 2015; Felício et al., 2018). Filipak (2014) cita que, a quantificação da lipoperoxidação é uma das técnicas mais comumente empregadas na determinação de estresse oxidativo em peixes, organismos estes que têm quantidades elevadas de lipídios com resíduos de ácidos graxos poli-insaturados.

Figura 8) Esquema do mecanismo de peroxidação lipídica (LPO).



Fonte: Adaptado de Boelsterli (2007).

5.4 Comportamento em zebrafish

A caracterização comportamental no modelo zebrafish é importante como biomarcador de toxicidade de compostos químicos. Estes testes podem aumentar a compreensão sobre toxicidade e o mecanismo de ação de xenobióticos (Siebel et al., 2015). Embriões e larvas de zebrafish são considerados modelos adequados para essas avaliações comportamentais, devido ao seu pequeno tamanho, facilidade de manuseio e rápido desenvolvimento (Portugues e Engert, 2009). Estudos realizados com zebrafish têm mostrado que esse organismo é um modelo experimental eficaz e sensível para ser usado em análise comportamental (Scheil et al., 2008; Vieira et al., 2009; Portugues e Engert, 2009 Colwill e Creton, 2011, Baley et al., 2016). Ensaios como os que utilizam mudanças alternadas de claro e escuro, com a finalidade de detetar alteração na atividade natatória, são comumente usados para avaliar a resposta do organismo ao estresse, mediante a exposição à xenobióticos (Burgues e Granato, 2007; Andrade et al., 2006). Um ponto comumente usado em

análises toxicológicas, dentro do contexto dos parâmetros comportamentais, é a locomoção, cuja maioria das análises utiliza a tecnologia automatizada. Análises de locomoção de larvas de zebrafish são indicadores comumente descritos na literatura científica para estudos dos efeitos de agentes tóxicos (Bichara et al., 2013). No caso do zebrafish, a locomoção, também pode ter correlação com danos neurológicos no sistema nervoso central (SNC), provocado pela ação de químicos nos estágios iniciais da vida do organismo (Irons et al., 2010; Padilla et al., 2011; Selderslaghs et al., 2010) e a toxicidade subletal de poluentes (Ulhaq et al., 2013). As alterações na neurogênese e na concentração de neurotransmissores no SNC, são correlacionáveis às alterações comportamentais. Silva et al (2008) relatam que o sistema colinérgico (referente ao sistema que depende da liberação de acetilcolina para atuar) é parâmetro para avaliar a ação de xenobióticos e alterações de padrões comportamentais.

Como já mencionado, com a inibição de AChE, a acetilcolina não é degradada, causando um acúmulo deste neurotransmissor nas sinapses nervosas e junções musculares. Esses comprometimentos levam a alterações comportamentais, como por exemplo, hiperatividade, asfixia e até a morte (Silva et al, 2008). Estudos sobre a ação de inseticidas organofosforados demonstraram a inibição de AChE, causando um efeito sobre a atividade locomotora (Baynes e Dominiczak, 2007). Dentre as possíveis alterações no comportamento locomotor, as principais delas incluem alteração na distância total percorrida, a velocidade média, o ângulo de giro, a frequência de movimento e a duração total de movimento, permitindo a identificação de nocividade de diferentes compostos químicos (Selderslaghs et al., 2013). Análises comportamentais são importantes também dentro de um contexto ecológico, pois muitos contaminantes perturbam a locomoção normal dos peixes em concentrações muito inferiores àquelas que causam a mortalidade dos organismos. Há registros na literatura de alteração de comportamento de embriões de peixes em concentrações 375 vezes menores que a LC10 (Klüver et al., 2015). Ainda quanto à relação ecotoxicológica e comportamental de peixes, larvas e embriões, o comprometimento da função locomotora influencia, principalmente, a busca por alimentos, reprodução e,, consequentemente a sobrevivência do organismo (Lovern et al., 2007). Por fim, as análises de comportamento podem fornecer medidas sensíveis de exposição a contaminantes. Estas respostas têm alta relevância ecológica, pois os efeitos podem ser traduzidos,

em longo prazo, à saúde e na sobrevivência das populações (Scott e Sloman, 2004). Uma vez que o comportamento é medido por respostas fisiológicas dos organismos em condições estressantes, os usos desses marcadores biológicos contribuem para elucidação do modo de ação de compostos químicos, elevando a fidelidade dos resultados observados (Lovern et al., 2007).

5.5 Comprimento e frequência cardíaca em zebrafish

Os estágios de desenvolvimento embrionários são demonstrados por Kimmel et al (1995). Embriões com 120 hpf têm medidas aproximadas pré-estabelecidas de comprimento total do corpo. São caracterizados como xenobióticos que afetaram o desenvolvimento embrionário, qualquer poluente capaz de provocar alteração na estrutura embrionária. Para Nagel (2002), alterações no comprimento e frequência cardíaca de zebrafish expostos aos químicos são parâmetros categorizados como de subletalidade e teratogênicidade. As alterações relacionadas ao comprimento larval em zebrafish estão fortemente associadas a deformidades morfológicas, assim como malformações esqueléticas, saco vitelino e edema do pericárdio. Já a avaliação dos batimentos cardíacos tem grande relevância, pois as alterações neste tecido podem representar condições patológicas do organismo (De Luca et al., 2014). Para Mcgrath (2008), o zebrafish é um modelo útil para se estudar doenças e alterações cardíacas, devido à semelhança ao coração de mamíferos, desenvolvimento rápido, e métodos genéticos disponíves. Estes organismos possuem mecanismos moleculares subjacentes à formação de vasos e morfogênese semelhantes aos dos vertebrados superiores, mostrando um notável grau de conservação anatômica e funcional (Isogai et al., 2001).

Outra característica importante da análises cardíacas de zebrafish é que o embrião se desenvolve em um ovo translúcido que possibilita a visualização das fases embrionárias, superando o problema da acessibilidade limitada de órgãos em outros organismos. A clareza óptica permite a observação, in vivo, de eventos fisiológicos ou patológicos durante o desenvolvimento do coração (Stainer et al., 1994). Como o desenvolvimento cardíaco embrionário é rápido é também rápido observar e quantificar a função cardíaca dos embriões (Narumanchi, et al., 2021). Um outro ponto importante relacionado ao zebrafish, é que o modelo possui um sistema cardíaco e vascular

relativamente simples, fácil de ser entendido, observado e estudado (Isogai et al., 2001). Além do que, é um dos modelos mais importantes para a biologia de desenvolvimento e regeneração do coração. Por estas facilidades de uso desse modelo biológico e pelo zebrafish possuir semelhanças genéticas com doenças humanas, ele vem sendo cada vez mais importante para estudos de doenças cardíacas humanas, na busca de novas terapias para a prevenção e tratamento da insuficiência cardíaca (Beffagna, 2019). As mudanças cardíacas são facilmente observadas por análises simples de microscópios, durante as primeiras etapas de vida deste bioindicador (Zhu et al., 2013; Ibrahim et al., 2020). O diuron em altas concetrações (2,50 mg/L e 10,00 mg/L) é capaz de diminuir a frequência cardíaca em peixe (Medaka japonês), após 7 e 13 dias de exposição (Ibrahim et al., 2020). Zhu e colaboradores (2013) também encontraram diminuição da frequência cardíaca de embriões (Gobiocypris rarus) expostos a altas concentrações (2.901, 4.138, 5.902, 8.418 e 12.000 mg/L) de DCA.

Por fim, o mal funcionamento cardíaco de embriões de zebrafish, principalmente durante o período embrio-larval, pode impedir o o indivíduo de chagar a fase adulta. Análises como essas, mostram as consequências da contaminação do ambiente aquático por praguicidas (Ibrahim et al., 2020). Por isso, é de extrema importância o desenvolvimento de estudos que visam avaliar as consequências diretas de contaminantes ambientais sobre o sistema cardíaco e vascular dos organismos endêmicos de locais impactados por esses compostos.

6 Justificativa

O uso de praguicidas está entre os principais instrumentos do modelo de agricultura. Na classe dos herbicidas, o diuron se destaca como um dos mais utilizados no território brasileiro, com cerca de 6,5 mil de toneladas aplicadas na agricultura, somente no ano de 2021 (IBAMA, 2023).

A problemática do diuron se estende além do herbicida, uma vez que, seus metabólitos DCA e DCPMU, também são persistentes no ambiente, potencialmente tóxicos e a legislação brasileira não prevê o monitoramento destes químicos nos compartimentos ambientais. Assim, o uso intensivo do diuron na agricultura, agregado aos seus próprios efeitos danosos, bem com de seus metabólitos, mostram a necessidade da continuidade de desenvolvimento de pesquisas que

identifiquem a toxicidade desse composto. Nesse cenário, análises modernas, como as que utilizam marcadores bioquímicos e comportamentais, se destacam pela sensibilidade de avaliar e identificar o perigo potencial de xenobióticos, além de permitir uma melhor compreensão do modo de ação e prevenir riscos de praguicidas amplamente utilizados na agricultura (Domingues et al., 2010).

Hoje, sabe-se que os animais aquáticos são fundamentais na identificação dos perigos ao meio ambiente e para avaliação do risco de agentes químicos contaminantes. Para Eissa (2010), análises em organismos aquáticos expostos a poluentes tem grande destaque para explorar os efeitos dos contaminantes e estressores ambientais, uma vez que o ambiente aquático é o principal destino desses produtos. Assim, o modelo escolhido, o peixe zebrafish, tem características adequadas para serem usados como organismo-teste de toxicidade (Nagel, 2002; Scholz et al., 2008). Outro fator de interesse ligado à escolha a do Danio rerio, é devido às implicações éticas, baseadas nos conceitos dos 3R's, que substituem os testes tradicionais para métodos alternativos na avaliação de substâncias químicas. E por fim, não existem estudos que avaliam o potencial tóxico do diuron, DCA e DCPMU sobre o aspecto bioquímico e comportamental, com concertações encontradas no meio ambiente, utilizando o modelo experimental zebrafish em sua fase embrio-larval.

7 Objetivos

Essa tese propõe uma avaliação dos possíveis danos induzidos pelos xenobióticos diuron, DCA e DCPMU em embriões e larvas de zebrafish. Para tanto, são apresentados os seguintes objetivos:

- Avaliar se as exposições ao diuron, DCA e DCMPU provocam alterações nos parâmetros bioquímicos de larvas de zebrafish, por meio de análises das atividades das enzimas acetilcolinesterase (AChE), catalase (CAT), glutationa-S-transferase (GST), lactato desidrogenase (LDH) e peroxidação lipídica (LPO);
- Avaliar se a exposições ao diuron, DCA e DCMPU causam mudanças de comportamento de larvas de zebrafish;

Avaliar se as exposições ao diuron, DCA e DCMPU causam alterações letais, subletais e teratogênicas em larvas de zebrafish;

- Avaliar se as exposições ao diuron, DCA e DCMPU alteram o comprimento e a frequência cardíaca de embriões e larvas de zebrafish;
- Avaliar se as exposições ao diuron, DCA e DCMPU provocam alterações histopatológicas no fígado, baço e cérebro em adultos de zebrafish;

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DIURON AND ITS METABOLITES INDUCE ALTERATIONS IN BIOCHEMICAL MARKERS AND BEHAVIOR OF ZEBRAFISH LARVAE

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ABSTRACT

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is an herbicide used in sugar cane cultivation. It is commonly found in aquatic ecosystem and is of high concern due to its ability to persist in the environment. Diuron metabolites include DCA (3,4-dichloroaniline) and DCPMU (3-(3,4-dichlorophenyl-1-methylurea). The objective of this study was to evaluate the effects of diuron and its metabolites in zebrafish (*Danio rerio*) larvae, from biochemical to individual level. Activities of the enzymes acetylcholinesterase (AChE), catalase (CAT), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and the levels of lipid peroxidation (LPO), swimming activity and body length were investigated after an exposure of 120h, and the heart rate was determined after 48h of exposure. The range of concentrations tested were: 0.003-3.000 mg/L diuron, 0.020-1.500 mg/L DCA and 0.020-2.100 mg/L DCPMU. Results showed that AChE activity was inhibited by diuron (3.000 mg/L) and DCPMU (0.129, 0.326, 0.828 mg/L). The swimming activity of fish larvae exposed to diuron (3.000 mg/L) was also impaired, which may be associated with AChE alterations. The CAT was induced by DCPMU and GST was induced by diuron. This suggests that CAT is acting to cope with oxidative stress induced by DCPMU and GST might have a role in the detoxification of diuron. In addition, larvae exposed to DCA (1.500 mg/L) had a reduction in their length and larvae exposed to diuron (3.000 mg/L), DCA (0.633 and 1.500 mg/L) and DCPMU (2.100 mg/L) presented bradycardia, suggesting cardiotoxicity. Overall, results indicate that diuron, DCA or DCPMU induce adverse effects during the early phases of zebrafish development, such as the impairment of neurotransmission and cardiovascular function and alterations in antioxidant enzymes and locomotor behavior. The findings indicate diuron as a more toxic compound to early stages of zebrafish than its metabolites, and it causes adverse effects at environmental relevant concentrations.

Keywords: *Danio rerio*; cardiotoxicity; fish embryo toxicity; neurotoxicity; oxidative stress; pesticides.

1. Introduction

Agriculture become, nowadays, one of the main activities responsible for the spreading of chemicals into the environment. Consequently, ecosystems and animals are permanently exposed to potentially harmful contaminants. Among pesticides used in agriculture and within the herbicides class, diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a compound widely used, mainly in sugarcane cultivation and has a wide spectrum of action (USEPA, 2003; APVMA, 2005). Diuron is absorbed by plant roots blocking the electron transfer to the photosystem II which inhibits weed photosynthesis and ensures higher agricultural yields (Wessels et al., 1959). Furthermore, it is known that this herbicide causes increased oxidative stress in non-target organisms by the increase of reactive oxygen species (ROS) production (Behrens et al, 2016). This herbicide has a slow degradation in soil (up to 230 days) (Hertfordshire, 2013) and a moderate solubility (42 mg/mL at 25 °C) allowing its leaching to aquatic bodies. Diuron can be degraded and biotransformed into metabolites such as DCA (3,4 dichloroaniline) and DCPMU (3-(3,4-dichlorophenyl-1-methylurea). which might be also toxic and consequently contaminate the ecosystem including the water, soil and the sediment (Gatidou and Thomadis, 2007). For these reasons, the potential harmful for non-target species and for the environment raise concern, requiring a range of studies to evaluate the toxic potential of these compounds.

Studies found diuron levels in surface water of about 6742 ng/L in the UK, 7800 ng/L in Brazil and 12 ng/L in USA (Thomas, 2002; Sapozhnikova et al., 2008; Saleh et al., 2016). In Brazil, a publication reporting pesticides in surface waters showed diuron in a range of 4 to 279 ng/L in a region of São Paulo with extensive sugarcane plantations (Acayaba et al., 2000). In a recent report, also in the region of São Paulo (Brazil), diuron was detected in surface waters in more than 91.9% of the samples analyzed and quantified in more than 66% of these samples (CETESB, 2021).

In previous studies with zebrafish early life stages, diuron at concentrations of 1, 2 and 3.8 mg/L caused changes on behavior (Velki et al., 2017) and exposure of 96h to diuron (0.1-5.0 mg/L) decreased survival, delayed hatching and increased malformations of zebrafish in a concentration-dependent manner (Zaluski et al., 2022). At biochemical level, an increase in glutathione (GSH) levels and a decrease in acetylcholinesterase (AChE) activity was observed in zebrafish larvae

exposed to diuron (Velki et al., 2019). In addition, diuron affected gene expression and activities of some of the cytochrome P450 (CYP) family enzymes in zebrafish larvae (Velki et al., 2019). In adult fish (*Sparus aurata*), diuron causes oxidative stress inducing increases of the reactive oxygen species (ROS) and changes in the ferric reducing antioxidant power (Sánchez-Muros et al., 2012). In adult Japanese medaka (*Oryzias javanicus*), diuron acts as an endocrine disruptor since exposures of 1-1000 µg/L during 21 days caused histopathological changes in fish gonads (ovaries and testes), (Kamarudin et al., 2020). Furthermore, diuron and its metabolites DCA, DCPMU and DCPU affected *Oreochromis niloticus* (Nile Tilapia) at concentrations of 10 ng/L – 200 ng/L. Results indicate that environmental concentrations of these contaminants caused significant changes in markers of oxidative stress and biotransformation enzymes, suggesting impairments on the physiological response related to biotransformation and antioxidant activity (Felício et al., 2018).

Despite the existence of several studies related with the effects of diuron, its toxicity for aquatic animals is not fully understood as well as the toxicity of its metabolites. In fact, which of these compounds (parent compound or metabolites) is more toxic is still not known (Gatidou and Thomadis, 2007; Boscolo Pereira et al., 2016; Felício et al., 2018). For instance, in the study performed by Felício et al. (2018), DCA metabolite was more toxic at higher concentrations, whereas diuron and DCPMU appeared to be harmful at low concentrations.

Although DCA is a well-studied compound and since it's the main degradation product of diuron and other substances, it is relevant to know if low and environmentally relevant concentrations affect the development and behavior of fish embryos and larvae, as well as the biochemical parameters. According to Primel et al (2007) this metabolite was found in the environment at concentrations up to 567 µg/L.

Due to ethical implications involving vertebrates for toxicological assessments, the use of embryos of aquatic organisms, including of fish, have become an important testing tool and a sensitive indicator of potential disturbance of aquatic environments (Kane at al., 2005; Sloman et al., 2006). The early life-stage test with *Danio rerio* has been established (Nagel, 2002). It emerged as a cost-efficient model widely used in scientific research, especially to be used in ecotoxicological assays which may support risk assessment of environmental contaminants (Choi et al., 2012).

Zebrafish is a sensitive model for ecotoxicological evaluations since it is easy to handle, has external fertilization and transparent eggs, which allows following the embryonic development (Osterauer, 2008).

The main objective of this study was to evaluate the effects of diuron and its metabolites, DCA and DCPMU, in the experimental model zebrafish in early life stages. The endpoints assessed ranged from biochemical to individual level and will be used to compare the toxicity of the parent compound and its metabolites. These endpoints included neurotoxicity, oxidative stress, embryonic development, heart rate and behavior.

2. Material and Methods

2.1. Chemicals

All the chemicals used, except the Bradford reagent, were purchased from Sigma-Aldrich Chemical Corp (St Louis, MO), including Diuron [3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea] (CAS no. 30-54-1; 98.0%), DCA [3,4-dichlorianiline] (CAS no. 95-76-1; 98.0%), DCPMU [1-(3,4-Dichlorophenyl)-3-methylurea] (CAS no. 3567-62-2; 99.9%) and DMSO [dimethyl sulfoxide] (purity, >99.7%, CAS no. 67-68-5). The Bradford reagent was purchased from Bio-Rad (Germany). While diuron stock solution was prepared in DMSO (10 mg diuron per ml DMSO), DCA and DCPMU were prepared in autoclaved water from zebrafish culture. Working solutions at different concentrations were prepared by appropriate dilution of the stock solutions in autoclaved water from zebrafish culture.

2.2. Fish husbandry and embryo collection

The zebrafish facility at Department of Biology, University of Aveiro, provided the eggs for all bioassays. At the facility, adults of zebrafish (*Danio rerio* Hamilton-Buchanan, 1822, ABwt lineage) were maintained in a flow-through water system (ZebTEC, Tecniplast®). Culture water was obtained through reverse osmosis and activated carbon filtration of tap water, complemented with salt (Instant Ocean® Sea salt, Spectrum Brands). Conductivity was kept at 800 ± 50 µS/cm, saturation of dissolved oxygen at 95% and pH at 7.5 ± 0.5. Zebrafish were maintained at 27.0 ± 1°C under a

light/dark photoperiod (12h:12h) and fed daily with commercially available artificial diet.

At the day before spawning, female and male zebrafish were placed in spawning tanks late in the afternoon. Spawning occurs in the following morning when lights turn on. Embryos were collected approximately 50 min later, rinsed and maintained in autoclaved water from zebrafish culture. Injured and unfertilized eggs were removed under a stereomicroscope (Stereoscopic Zoom Microscope - SMZ 1500, Nikon Corporation). All bioassays started in the first 3 hours post-fertilization (hpf).

2.3. Fish embryo exposure assays

Exposure of zebrafish eggs to increasing concentrations of diuron, DCA or DCPMU was conducted in accordance with the fish embryo acute toxicity test (FET) guideline 236 (OECD, 2013) with some alterations. The concentrations tested were based on environmental concentrations and on the lethal concentration of 10% (LC_{10}) found in the literature (Loss et al., 2013; Primel et al., 2007; CETESB, 2021). Indeed, for *Danio rerio*, the established LC_{50} value for 96h of exposure to diuron is 6.3 mg/L and the LC_{10} is 3.7 mg/L (Velki et al., 2017). The LC_{50} values range from 2.4 – 2.7 mg/L (Busquet et al., 2014) and LC_{10} of 1.6 mg/L (Von Hellfeld et al., 2020) for 96h exposures to DCA. Data on LC_{10} and LC_{50} values of DCPMU for zebrafish were not found in the literature. The DCPU metabolite was not evaluated since the molecule is no longer available for purchase, making its evaluation impossible. Thus, the concentrations tested were 0.003, 0.012, 0.048, 0.189, 0.754 and 3.000 mg/L for diuron, 0.020, 0.112, 0.633, 0.047, 0.267 and 1.500 mg/L for DCA and 0.020, 0.051, 0.129, 0.326, 0.828 and 2.1 mg/L for DCPMU. A negative control (NC) (flow-through system autoclaved water) was used in all bioassays. In addition, in the diuron bioassay a solvent control (SC) was used (0.01% of DMSO, the same concentration as used in all treatments with diuron,). For each treatment, six replicates (N=6) were performed by placing 13 eggs in glass petri-dishes filled with 15 ml of the respective testing solution. Test solutions were renewed at 48h to avoid depletion of chemical concentration. All tests were performed during 120h in a temperature controlled room (27 ± 2 °C) with a photoperiod of 12h:12h light:dark. Every day, mortality, hatched embryos and malformations were checked under the stereomicroscope and dead embryos were removed. Normal

morphological development was checked following zebrafish stages (Kimmel et al., 1995). Heart rate of embryos was determined at 48h of exposure in 6 organisms per treatment (N=6, one embryo per replicate). The heartbeats were counted under a stereomicroscope for 15 seconds, in triplicate in each organism and the heart rate (beats per minute) was calculated.

At the end of exposure, three organisms of each replicate were used for behavior analysis (3 organisms per each of the 6 replicates, N=6) and the remaining (8-10 organisms per replicate, N=6) were pooled, snap frozen in liquid nitrogen and stored at -80°C until further biochemical analysis. At the end of behavior analysis, the larvae were anesthetized with tricanine (MS-222, Sigma, USA) and used to measure the total length of the zebrafish larvae (3 organisms per each of the 6 replicates, N=6).

2.4 Behavior analysis

The behavior of organisms exposed to Diuron, DCA and DCPMU was assessed using Zebrabox® (Viewpoint, FR). At the end of exposure, three zebrafish larvae per replicate were placed individually in 24-well plates filled with 1 ml of water from zebrafish culture. The larvae selected for behavior analysis were chosen randomly, ensuring the absence of malformations. Larvae from 1.5 mg/L DCA exposed group, were not considered in the behavior analysis due to the presence of a high percentage of malformations. An infra-red light, not perceived by the larvae (constant at 2.3 mW/cm^2) was used for video recording purposes. Zebrabox white light was set at an intensity of 10% (0.26 mW/cm^2) and used during light periods. Behavior was assessed during alternating dark/light periods of 3 min after an initial 7 min acclimation period in dark conditions. The duration of swimming (seconds) and swimming distance (mm) along behavior assessment is automatically recorded by the ZebraLab® Software (Viewpoint, FR) in integration periods of 1 min, which allowed to determine the total swimming distance and total swimming time (Franco et al., 2019).

2.5 Biochemical analysis

Pooled embryos were homogenized by sonication in potassium phosphate buffer (0.1 M; pH 7.4) using a ratio of 100 µl of buffer per larvae. From each replicate, two aliquots were taken from

the homogenate: 50 µl for protein quantification in the homogenate and 200 µl for determination of lipid peroxidation (LPO) adding 4 µl of 4% 2,6-di-tert-butyl-4-methylphenol to avoid further oxidation of lipids. The remaining homogenate was centrifuged (10.000 *g*, 20 min, 4 °C) to obtain the post-mitochondrial supernatant (PMS) which was kept at −80 °C until further evaluation of enzymatic activities and protein concentration in PMS fraction. The PMS was used to determine the enzymatic activities of catalase (CAT), glutathione-S-transferase (GST), acetylcholinesterase (AChE) and lactate dehydrogenase (LDH).

All spectrophotometric determinations were performed in a microplate reader (Multiskan Spectrum, Thermo Scientific). The concentration of protein in both homogenate and PMS fractions was determined in triplicate by the Bradford method, adapted to microplate and at a wavelength of 595 nm (Bradford, 1976). While protein concentration in the homogenate was used to normalize the LPO levels, the protein concentration of PMS fraction was used to normalize the activity of the different enzymes.

The AChE was determined according to the method of Ellman et al. (1961) adapted to microplate by Guilhermino et al. (1996). Acetylthiocholine was used as substrate. Conjugation of DTNB with thiocholine was followed through the increase of absorbance at 414 nm for 5 min. AChE activity is expressed as nmol of substrate hydrolysed per min per mg of protein ($\varepsilon = 13.6 \times 10^3$ $M^{-1}.cm^{-1}$).

The activity of CAT was determined using a protocol established by Clairborne (1985), which measures H_2O_2 consumption by following the decrease in absorbance at 240 nm at 25 °C. CAT activity is expressed as µmol of substrate hydrolysed per minute per mg of protein ($\varepsilon = 4.0$ $M^{-1}.cm^{-1}$).

The GST activity was measured following the methodology of Habig and Jacoby (1981) adapted to microplate by Frasco and Guilhermino (2002). For the enzymatic measurement, conjugation of GSH and CDNB (1-chloro-2,4-dinitrobenzene) was followed at 340 nm. Enzymatic activity is expressed as nmol of substrate conjugated per minute per mg of protein ($\varepsilon = 9.6 \times 10^3$ $M^{-1}.cm^{-1}$).

The activity of LDH was measured following oxidation of NADH when pyruvate is

converted to lactate at 340 nm, as described by Vassault (1983) and adapted to microplate by Diamantino et al. (2001). Activity of LDH is expressed as μmol of substrate hydrolysed per minute per mg of protein (ε =6.3 × 10^3 M^{-1}.cm^{-1}).

The levels of LPO were measured using a protocol established by Ohkawa et al. (1979). To each sample homogenate, 100 μl of 100% trichloroacetic acid (TCA) and 1 ml of 0.73% thiobarbituric acid (TBA) (in 60 mM Tris-HCl; 0.1 mM diethylene triamine pentaacetic acid (DTPA); pH 7.4) was added. Then, the samples were vortexed and placed in oven at 100 °C during 1h. Samples were then centrifuged at 14000 g for 5 min and supernatant was used to measure thiobarbituric acid reactive substances (TBARS) at 535 nm and 25 °C. LPO is expressed as nmol TBARS per mg of protein (ε = 1.56 × 10^5.M^{-1}).

2.6 Statistical analysis

Student t-test was used to verify differences between negative and solvent control in all parameters assessed for the bioassay with diuron. As no differences were observed between negative and solvent control, solvent control was used to perform further statistical analysis on diuron assay. To verify differences between the respective controls and treatments, datasets from diuron and the 2 metabolites were analysed using One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test, when the criteria of normality and homocedasticity were satisfied. In remaining cases, non-parametrical Kruskal-Wallis test was used. For all statistical tests the significance level was set at $p<0.05$. Data was analysed and plotted in GraphPad Prism (version 2.0, GraphPad Software Inc., San Diego, CA).

3. RESULTS

3.1 Mortality, malformations and larvae length

At the end of the assays, in the zebrafish control groups, mortality remained below 2% and no malformations were observed, thus satisfying the test validation criteria specified in the FET guideline (OECD, 2013). As expected, the fish groups exposed to diuron and its metabolites at the chosen sub-lethal concentrations exhibited a mortality rate below 8%. In general, malformations

were less than 3% in diuron-exposed groups, less than 2% in the groups exposed to 0.020 mg/L – 0.112 mg/L DCA, and less than 11% in the group exposed to 0.633 mg/L DCA. In the group exposed to 1.500 mg/L of DCA, malformations reached 95%, therefore animals from this treatment were not considered for behavior and biochemical analysis. There were no malformations observed in the zebrafish exposed to DCPMU.

The growth in terms of total body length was measured at 120 h of exposure. The increasing concentrations of diuron and DCPMU tested in the bioassays with zebrafish did not significantly altered the total length of the zebrafish larvae (Fig. 1, $p>0.05$). Total body length of larvae from the control group of the assay with DCA was 4.21 ± 0.1 mm (mean ± SD). Larvae exposed to 1.500 mg/L DCA showed a total length of 3.99 ± 0.06 mm (Fig. 1), which was significantly lower than the total length of larvae from control group ($p<0.05$). This represents a reduction of 5.22% of total body length in embryos exposed to DCA (1.500 mg/L) in comparison with the larvae from control group.

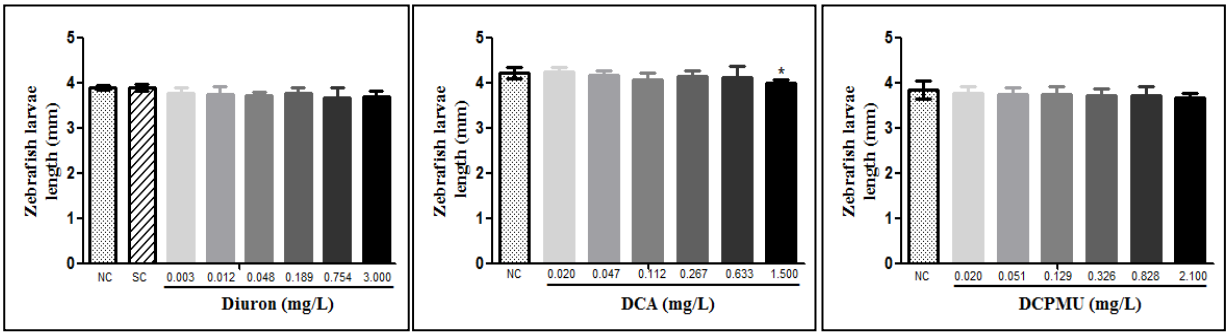

Figure 1. Length of *Danio rerio* larvae after being exposed to sub-lethal concentrations of 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (Diuron), 3,4-dichloroaniline (DCA) and 1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU) for 120h. Bars represent the mean and error bars represent standard deviation. NC correspond to negative control and SC to solvent control. * represents the significant differences from the respective control (* $p< 0.05$, Dunnetts' Test).

3.2 Embryo heart rate

The heart rate in zebrafish embryos from control groups and groups exposed during 48h to diuron, DCA and DCPMU are presented in Figure 2. Data shows that, compared to the respective control groups, embryos exposed to diuron (3.000 mg/L), DCA (0.633 and 1.500 mg/L) and DCPMU (2.100 mg/L) presented a significant decrease in the heart beats per minute ($p<0.05$).

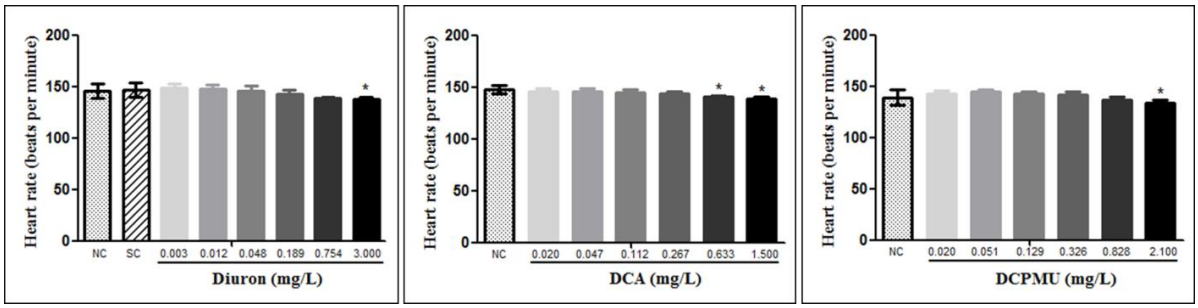

Figure 2. Heart rate (beats per minute) of *Danio rerio* embryo after 48h of exposure to sub-lethal concentrations of 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (Diuron), 3,4-dichloroaniline (DCA) and 1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU). Bars represent the mean and error bars the standard deviation. NC correspond to negative control and SC to solvent control. * represents the significant differences from the respective control (* $p < 0.05$, Dunnetts' Test).

3.3 Zebrafish larvae behavior

Exposed zebrafish larvae were subjected to alternating dark/light periods of 3 min after an initial acclimation period of 7 min in dark conditions to assess locomotor activity in all bioassays. When analyzing the total swimming distance and total duration of swimming (Figure 3), fish exposed to the highest concentration of diuron (3.000 mg/L) presented significantly lower swimming distances and shorter durations of swimming when comparing to respective control larvae ($p<0.05$).

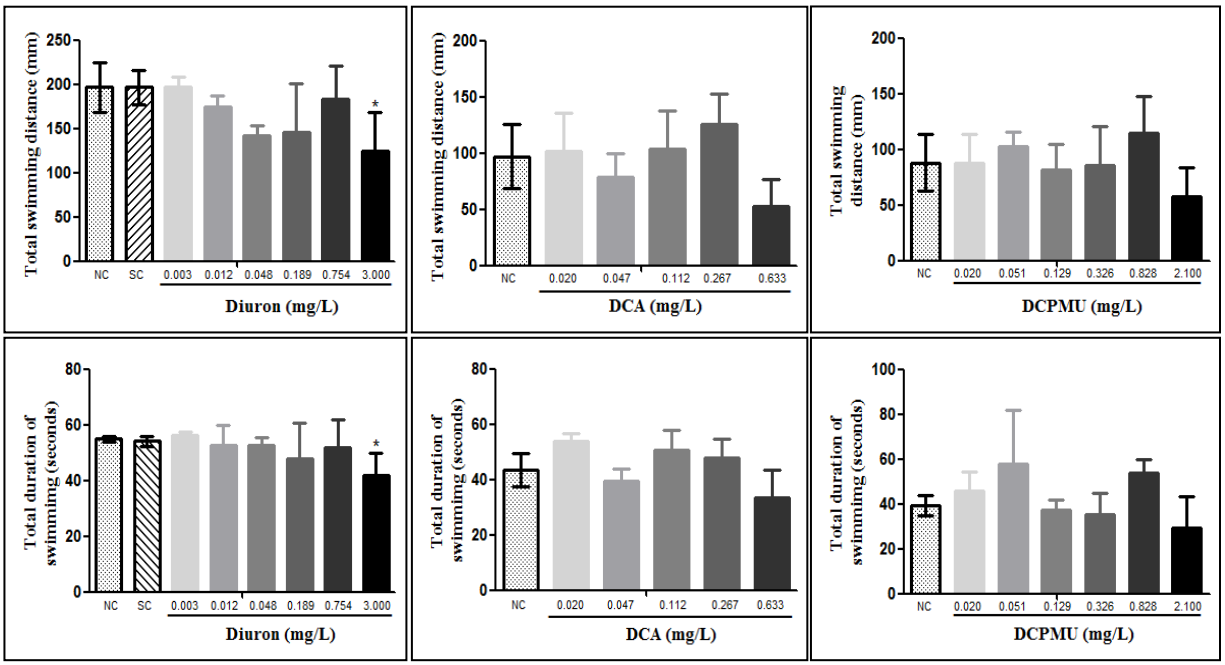

Figure 3. Total swimming distance and total duration of swimming of *Danio rerio* larvae after being exposed 120h to sub-lethal concentrations of 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (Diuron), 3,4-dichloroaniline (DCA) and 1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU). Bars represent the mean and error bars the standard deviation. NC correspond to negative control and SC to solvent control. * represent significant differences from the respective control (* $p<0.05$, *** $p<0.001$, Dunnetts' Test, respectively).

3.4 Biochemical markers in zebrafish larvae

In the Figure 4 are presented the results of the biochemical parameters analyzed after 120h of exposure to diuron, DCA and DCPMU. The AChE activity was significantly decreased ($p<0.05$) in larvae exposed to diuron (3.000 mg/L) and DCPMU (0.129, 0.326 and 0.828 mg/L) when compared to the activity observed in the control group. However, fish exposed to 0.112, 0.267 and 0.633mg/L of DCA, presented a significantly higher AChE activity ($p<0.05$).

In general, a significant decrease ($p<0.05$) was observed in CAT activity with increasing concentrations of diuron and DCA. On the other hand, CAT activity was significantly higher ($p<0.05$) in larvae exposed to increased DCPMU concentrations concerning exposure to DCA, the CAT activity in larvae presented a bell-shaped concentration-response curve; while intermediate concentrations 0.112 and 0.267 mg/L of DCA presented a significantly increase in CAT activity ($p<0.05$).

Diuron significantly increased GST activity ($p<0.05$) in zebrafish larvae exposed to almost all concentrations tested. Despite not significant ($p>0.05$), the activity of GST observed in larvae

exposed to the other metabolite, DCPMU, were slightly higher than GST activity in larvae from control group. LPO levels (data not shown) in the larvae exposed to diuron or its metabolites (DCA and DCPMU) were similar to the observed in larvae from the respective control group ($p>0.05$).

The LDH activity in exposed larvae was not altered by diuron nor by DCPMU, but in general, presented higher levels when exposed to DCA. The larvae exposed to 0.112 mg/L of DCA treatment presented significant higher levels ($p<0.05$) than control larvae.

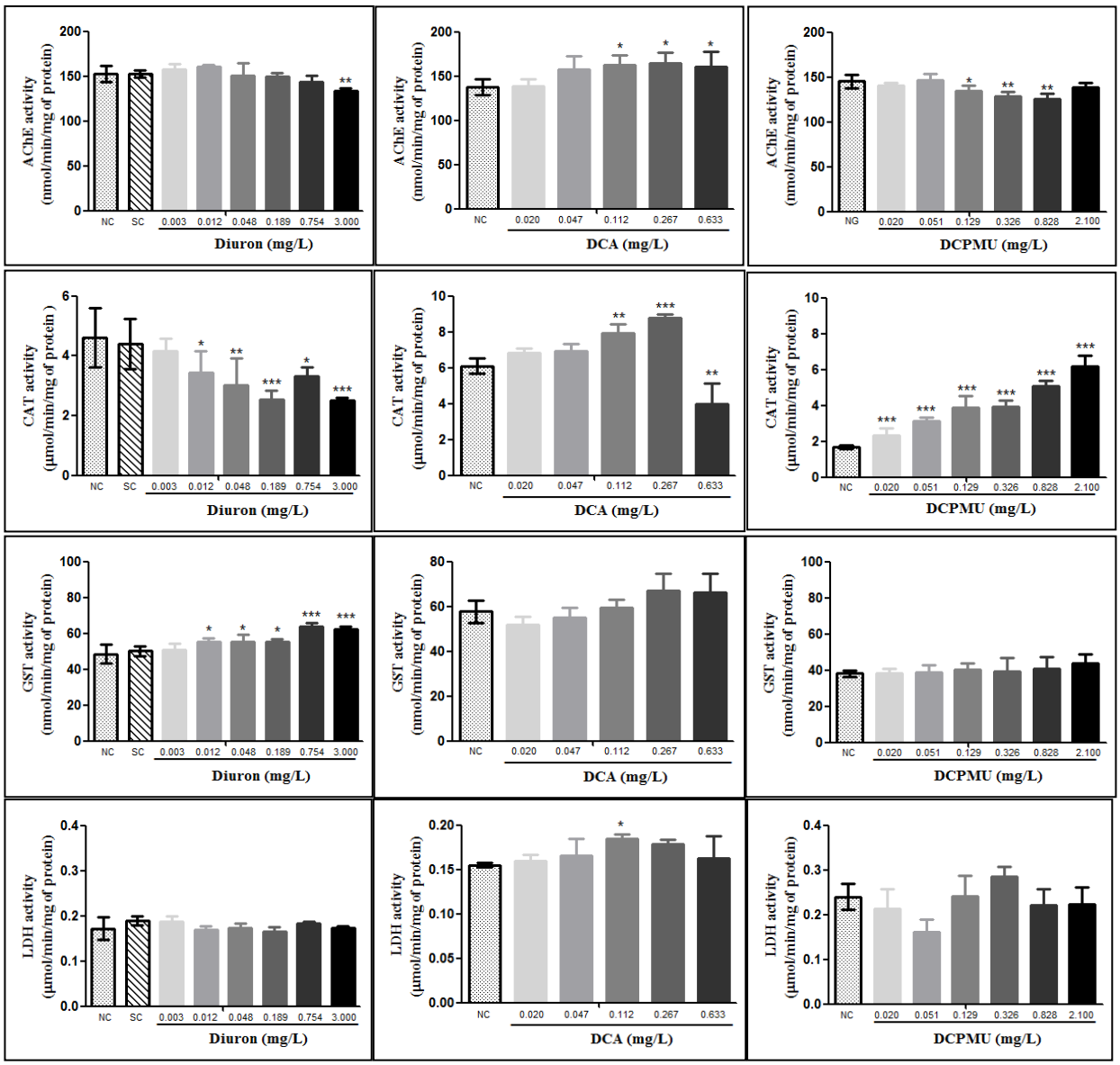

Figure 4. Activity of the enzymes acetylcholinesterase (AChE), catalase (CAT), glutathione-S-transferase (GST) and lactate dehydrogenase (LDH) in *Danio rerio* larvae after being exposed during 120h to sub-lethal concentrations of 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea (Diuron), 3,4-dichloroaniline (DCA) and 1-(3,4-Dichlorophenyl)-3-methylurea (DCPMU). Bars represent the mean and error bars the standard deviation. NC correspond to negative control and SC to solvent control. *, **, *** represent significant differences from the respective control (* $p<0.05$, ** $p< 0.01$, *** $p< 0.001$, Dunnetts' Test, respectively).

4. Discussion

In order to determine whether early life stages of zebrafish are affected by diuron, DCA and DCPMU, individual organism parameters such as, total larvae length, heart rate and behavior were analysed. Among the biochemical markers widely used in ecotoxicological evaluation, this study focus on the occurrence of responses of the enzymes related with neurotransmission and muscle development (AChE), with oxidative stress and detoxification of xenobiotics (CAT, GST), oxidative damage on lipids (LPO), and with enzymes related to anaerobic energy production (LDH) (Howcroft et al., 2010; Monteiro et al., 2006).

The growth of zebrafish was not affected by diuron or DCPMU, it was only affected by DCA (1.5 mg/L). Similarly, Zhu et al. (2013) observed a decrease in total body length of rare minnow larvae (*Gobiocypris rarus*) exposed during 72h to DCA concentrations above 2.9 mg/L, reaching a reduction of 77.5% in larvae exposed to the highest concentration (12 mg/L DCA). These authors explained this reduction based on the increase in the malformations percentage, which are in good agreement with our results, since in the present study the larvae exposed to 1.5 mg/L also presented a high level of malformations.

To determine if cardiovascular function at the larval stage of zebrafish is affected by diuron, DCA, and DCPMU, heart rate was used as an endpoint. Regarding the physiological outcome of heart rate, diuron (at 3.0 mg/L), DCA (0.633 and 1.5 mg/L) and DCPMU (2.1 mg/L) caused bradycardia. In another fish species (*Japanese Medaka*) a decrease in heart rate was also observed after 7 and 13 days of exposure, at concentrations of 2.5 mg/L and 10.0 mg/L of diuron, respectively (Ibrahim et al., 2020). In agreement with our study, Zhu et al. (2013) also found a decrease in the heart rate in fish rare minnow (*Gobiocypris rarus*) embryos and larvae exposed for 72h to DCA at concentrations above 2.901 and 1.223 mg/L DCA, respectively. Optimal cardiac functioning is important for embryonic development, as malfunction during this period might lead to mortality or severe impairment in adulthood (Ahmed and Delgado-Olguin., 2019; Ibrahim et al., 2020).

At sub-cellular level, different pesticides have been shown to alter the activity of several enzymes

related with neurotransmission and metabolic processes and to induce oxidative stress. It was demonstrated that pesticides, can inhibit AChE activity in different fish species, including in zebrafish (Pereira et al., 2012; Quintaneiro et al, 2017; Gaaied et al, 2019). The present study showed alterations of AChE activity in larvae exposed to all compounds, suggesting a neurotoxic effect of diuron and its metabolites for zebrafish early life stages. These alterations were compound dependent. In fact, while higher concentrations of diuron and DCPMU inhibited AChE activity in zebrafish larvae, DCA induced the activity of this enzyme. Velki and collaborators (2019) did not observe alterations on AChE activity of zebrafish larvae exposed to diuron, despite having observed an inhibition on *in vitro* testing. However, several authors reported an AChE inhibition in different fish species with exposure to diuron. For instance, Bretaud et al., (2000) observed an inhibition in the brain of juvenile goldfish (*Carassius auratus*) exposed to 500 µg/L of diuron for 6-48h, El-Nahhal, (2018) also reported an inhibition in the brain and blood serum of the fish larvae of Nile tilapias (*Oreochromis niloticus*). It is known that inhibitions of the AChE activity led to an increase of acetylcholine in the central nervous system and muscular junctions causing cholinergic toxicity, which can result on impairments of muscle contractions due to the overstimulation of the parasympathetic system (Lott and Jones, 2022). Impairments on muscular contractions might result on alterations of the behavior of the organisms and locomotor activity, which might compromise search for shelter and food and consequently survival (Lovern et al., 2007). Indeed, in the present study, diuron affected behavior by decreasing the total swimming distance and duration at same concentration (3.000 mg/L) where inhibition of AChE was observed. These results suggest that the inhibition of AChE, an enzyme involved with neural and muscular functions of the organism, might be related to the changes observed on the behavior pattern of zebrafish larvae exposed to diuron. In good agreement with the present study, Zaluski and collaborators (2022) reported a decrease of the time spent swimming of zebrafish larvae exposed to diuron 0.1 mg/L, despite no changes were observed on the distance swimming. Furthermore, Velki et al. (2017) also found similar alterations on behavioral parameters of embryo and larvae exposed to diuron during 24h, such as a decrease of the embryo spontaneous coiling movements and a reduction of thigmotaxis in larvae. Still, according to the same authors,

diuron affects neurotransmission and consequently, decrease the rate of spontaneous movements (Velki et al., 2017). However, the mechanisms involved in diuron altering behavior are still unknown (Velki, et al 2017). The possible hypothesis, according to the authors, is that diuron affects glycinergic neurotransmission and, consequently, decreases the rate of spontaneous movements of zebrafish embryos (Velki et al., 2017).

Concerning DCPMU, despite the inhibition of AChE activity induced by the exposure to 0.129, 0.326 and 0.828 mg/L no alterations on locomotor behavior of the larvae was verified.
The diuron metabolite, DCA is known to act as a non-specific membrane irritant or metabolic inhibitor (Scheil et al., 2008) and only a limited number of studies described the effects of 3,4-DCA on animal behavior. Its mechanism of action does not typically involve AChE inhibition (e.g. Monteiro et al., 2006). The induction of AChE observed with the exposure to DCA, low in percentage as compared to control, did not induce alterations in the behavior of larvae. The AChE induction is not a typical response to a toxicant and might be related with DCA apoptosis induction. Apoptosis has been pointed out to upregulate AChE genes (e.g. Zhang et al., 2002) and as a mechanistic explanation for AChE induction (e.g. Andrade et al., 2016).

Aquatic contaminants, such as pesticides have been associated with an increase in reactive oxygen species (ROS) in organisms (Sobrino-Figueroa 2013). Among the detoxification biomarkers, antioxidant enzymes are responsible for the body's defense against ROS. More specifically, CAT degrades hydrogen peroxide into water (Valavanidis et al., 2006, Nandi et al., 2019). In the present study, diuron inhibited CAT activity in a dose dependent trend. The inhibition of CAT activity can be caused by an excess of ROS in the organism, which might result from alterations in enzyme synthesis, inactivation or assembly of its subunits (Wang et al, 2016) and might cause oxidative damage. Furthermore, while CAT inhibition observed in larvae exposed even to low concentrations of diuron (LOEC of 12.0 μg/L) suggests an impairment of normal ROS detoxification, no oxidative damage on lipids was observed since the levels of LPO were similar to the ones observed in control larvae. In another work with zebrafish larvae, diuron (1-10 mg/L) did not affect CAT expression in embryos and larvae zebrafish during 1 or 2h (Velki et al., 2019). In contrast with our results Kao et al., (2019), found up-regulated levels of

antioxidant proteins, including CAT, in zebrafish embryos exposed to similar concentrations of diuron than those used in the present study. However, the exposure to diuron was shorter, up to 48h, which might have allowed the embryos to respond and cope with the oxidative stress induced. On the other hand, DCPMU induced CAT activity in larvae exposed during 120h, which suggest the activation of the antioxidant system to protect cells from oxidative stress. The induction of CAT might occur to eliminate ROS from cells, namely H_2O_2, and counteract the excess of its production in zebrafish larvae due to the exposure to DCPMU. This response might result in an adaptive mechanism to allow the zebrafish larvae to tolerate DCPMU by decreasing ROS and preventing the occurrence of oxidative damage. Indeed, the outcome of oxidative damage on lipids, LPO, was not altered by the exposure to DCPMU. Concerning DCA, this diuron metabolite promoted a bell-shaped response in CAT enzymatic activity of zebrafish larvae. At the lower concentrations of DCA, CAT was induced to cope with enhanced ROS levels by scavenging H_2O_2; whereas at the higher concentrations the CAT was inhibited compromising the detoxification of ROS. The bell-shaped trend observed in CAT activity is a typical response of antioxidant enzymes in organisms exposed to contaminants, including pesticides (e.g. Bellas et al., 2022). In a study with female fish of *Chapalichthys pardalis* exposed to 0.5 mg/L DCA for presented a decrease on CAT activity in liver (Carbajal-Hernández et al., 2017). In other study with the Nile tilapia (*Oreochromis niloticus*), 0.040 µg/L of diuron and its metabolites, DCA and DCPMU induced a decrease in the CAT activity in gills and an increase with 0.20 µg/L DCA in the liver (Felício et al., 2018).

The GST is an enzyme involved in the biotransformation of xenobiotics, including pesticides to promote their detoxification. GST acts on the first cellular responses to the entry of contaminants (Iyer, 2002; Li, 2009) and is used as a stress bioindicator (Van der Oost and Vermeulen, 2003). In this study, an induction of GST in fish exposed to diuron and DCA was observed, suggesting that GST activates the phase II of biotransformation to detoxify mainly of the herbicide diuron and its metabolite. Furthermore, GST also play an indirect role as an antioxidant enzyme to maintain the redox status of the organism (Van der Oost and Vermeulen, 2003). In fact, in a study evaluating the effects of diuron, DCA and DCPMU in the terrestrial

nematode *Caenorhabditis elegans*, GSH levels significantly increased with diuron and DCA (Lima et al., 2022). The authors suggested that the increase observed likely reflects an attempt to restore the organisms' redox status. In other hand, a decrease in GST activity was reported in the gills of adult fish (*Oreochromis niloticus)* exposed to DCA and DCPMU for seven days at concentrations of 40 and 200 ng/L (Felico et al., 2018). In another study, regarding DCA effects on the fish *Pomatoschistus microps*, significant decreases on GST activity were also found (Monteiro et al., 2006). In accordance with the results of the present study, Park et al. (2020) also found an induction of GST activity in zebrafish larvae exposed to DCA. Decrease of the antioxidant enzymes activity might indicate the compromising of the antioxidant system, while their increase is a positive response to cope with ROS production (Regoli and Giuliani, 2014). Results of the present study suggest that probably the detoxification of diuron is happening by GST, which may be preventing the eventual formation of ROS explaining the non-effect observed in LPO levels, since CAT was inhibited by diuron exposure and not contributing to ROS detoxification. In the other hand, the induction observed in CAT activity of fish exposed to intermediate concentrations of DCA and to DCPMU suggest that the antioxidant system was activated to cope with these diuron metabolites.

The LPO is an indicator of oxidative damage on lipids induced by ROS that are not cleared by the antioxidant stress system (Van der Oost and Vermeulen, 2003). In fish, some studies have shown that heavy metals such as mercury, insecticides, and ethanol led to an increase of LPO levels (Vieira et al., 2009; Xing et al., 2012). In the study of Felício et al. (2018) with Nile tilapia exposed to diuron, DCA, DCPU and DCMPU at concentrations of 40 and 200 ng/L, authors found an increase of LPO levels in the gills after exposure to all compounds, indicating that metabolites and diuron can induce oxidative damage in lipids. Another study with Nile tilapia exposed to a mixture of the pesticides diuron and hexazinone at concentrations of 125 to 500 µg/L for 72 h, no alterations on LPO levels were observed (Franco-Bernardes et al., 2015). Considering our results, nor the exposure to diuron or its metabolites led to oxidative damage of lipids, suggesting that the oxidative apparatus was able to cope with the oxidative stress induced by these xenobiotics.

The LDH is an essential enzyme in cell metabolism, participates in the process of transforming glucose into energy in cells through the anaerobic pathway (de Coen et al., 2001). It has great relevance in conditions of stress induced by environmental contaminants when energy may be needed in a short period of time (Monteiro et al., 2006). Thus, changes in LDH activity are used as an indicator of exposure to chemicals (de Coen et al., 2001). In the present study no significant alterations were found on the LDH activity in zebrafish embryos, suggesting that the induced stress by diuron, DCA and DCPMU did not led to an increase in energy production through the anaerobic metabolism. In good agreement with found results, in a study with DCA (0.50–1.49 mg/L) for 96h using the estuarine fish, *Pomatoschistus microps*, the authors suggested that the anaerobic pathway of energy production was not used to cope with eventual increase in energetic needs, since no alterations were observed on LDH activity (Monteiro et al., 2006). However, in another study using the *Daphnia magna* model, DCA at a concentration of 10 µg/L for 21 days increased LDH activity suggesting that the alterations induced in the LDH may reflect possible changes in overall cellular metabolism and energy production (Guilhermino et al., 1994).

In summary, results suggest that diuron, DCA and DCPMU exert adverse effects on zebrafish early life stages at environmental concentrations, either at individual or biochemical level. At individual level, all the compounds affected the cardiovascular system by inducing bradycardia and the diuron affected behavior by reducing locomotor activity. The most toxic compound at individual level seems to be the DCA since the lower LOEC observed was 0.633 mg/L of DCA for the heartrate parameter. At biochemical level, the three compounds affected the AChE activity suggesting impairments in neurotransmission. Furthermore, diuron and/or its metabolites induced the activity of CAT (DCA and DCPMU) and GST (diuron and DCA) suggesting the activation of the antioxidant system and promoting detoxification processes. The most toxic regarding the biochemical level seems to be the diuron with a LOEC of 0.012 mg/L (GST and CAT activity). Overall, these results suggest that diuron and its degradation products, DCA and DCPMU, may be substances of concern in ecosystems that receive agriculture effluents due to its potential adverse effects on zebrafish larvae.

5. Acknowledgments

The work of BCPS was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES – prInt Programa Institucional de Internacionalização) Processo 88887.569799/2020-00. Thanks are due to Foundation for Science and Technology and Ministério da Ciência, Tecnologia e do Ensino Superior (FCT/MCTES) for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020+LA/P/0094/2020). The work of CQ is funded by national funds (OE), through FCT in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decret-Law 57/2016, of August 29, changed by Law 57/2017, of July 19, with doi: 10.54499/DL57/2016/CP1482/CT0160.

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Multi-parameter analysis of Diuron and its Metabolites (DCA and DCPMU) in different stages of Zebrafish (*Danio rerio*)

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ABSTRACT

Diuron (3-(3,4-Dichlorophenyl)-1,1-dimethylurea), a prominent herbicide in Brazil's agricultural landscape, particularly in sugarcane farming, has raised concerns due to its persistent presence in the environment and the formation of potentially toxic metabolites, such as 3,4-dichloroaniline (DCA) and 3-(3,4-dichlorophenyl)-1-methylurea (DCPMU). These concerns encompass both environmental contamination and human health risks. Taking advantage with the genetic and metabolic similarities between zebrafish (*Danio rerio*) and humans, this study conducts a multiparameter evaluation of Diuron and its metabolites at environmentally relevant concentrations ranging from 0.5 to 100.0 µM in Brazil, developing country. For the embryonic-larval phase, the OECD No. 236 protocol for the Fish Embryo Toxicity (FET) test was employed, with an extension to 144 hours post-fertilization (hpf). Oxygen consumption within 24 hpf embryos following immediate exposure was quantified. Enzyme activity of acetylcholinesterase (AChE) was assessed in embryos exposed for 96 hpf, alongside genotoxic analysis using the Comet Assay. Adult zebrafish underwent acute exposure following the OECD No. 203 protocol for the Fish Acute Toxicity Test, spanning 96 hours. Evaluation of mutagenicity through the Micronucleus Test (MN) was conducted, supplemented by examinations of cytotoxic and genotoxic effects, including epigenetic considerations through Nuclear Abnormalities (NA) in erythrocytes, substantiated by histopathological investigations. Notably, 10.0 µM DCA alters AChE enzyme activity, suggesting neuromuscular toxicity. Diuron exposure triggers DNA damage across all tested concentrations, with nuclear abnormalities becoming prominent beyond 10.0 µM. DCA exhibits genotoxic potential within the 0.5-5.0 µmol/L, inducing nuclear pleomorphism at 5.0-10.0 µM concentrations. DCPMU induces DNA damage across all tested concentrations, accompanied by nuclear abnormalities within the 1.0-10.0 µM range. Adult zebrafish subjected to Diuron, DCA and DCPMU exposure exhibit no significant organ alterations. Erythrocyte analysis reveals no mutagenicity. Although non-mutagenic genotoxicity has been observed, chromosomal instability is of concern. This study significantly advances our understanding of the implications of Diuron and its metabolites on zebrafish and underscores the necessity of multiparametric evaluation in elucidating herbicide and metabolite impacts on environmental and human health. This endeavor informs regulatory measures for the safe use of Diuron and similar herbicides, with substantial implications for developing nations grappling with escalating water contamination due to pesticide use.

Keywords: Embryotoxicity, Genotoxicity, Mutagenicity, Acetylcholinesterase Activity**.**

1. INTRODUCTION

Pesticides are utilized in agricultural production to manage, prevent, and eliminate pests, thereby increasing quality and productivity (Atwood and Paisley-Jones, 2017). Brazil, a developing country that relies heavily on agricultural exports, is a major consumer of pesticides, accounting for 20% of the global market (equivalent to approximately US$ 10 billion per year) (Barizon et al., 2020). Diuron is a systemic herbicide from the phenylurea group that is commonly used on large plantations of sugar cane, soybeans, corn, eucalyptus, and various fruit crops such as bananas, pineapples, and grapes. It is also applied in urban areas (EFSA, 2005).

Diuron is used in both the pre-emergent and post-emergent phases, inhibiting the photolysis of water and subsequently preventing photosynthesis (Field et al., 2003), thereby regulating weed growth. However, the worldwide application of this herbicide is accompanied by environmental consequences due to its moderate to high permanence in sediments, soils, and surface waters (Field et al., 2003). The herbicide has been identified in various environmental compartments, particularly aquatic environments, raising concerns about potential human and animal exposure through various means, such as water, food, and occupation (Hernández et al., 2013).

In 2019, Diuron was found in 91.9% of surface and groundwater samples in São Paulo, Brazil by Companhia Ambiental do Estado de São Paulo (CETESB), with the highest concentrations reaching 0.32 µg/L (1.37 nM) (CETESB, 2021). Studies conducted in Rio Grande do Sul, Brazil have identified Diuron in surface water, up to 790 ng/L (3.39 nM), and drinking water, with a maximum limit of 490 ng/L (6.2 nM) (Caldas et al., 2019). In São Luis, Maranhão, Brazil, the presence of Diuron in groundwater was observed, with concentrations ranging from <0.03 to 0.67 µg/L (0.1288 to 2.87 nM) (Viana et al., 2019). Herbicide concentrations were detected in the Aquidauana River, Mato Grosso do Sul, Brazil (Viana et al., 2019) at 0.0005 µg/L (0.00214 nM) and up to 1,000 ng/L (4.29 nM) in the Piracicaba, Capivari, and Jundaí rivers, São Paulo, Brazil (Madeira et al., 2023). Groundwater in the Ribeirão Preto region of São Paulo showed concentrations of 7.12 mg/L (30.53 µM) of the herbicide (Di Bernardo Dantas et al., 2011).

It is well known that Diuron contamination has been detected in several Brazilian territories, and in this context, the Instituto Brasileiro de Ambiente e Nacional de Recursos Naturales (IBAMA)

(Brazilian Institute of the Environment and Renewable Natural Resources) classifies Diuron as a Class II product, indicating that it is a highly dangerous product for the environment (IBAMA, 2021). In the context of drinking water supply, the Brazilian Ministry of Health establishes a maximum concentration of 90 μg/L (0.361 μM) of this herbicide in drinking water, in accordance with Regulation 2.914/2011(Brasil, 2011).

However, concentrations detected for diuron in aquatic environments may not reflect the true problem of persistence of this compound in the environment. biodegradation processes that result in the formation of metabolites, including 3,4-dichlorophenylmethylurea (DCPMU) and 3,4-dichloroaniline (DCA). Both DCPMU and DCA are considered environmental pollutants due to their persistence in soil and water (Field et al., 2003). Both DCA and DCPMU metabolites were found in the Piracicaba, Capivari, and Jundiaí rivers. The concentration of DCA was limited to 0.23 mg/L (1.419 μM), while the concentration of DCPMU was limited to 10 ng/L (1.419 μM) (Madeira et al., 2023).

Diuron is classified as a class III herbicide, which means it is dangerous to the environment, according to the Global Harmonized Classification System (GHS) (IBAMA, 2021). Its main degradation product, DCA, is a xenobiotic that is targeted for decontamination studies (Quirantes et al., 2017). Additionally, it is toxic when ingested, inhaled, or when it comes into contact with the skin (PubChem, 2022). The metabolite DCPMU, or 3,4 dichlorophenyl-N-methylurea, is a compound that has been rarely studied in classical *in vivo* and *in vitro* models. However, Mohammed et al. (2018) found that DCPMU is the only diuron metabolite found in incubations in mammalian liver microsomal preparations.

Toxicological characterization in experimental models is important to contribute to knowledge about the herbicide Diuron and its degradation products, as DCA and DCPMU. In a more realistic context, since the aquatic environment is the first destination of these compounds, and alternative models such as fish are of great relevance for toxicity analyzes (Khabib et al., 2022). Recent studies have identified that Diuron (300 ng/L – 1,287 nmol/L and 3 μg/L – 12,87 nmol/L) cause aneuploidy when exposed 11 weeks, in addition to alterations in hemocyte parameters, such as reactive oxygen species in adult oysters (*Crossostrea gigas*)(Bouilly et al., 2007). In oyster larvae (6

hours post-fertilization), embryotoxic and genotoxic effects of the herbicide and its metabolites were observed (Behrens et al., 2016). Another experiment carried out with fish (*Clarias gariepinus*) in short exposure (96 hours) to Diuron (0,077 M to 0,025 M) showed mutagenicity, biochemical changes in the blood and liver dysfunction (Popoola et al., 2018). Exposure of Nile tilapia fish (*Oreochromis niloticus*) to Diuron and its metabolites DCA, DCPU and DCPMU (acute semi-static exposure, 40 ng/L to 200 ng/L), resulted in damage to antioxidant activity and biotransformation at concentrations evidenced by markers of oxidative stress and lipid peroxidation (Felício et al., 2018).

In assessing the toxicity of herbicides, their active ingredients and metabolites, the alternative water model, zebrafish (*Danio rerio*) stands out (Gonçalves et al., 2020). The zebrafish's rapid development and high reproduction rate offer an approach for carrying out toxicological tests. Furthermore, given the 71% genomic similarity of zebrafish to humans, it is possible to evaluate endpoints relevant to human health (Tal et al., 2020). In accordance with the ethical principles of the "3 Rs" and recognition by regulators worldwide, as well as support from Brazilian legislation, the zebrafish is a valuable tool for substance release and hazard assessment (Russell, 1959; Canedo et al., 2022). Zebrafish and human metabolic, toxicokinetic, and toxicodynamic profiles exhibit significant similarities and conservation (de Souza Anselmo et al., 2018). The ease of maintenance, small size, and capacity of the zebrafish to evaluate toxicity through different exposure pathways make it a highly effective and ethical *in vivo* model for toxicological research (Nagel, 2002). Its application yields significant results in comprehending the impact of diverse substances on the environment and human health (Zinsstag et al., 2011).

The objective of this research is to conduct a comprehensive assessment of general toxicity of Diuron and its metabolites, DCA and DCPMU, in different development life stages of zebrafish (*Danio rerio*). This study highlights the need for a multi-parametric evaluation to strengthen regulatory protocols for the safe use of this herbicide, not only in Brazil, but also in comparable developing countries that are experiencing increasing water contamination by Diuron.

2. MATERIALS AND METHODS

2.1 Chemicals

The herbicide Diuron (CAS no. 330-54-1, purity ≥ 98%, catalog no. D2425, Sigma-Aldrich Co, St Louis, MO) and its metabolites DCA (CAS no. 95-76-1, purity ≥ 98%, catalog no. 437778, Sigma-Aldrich Co., St Louis, MO) and DCPMU (CAS no. 3567-62-2, purity ≥ 98%, catalog no. S613053, Sigma-Aldrich Co., St Louis, MO) were used in standard solutions. The concentrations analyzed ranged from 0.5 to 100.0 µM, which are proportional to those found in the environment, including soils, bodies of water, and biological samples from animals and humans(Field et al., 2003; Da Rocha et al., 2013). Dimethyl sulfoxide (DMSO) and Tween® 20 (Sigma-Aldrich Co., St Louis, MO) were used to prepare stock solutions of diuron and its metabolites at nominal concentrations. The solutions were then diluted in osmosis water to create six different concentrations of each substance. The negative control was ISO-7346 water and the solvent control was 0.01% DMSO and Tween® 20 in ISO-7346 water. Prior to adding 0.01% DMSO and Tween® 20 at study concentrations, tests were conducted with the solvents. No toxic effects were observed on the exposed organisms. In the fish embryo toxicity test (FET), a 2.0 µM solution of hydrogen peroxide (H_2O_2) was used as the positive control group. The hydrogen peroxide model significantly affects development, causing delayed hatching, edema, and suspected liver damage (Li et al., 2021). The oxygen consumption rate (OCR) test employed potassium cyanide (KCN) diluted in NaOH as a positive control. KCN inhibits cytochrome c oxidase, acting as a mitochondrial uncoupler and halting mitochondrial respiration (Cammer, 1982).

2.2 The fish embryo toxicity test (FET)

2.2.1. Fish maintenance

The matrices used to obtain the eggs were provided by the Zebrafish Vivarium of the Faculty of Medicine of Botucatu (FMB). Male and female animals were kept in 6-liter glass aquariums under controlled conditions, with a temperature of 26±1 °C, pH of 7.5 ± 0.5, dissolved oxygen at 95% saturation, conductivity of 750 ± 50 µs/cm, and lighting varying between 14 hours of light and 10 hours of darkness. The zebrafish were fed three times a day with Maramar® Tropical Granules,

Alcon® Spirulina Feed, and Maramar® high hatching Artemia Eggs. Male and female zebrafish were separated in the spawning tank until the following morning to obtain eggs. The next morning, the separation was removed, and with the stimulus of light, mating began to obtain zebrafish embryos. The eggs were collected post-spawning using 0.6 µm pore sieves and washed with distilled water to remove impurities. Subsequently, they were transferred to Petri dishes containing autoclaved water from the zebrafish flow-through system. Viable (fertilized) eggs were selected using an inverted microscope (Motic®, AE2000).

2.2.2. Experimental design

The study followed OECD No. 236 (2013) guidelines and was conducted at the Zebrafish Animal Laboratory, located at UNIPEX - Experimental Research Unit of Botucatu Medical School, São Paulo State University (UNESP), Brazil. The Ethics Committee on Animal Use approved the project (Certificate No. 1304/2019). Each concentration was tested on 20 zebrafish embryos for diuron, DCA, and DCPMU. Embryos were distributed into sterile 24-well plates and exposed to autoclaved water from a zebrafish flow-through system as an internal control. The FET test assay was performed in triplicate (n=3) with independent assays. The embryos were monitored and evaluated at 8, 24, 48-, 96-, 120-, and 144-hours post-fertilization (hpf) under an inverted microscope (Motic, AE2000) following the OECD TG236 protocol with an extension until 144 hpf. To compare with the treated organisms, we utilized the description of typical zebrafish development by Kimmel et al. (1995), which includes endpoints such as mortality, sublethality, and teratogenicity. We captured images of embryos and larvae at various times post-fertilization using a digital camera (Olympus MVX10) attached to a stereomicroscope (SMZ800, Nikon).

2.3 Oxygen Consumption Rate (OCR) test

The zebrafish embryos were subjected to an oxygen consumption rate (OCR) test at 24 hours post fertilization (hpf) using the Oroboros 02k Oxygraph (Oroboros Instruments, Austria) following the protocol adapted from Gnaiger's (2020) research. The test was conducted at a temperature of 27°C and a speed of 500 rpm. After stabilizing and calibrating the oxygen consumption curve with 1

mL of MAS-1 mitochondrial respiration medium in the cuvette, 20 embryos were inserted. Following a 3-minute incubation period, the substrates that activate the Krebs cycle and the electron transport chain (ETC) were added, including succinate (CAS no. 123-24-1, catalog no. W237701, Sigma-Aldrich Co.), malate (CAS no. 617-55-0, catalog no. 374318, Sigma-Aldrich Co.), and pyruvate (CAS no. 133-24-6 and ADP (CAS No. 16178-48-6, catalog No. 018971 mM) were added at 2 mol/L, followed by 35 mM digitonin (CAS No. 11024-24-1, catalog No. 300410, Sigma-Aldrich Co.) to permeabilize the chorion and biological membranes. The test substances, diuron, DCA, and DCPMU, were added in triplicate at concentrations of 0.5, 1.0, and 5.0 µM, along with negative and positive controls (KCN (CAS No. 151-50-8, catalog No. 60178, Sigma-Aldrich Co.), 0.4 mol/L diluted in NaOH, pH >10). Basal respiration was analyzed after 5 minutes. To inhibit ATP synthase and enable respiration without phosphorylation, 45 mM of oligomycin (CAS No. 1404-19-9, catalog No. 495455, Sigma-Aldrich Co.) was added. This was followed by the addition of 50 mM FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, no TIP2k, catalog No. C2920, Sigma-Aldrich Co, purity ≥ 98%), a mitochondrial uncoupler that depolarizes mitochondrial membranes, increases the OCR, and allows the analysis of uncoupled respiration. Finally, for residual consumption, rotenone (CAS No. 83-79-4, catalog No. R8875, Sigma-Aldrich Co.) and antimycin A (CAS No. 83-79-4, catalog No. A8674, Sigma-Aldrich Co.) 25 mM were added since they inhibit complexes I and II and stop respiration. The OCR was measured at stabilized intervals of 3 minutes by subtracting the residual consumption after the addition of rotenone and antimycin A to obtain a specific value for mitochondrial respiration.

2.4 Acetylcholinesterase (AChE) activity assay

At the end of each exposure assay, a pool of 30 larvae per treatment concentration was used to measure AChE activity after 96 hours. Protein quantification was performed using the Bradford method (Bradford, 1976), adapted for a 96-well plate. AChE activity was determined using the method of Ellman et al. (1961), adapted to a 96-well plate by Guilhermino et al. (2011), with acetylthiocholine as the substrate. The conjugation of 5,5’-ditiobis (2-nitrobenzoic acid), DTNB (CAS no. 69-78-3, purity ≥ 98%, catalog no. 22582, Sigma-Aldrich Co) with 2-(Dimethylamino)

ethanethiol hydrochloride, thiocholine (CAS no. 13242-44-9, purity ≥ 98%, catalog no. D2425, Sigma-Aldrich Co) was monitored by measuring the increase in absorbance at 414 nm for 5 minutes. AChE activity is expressed as nmol of substrate hydrolyzed per minute per mg of protein ($\varepsilon = 13.6 \times 10^3\ M^{-1}cm^{-1}$).

2.5 Comet assay

Twenty selected embryos were exposed to each experimental condition using six-well culture plates. The experimental conditions included three concentrations of each tested substance (0.5, 1.0, and 5.0 µM), a positive control (0.001% v/v hydrogen peroxide H_20_2, CAS no. 7722-84-1, catalog no. 1.08600, Sigma-Aldrich Co., St Louis, MO, 29-32%), and a negative control (ISO-7346 water and 0.01% v/v DMSO and Tween 20). Three independent replicates were conducted. After 96 hours of static exposure, the larvae were euthanized and resuspended in 100 µL of PBS buffer. The next step involved a maceration process for cell isolation. Cell viability was assessed using the Trypan Blue method after filtration in a cell strainer and centrifugation. Samples with a viability equal to or greater than 80% were accepted. 50 µL of each cell suspension were added to 150 µL of a 1% LMP agarose (Low Melting Point, catalog no. 16520050, Invitrogen, USA) solution at 27 °C. The mixture was homogenized and distributed on pre-gelatinized slides containing 1.5% NMP agarose (Normal Melting Point, UltraPure™ Agarose, catalog no. 18300012, Invitrogen, USA). The process involved gelatinization followed by lysis in a solution containing pH 10, NaCl 2.5 M, Tris 10 mM, EDTA 100 mM, Triton X-100 1% v/v, and DMSO 10% v/v. Electrophoresis was carried out in a solution with pH 13, EDTA 1 mM, and NaOH 300 mM for 20 minutes at 4 °C. The slides were then neutralized in 0.1 mol/L Tris-HCL buffer (CAS no. 77-86-1, catalog no.). The cells were stained with SYBR™ Gold Nucleic Acid Gel Stain (1:10,000, catalog no. S11494, Invitrogen, USA) at a concentration of 0.1% v/v and analyzed under an epifluorescence microscope (Eclipse E200, Nikon, Japan). The intensity of the comet tail (Tail Intensity) was quantified using Comet IV software version 4.3 from Perceptive Instruments Ltda.

2.6 Fish, Acute Toxicity Test

2.6.1 Fish maintenance

Sexually mature zebrafish (approximately 3 months old) were provided by the Special Toxinology Laboratory Applied at the Butantan Institute (São Paulo/SP) and were maintained under controlled conditions in 9 L aquaria, with water reconstituted and standardized according to ISO 7346-1 and 7346-2 (ISO, 1996), at a temperature of 27 ± 1 °C and a photoperiod of 14/10 (light/dark). Throughout the experiment, parameters such as pH, ammonia, and oxygen were also monitored and controlled. The animals were fed three times a day with Maramar® Tropical Granules, Alcon® Spirulina Feed, and Maramar® high hatching Artemia Eggs. The experimental group consisted of adult zebrafish with a mean weight of 0.34 ± 0.04 g and a mean length of 3.01 ± 0.3 cm, which were acclimatized for one month prior to the start of the experiment. The mortality rate during maintenance was less than 2%.

2.6.2 Experimental design

Thirteen adult fish were included in the test, with 13 animals for each concentration of tested substances (Diuron, DCA, and DCPMU), and finally 13 for the solvent control (DMSO 0.01%), all kept in 8 L glass aquaria. The exposure was semi-static, with 50% of the water and substances replaced after 48 hours of exposure. The exposure duration was 96 h in accordance with OECD No. 203 (2019). The parameters of temperature (°C), pH, dissolved oxygen (mg/L), and total hardness (mg/L) were monitored daily during exposure to assess water quality.

2.6.3 Histological evaluation

Histological sections with a thickness of 4 µm were stained with H&E and analyzed for alterations in the liver, brain, and spleen using a conventional optical microscope (Olympus Optical Co., Ltd., Japan). Histological findings for each organ were graded using a qualitative and semi-quantitative method. Alterations were rated on a scale of 0 (normal) to 3 (severe). The alteration index was then calculated by summing the intensity of observed damages for each established parameter, multiplied by the level of importance of each parameter.

2.6.4 Micronucleus and Nuclear Abnormalities Test

After acute exposure, seven adult zebrafish were randomly selected and euthanized using an overdose of benzocaine (3mg/L, CAS no. 94-09-7, catalog no. E1501, Sigma-Aldrich Co., St Louis, MO). The caudal fins were then removed for blood collection. Three blood smears per fish were fixed in absolute methanol, placed on glass histological slides, and stained with acridine orange (5 mg/mL acridine orange, CAS no. 65-61-4, catalog no. A8097, Sigma-Aldine Co. A8097, Sigma-Aldrich Co., St Louis, MO) before being analyzed under a fluorescence microscope (Olympus BX53). Observations were recorded in microphotographs using cellSens software (Olympus, Center Valley, PA, USA) and analyzed with Fiji-ImageJ (National Institutes of Health, Bethesda, Maryland, USA). The identification of micronuclei in erythrocytes followed specific criteria, including the presence of extranuclear bodies with spherical or ovoid shapes in the cytoplasm. The evaluation of erythrocytes with nuclear anomalies followed the protocol described by Canedo et al. (2021). The frequency of nuclear abnormalities, such as micronuclei, was determined using the equation FAN=AN/N. FAN represents the frequency of anomalies, while AN/N represents the ratio between the number of anomalies and the erythrocyte nuclei counted for each zebrafish examined (1,000 nuclei).

3. STATISTICAL ANALYSES

The data obtained from the tests, which included more than two unpaired groups, underwent a normality test (Shapiro-Wilks). For non-parametric data, we applied analysis of variance (Kruskal-Wallis), followed by Dunn's multiple comparison post-test. If the data was parametric and normal, we used analysis of variance (One-way ANOVA), followed by Dunnet's post-test to determine the significance of the exposures compared to the negative control group. The statistical significance of the data was determined by a p-value of ≤ 0.05. The analysis was performed using GraphPad Prism 5.01 software (San Diego, CA 92108, USA, ©GraphPad Software, Inc).

4. RESULTS

4.1 Embryos analysis

Mortality was observed in zebrafish embryos exposed to concentrations of 50.0 and 100.0 µM of diuron, DCA, and DCPMU since 8 hpf (Figure 1). Significant mortalities were also observed at concentrations of 10.0 µM of diuron from 48 hpf, 5.0 µM and 10.0 µM of DCA from 8hpf, and 10.0 µM of DCPMU from 8hpf (Figure 1). Malformations consistent with pericardial edema, yolk sac edema, and skeletal deformities were observed in zebrafish larvae exposed to DCA 10.0 µM and DCPMU 10.0 µM (Figure 2). Figure 3 shows microphotographs of zebrafish larvae controls and those exposed to diuron, DCA, and DCPMU at 120 hpf.

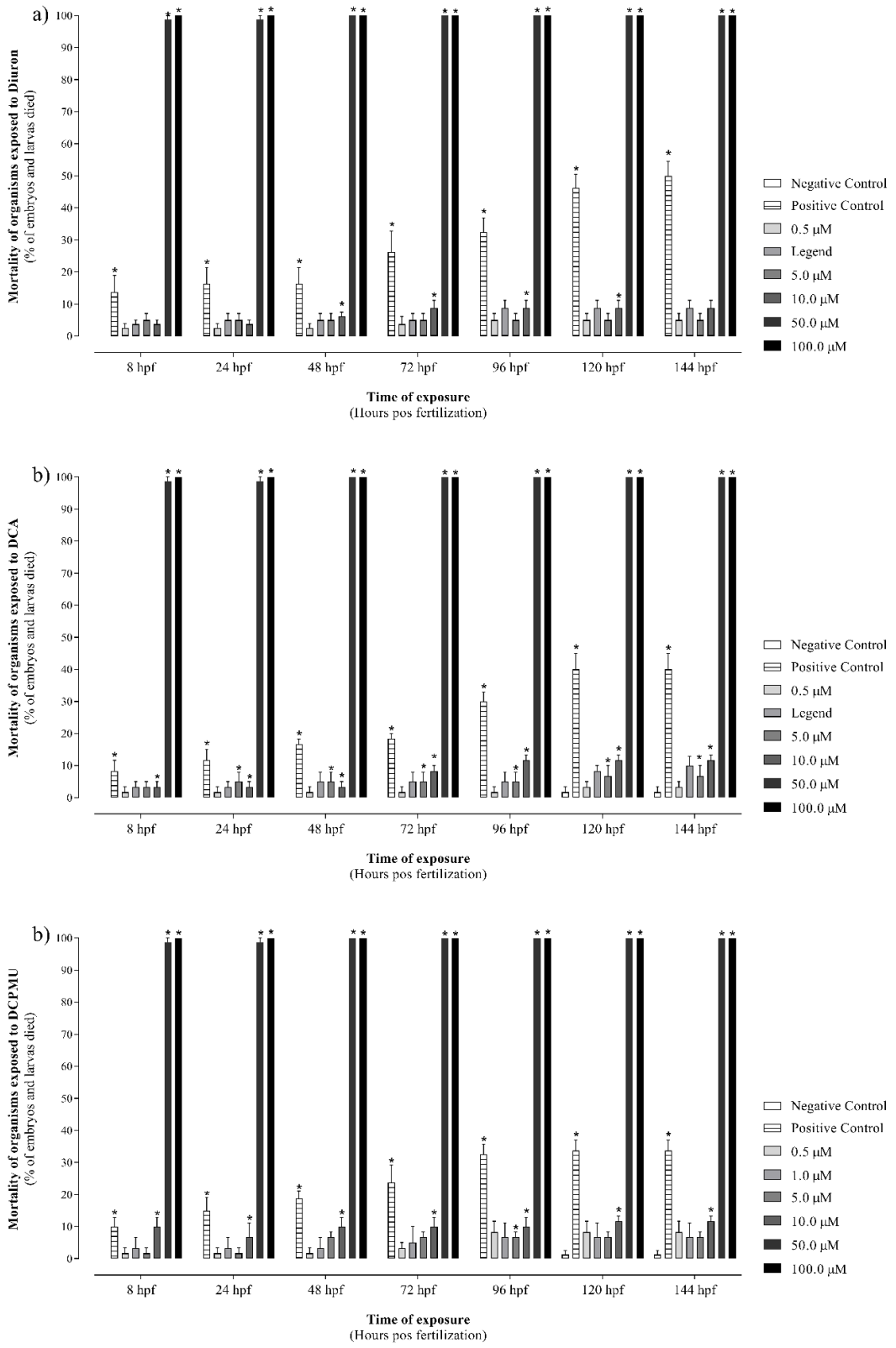

Figure 1) Graphical representation of the mortality of zebrafish embryos and larvae, expressed by the proportion of clotted eggs and absence of heartbeats due to the concentration and time of exposure to Diuron, DCA and DCPMU. a) Diuron, b) DCA and c) DCPMU. Mortality was statistically significant ($p \leq 0.05$) compared to the negative control when represented by (*). The bar graphs represent the mean and standard deviation. (n= 20 embryos per condition/3 replications). Plotted in GraphPad Prism 5.01 software.

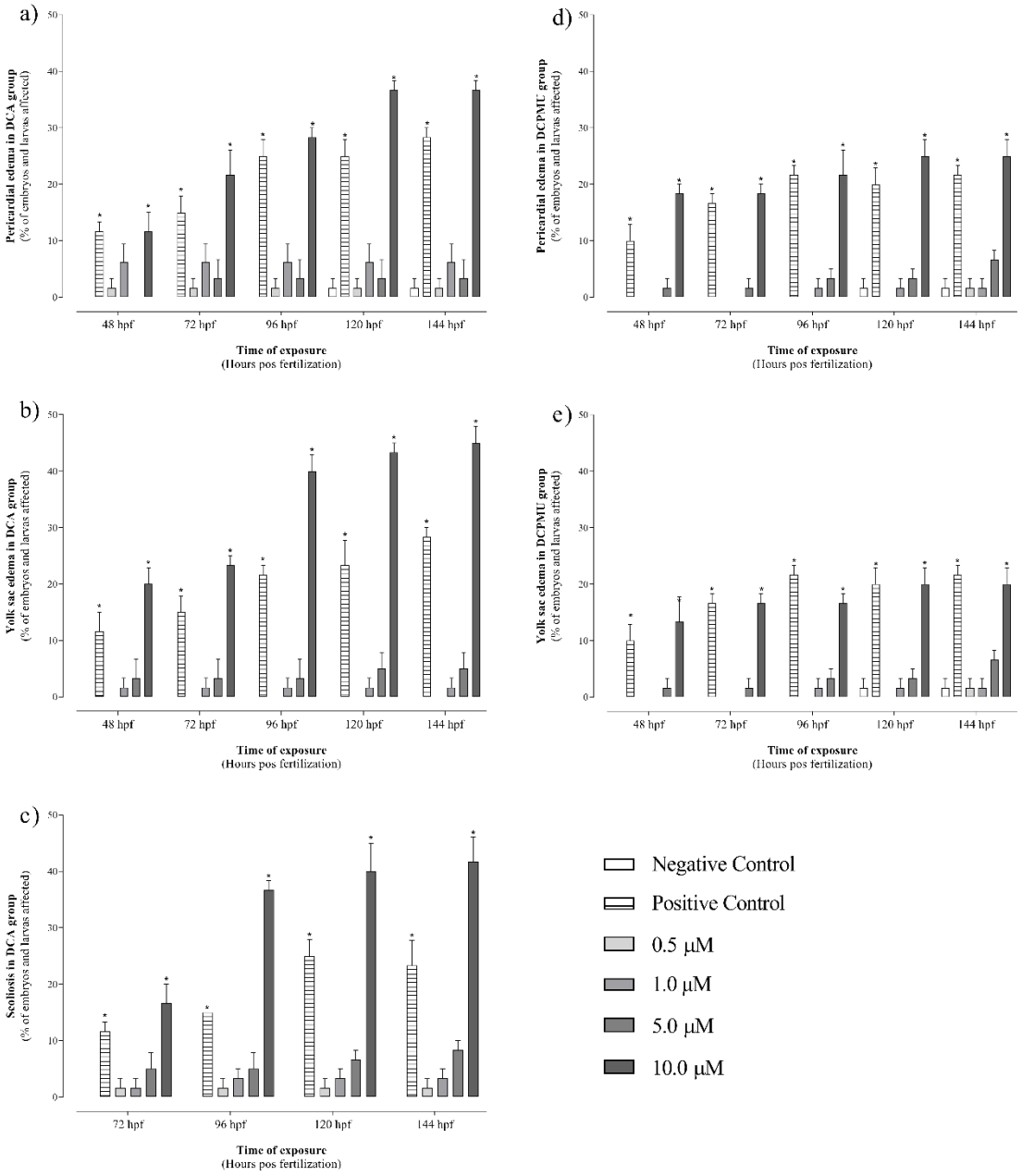

Figure 2) Graphical representation of the rate malformations of zebrafish embryos and larvae by concentration and time of exposure to DCA and DCPMU. a), b) and c) DCA, d) and e) DCPMU. The malformations were statistically significant ($p \leq 0.05$) compared to the negative control when represented by (*). The bar graphs represent the mean and standard deviation. (n= 20 embryos per condition/3 replications). Plotted in GraphPad Prism 5.01 .

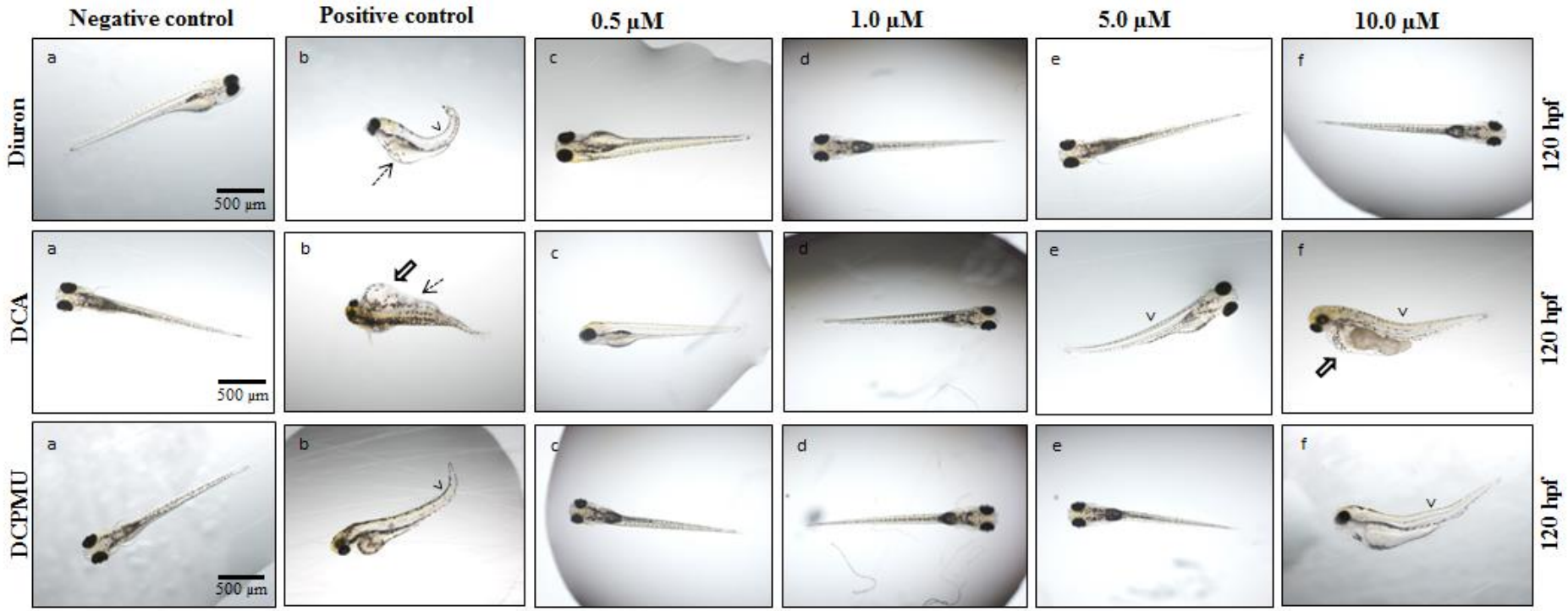

Figure 3) Representative micrographs of the development of zebrafish larvae at 120 hpf exposed to Negative Control (a), Positive Control (b) 0.5 µM (c), 1.0 µM (d), 5.0 µM (e) and 10.0 µM (f) of Diuron, DCA and DCPMU (1.6x magnification for larvae on Olympus MVX10 microscope). Legend of the changes found are represented in: Yolk sac edema (dashed arrows). Pericardial edema (open arrows). Skeletal deformity (arrowheads). Micrographs are representative of 3n experimental independent.

Figure 4 displays the OCR rates for each phase of mitochondrial respiration. The results show that the addition of Diuron, DCA, and DCPMU did not significantly alter OCR during the basal respiration of 24-hpf zebrafish embryos at different concentrations, compared to the negative control and between the various concentrations examined. However, there was an increasing trend in the OCR of basal respiration at 1.0 µM of Diuron and all concentrations of DCA and DCPMU (0.5-5.0 µM). When evaluating non-phosphorylating respiration with oligomycin, an ATP synthase inhibitor (Rahn et al., 2013), there were no significant changes regarding the negative control and the different concentrations of Diuron, DCA, and DCPMU tested (0.5 to 5.0 µM). When using FCCP for uncoupled respiration, it acts as an uncoupler of oxidative phosphorylation, causing the ETC to reach its maximum capacity (Obeidat et al., 2018). No significant differences were observed between the negative control and the exposure to the compounds, nor between the various concentrations.

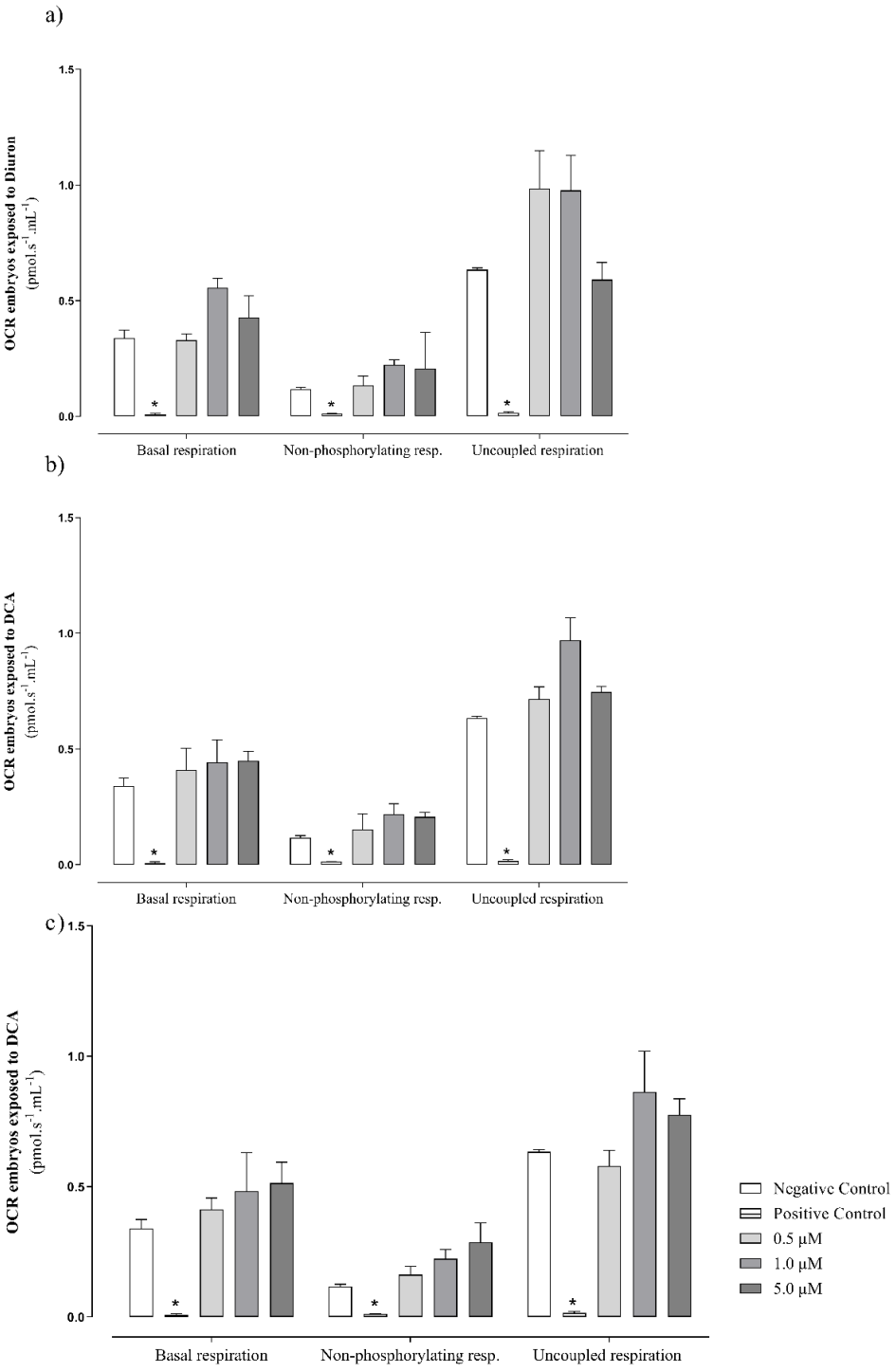

Figure 4) Representative graphs of oxygen consumption rates (OCR) during mitochondrial respiration were generated by adding mitochondrial modulators to zebrafish embryos (24 hpf) exposed to differing concentrations of Diuron, DCA, and DCPMU. The figures depict the oxygen consumption rate (OCR) (pmol.s-1.mL-1) obtained for each concentration and substance analyzed during respiration monitoring, including negative and positive controls (KCN 10 mM in NaOH pH>10) (with mean and standard deviation values) for each phase. Basal respiration is measured by adding Diuron, DCA, and DCPMU (0.5-5.0 µM) followed by adding 7.5 µM oligomycin (45 mM) to establish non-phosphorylating respiration. Next, 10 µM FCCP (50 mM) is added to measure uncoupled respiration. The following are the OCR results of embryos exposed to Diuron at 24 hpf (a), DCA at 24 hpf (b), and DCPMU (c). The rate of oxygen consumption displayed statistical significance ($p \leq 0.005$) compared to the negative control when represented by (*). The bar graphs represent the mean and standard deviation.. (n= 20 embryos per condition/3 replicates). Plotted in the GraphPad Prism 5.01 software.

Figure 5 presents the results of the AChE enzyme analysis after 96hpf of exposure to diuron, DCA, and DCPMU. The AChE activity decreased in larvae exposed to DCA (10.0 µM) compared to the control group.

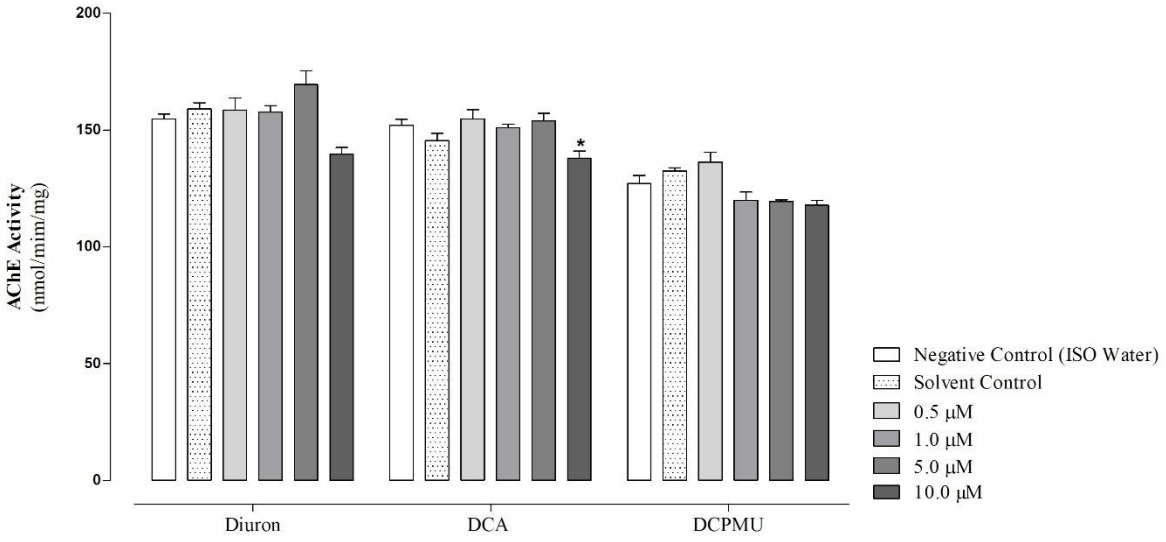

Figure 5) AChE activity in analyses of cell pools from larvae exposed (96 hpf) to different concentrations of Diuron, DCA and DCPMU. The AChE displayed statistical significance ($p \leq 0.05$) compared to the negative control when represented by (*). The bar graphs represent the mean and standard deviation. (n= 20 embryos per condition/3 replicates). Plotted in the GraphPad Prism 5.01 software.

Regarding the genotoxicity analyses conducted during the embryonic and larval period (96 hpf), the cell suspensions obtained from maceration of the larvae showed high cell viability (93 ± 6%). These results indicate that exposure to the compounds resulted in DNA damage. The comet assay showed a statistically significant increase ($p < 0.0001$) in the intensity of the comet tail, indicating higher levels of damaged DNA, at all tested concentrations (0.5 - 5.0 µmol/L) of Diuron, DCA, and DCPMU compared to the negative control (Figure 6).

Nucleoid tail intensities were classified according to DNA fragmentation levels (Figure 6d). These levels ranged from little or no damage (0-10%) to severe damage (> 75%). The results for each concentration can be found in Table 1 of the Supplementary Material. The data indicate that all exposures resulted in a decrease in the number of nucleotides with minimal or no damage (0-10%), suggesting that Diuron, DCA, and DCPMU can cause DNA fragmentation. At a concentration of 1.0 µmol/L, both Diuron and DCA showed a significant percentage of damaged cells with a low index.

The majority of cells exposed to Diuron concentrations of 1.0 and 5.0 µmol/L and DCA concentrations of 0.5 to 5.0 µM exhibited medium damage (25 to 50%). High damage (50-75%) was observed at concentrations of 0.5 and 5.0 µM for both Diuron and DCA.

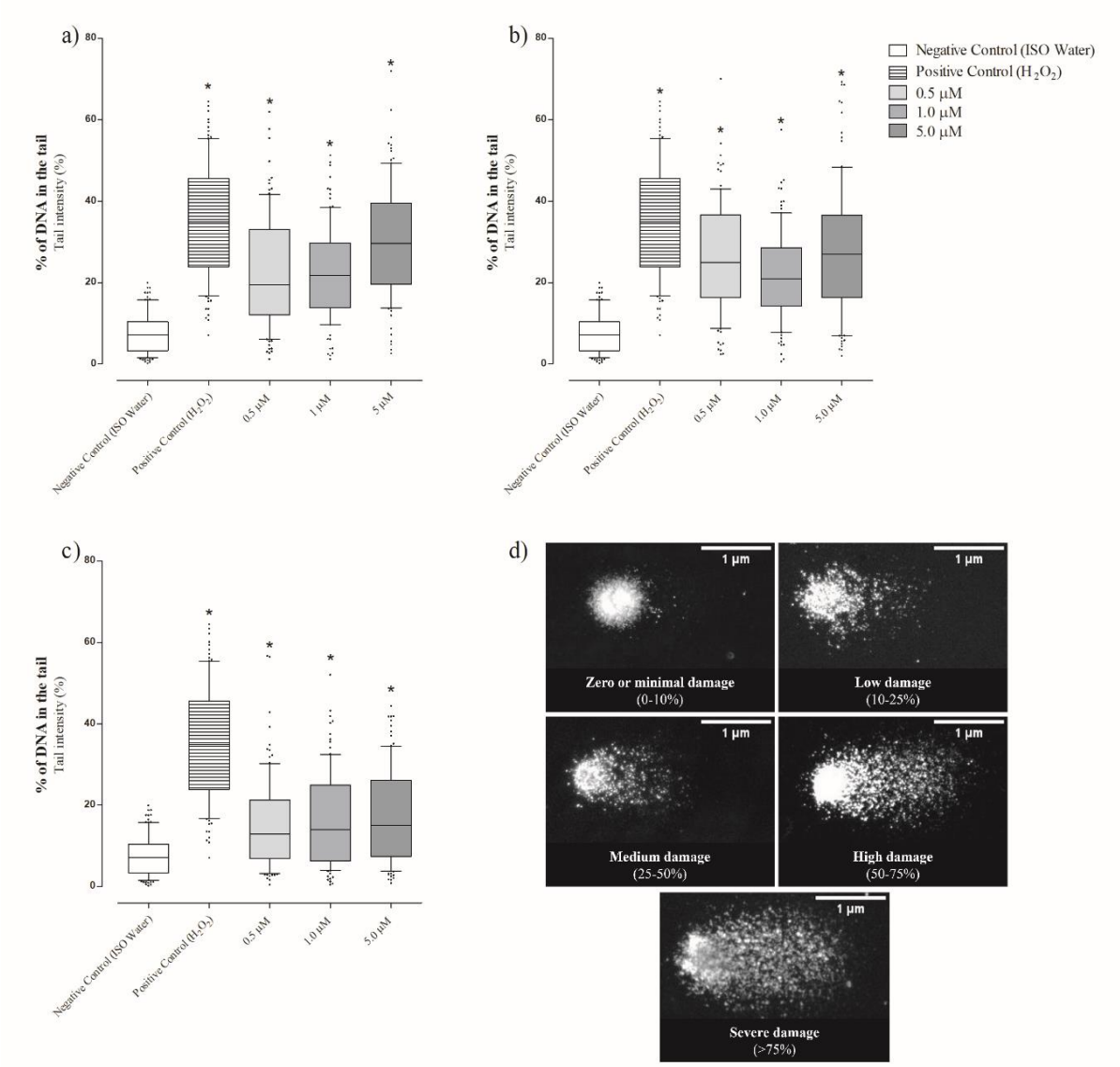

Figure 6) Representative graph of comet tail intensity (%) in analyses of cell pools from larvae exposed (96 hpf) to different concentrations of Diuron (a), DCA (b) and DCPMU (c). The graphs express the intensity of the tail (%) obtained at each concentration for each substance analyzed (boxplot with median and quartiles indicated, percentile intervals 10-90). The intensity was significant ($p \leq 0.005$) compared to the negative control when indicated by (*). (n= 20 larvae per condition/3 replicates) (100 nucleoids per slide per condition). The Comet IV software (version 4.3, Perceptive Instruments Ltda) was employed to capture the microphotographs, which accurately reflect the damage intensity observed in the exposures. Prepared in GraphPad Prism 5.01 software. d) Representative micrographs of the various levels of DNA damage were observed in a pool of larvae exposed to diuron, DCA, and DCPMU for 96 hours. The images are indicative of the recorded DNA damage levels during the experiment and were obtained utilizing the software Comet IV.

4.2 Adult analysis

All animals exposed to diuron at different concentrations survived, however animals exposed to concentrations of 100.0 µM became apathetic. In groups DCA and DCPMU, all animals exposed

to the highest concentrations, 50.0 and 100.0 µM of DCA and DCMPU died. The organisms exposed to the lowest concentrations of DCA and DCPMU (0.5, 1.0, 5.0, 10.0 µM), mostly survived (data no show). As for the histological analyzes of the removed tissues, liver, spleen and brain of adult zebrafish exposed to diuron, DCA and DCPMU for 96 hours, revealed, in general, normal morphology: tissues and cells of normal distribution, prominent nuclei, outlined, intact, clear, dense and well-preserved cytoplasm. There were no evident histological changes in the liver, spleen and brain of adult zebrafish exposed to diuron, DCA and DCPMU (data no show). However, the groups exposed to 10.0 µM DCA or DCPMU showed slight cytoplasmic vacuolation of hepatocytes. Structural alteration and hyaline droplets (figure 7).

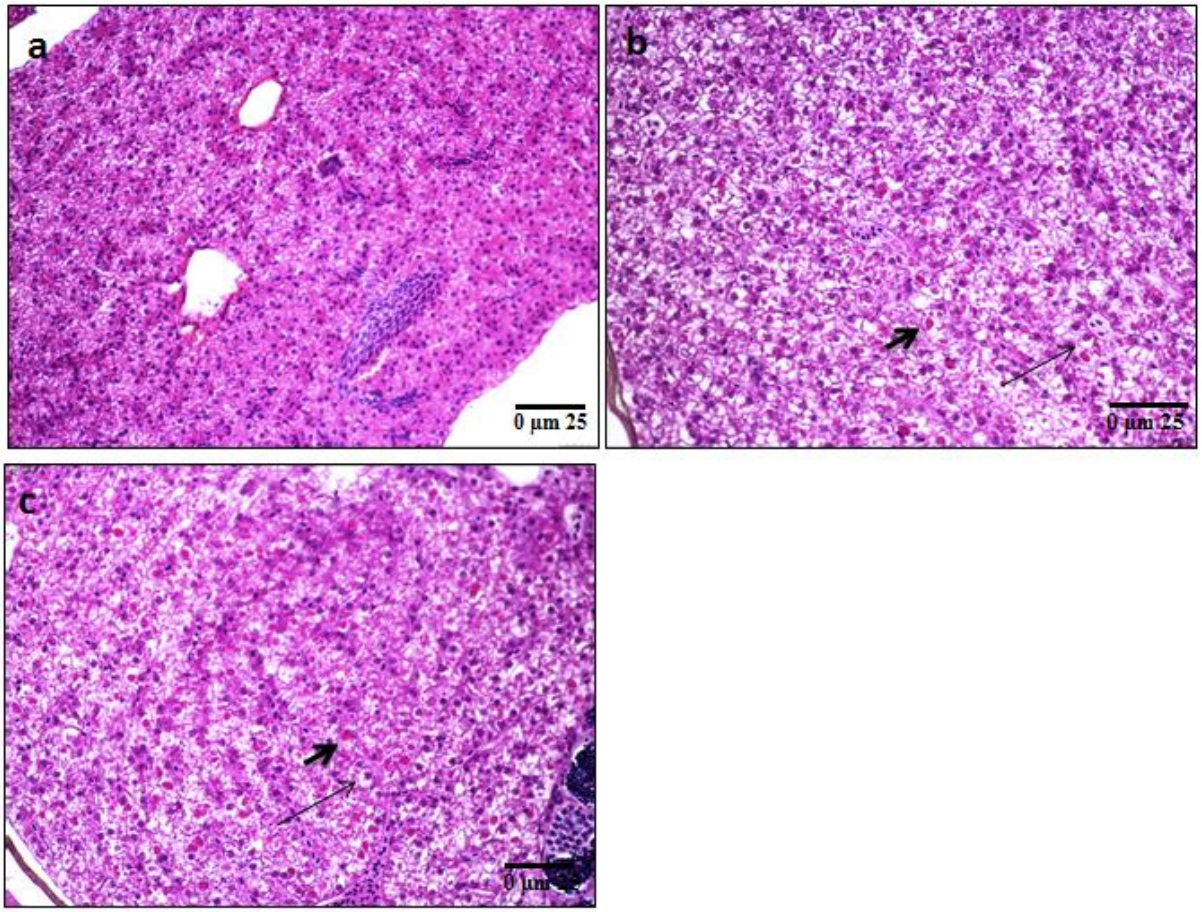

Figure 7) Histological micrographs of the effects of DCA and DCPMU in liver zebrafish adults after 96 h of exposure (H&E, 25 µm). Sections of Negative control (a); 10.0 µM of DCA (b); 10.0 µM of DCPMU (c). (40x on Olympus MVX10 microscope). Legend of the changes found are represented in: Vacuolization (thin arrows); hyaline droplets (thick arrows).

The frequency of micronuclei in adult zebrafish erythrocytes did not differ significantly across any of the exposures. Additionally, the frequency of micronuclei in exposed adult zebrafish erythrocytes did not exceed the naturally observed negative control levels of 0.5% (Canedo et al., 2021) (data not shown).

Significant differences were observed in the shapes of the nuclei, including lobulated, bilobed, blistered, notched, nuclear constrictions, and kidney-shaped (Figure 9b), when compared to the control groups (refer to Table 2 - Supplementary Material). Concentrations of Diuron (5.0 - 100.0 µM), DCA (1.0 and 10.0 µM), and DCPMU (1.0 - 10.0 µM) were significantly higher than those of the negative control, surpassing the baseline limit of blistered nuclei (1.6%) as reported by Canedo et al. in 2021. The study found notable concentrations of specific substances, including 50.0 µM Diuron, 10.0 µM DCA, and 1.0 µM DCPMU, all of which surpassed the blistered nuclei threshold of 1.1% (Canedo et al., 2021).

Additionally, the concentration of 10.0 µM Diuron, 10.0 µM DCA, and 1.0 to 5.0 µM DCPMU, resulted in a significantly higher proportion of erythrocytes with blisters or sprouts compared to the negative control, surpassing 0.7% of zebrafish erythrocytes (Canedo et al., 2021). Bilobulated nuclei were only significant at a concentration of 50.0 µM DCA and 1.0 µM DCPMU. Nuclear abnormalities, such as nuclear constriction and kidney-shaped nuclei, were observed at specific concentrations: 100.0 µmol/L of Diuron and 5.0 to 10.0 µM of DCPMU, respectively.

Figure 9a shows a significant increase in the percentage of nuclei exhibiting nuclear abnormalities (NA) at a concentration of 50.0 µm of Diuron, indicating an impact on nuclear morphology at high concentrations. The metabolites DCA and DCPMU also showed noteworthy NA percentages at concentrations of 10.0 µM and 1.0-10.0 µM, respectively.

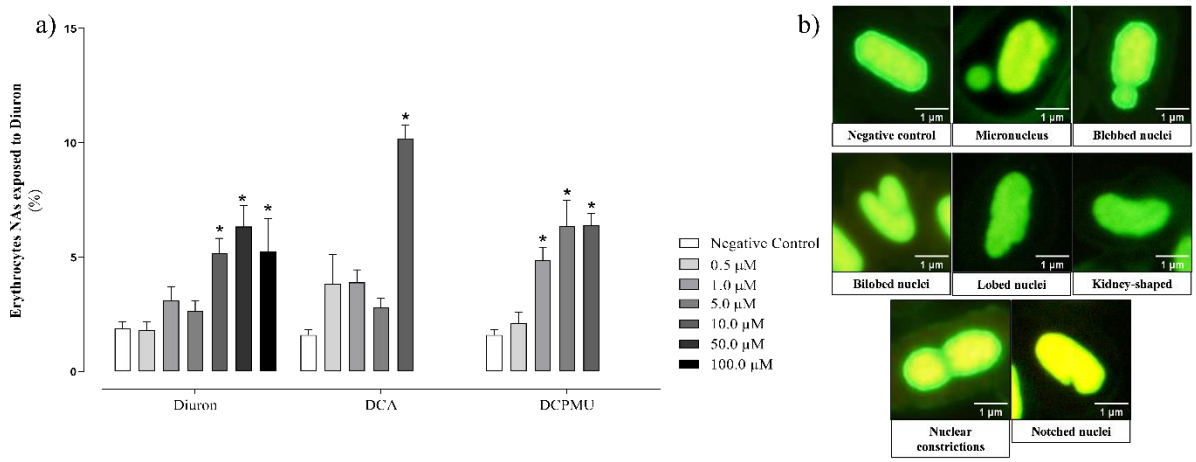

Figure 9) a) Graph depicting the cumulative count of nuclear irregularities observed in adult zebrafish red blood cells following a 96-hour exposure to Diuron, DCA, and DCPMU in a semi-static environment. The graph displays the frequency (percentage) of nuclear abnormalities per 1,000 cells at each substance's concentration. The frequency was notable ($p \leq 0.005$) compared to the negative control, marked by (*). The bar graphs represent the mean and standard deviation. (n= 7 fish per condition, 1,000 cells per fish). b) Representative microphotographs describing the NAs found in the erythrocytes of adult zebrafish exposed for 96 hours to Diuron, DCA and DCPMU are presented. Prepared in GraphPad Prism 5.01 software. b) The micrographs are representative of the NAs found in the exposures, ×1,000 magnification.

5. DISCUSSION

During all life stages examined and at all concentrations evaluated, the herbicide Diuron and its metabolites induced effects in zebrafish. Diuron was the least embriotoxic, but the most genotoxic. The DCA metabolite affected AChE activity. Furthermore, Diuron, DCA and DCPMU showed mortality at high concentrations and caused DNA damage and nuclear abnormalities. Given the comprehensive scenario and the fact that these compounds are already present in aquatic environments and have been quantified in biological samples in many developing countries, including Brazil, the situation is concerning.

To determine whether early life stages of zebrafish are influenced by Diuron, DCA and DCPMU, were analyzed by FET test parameters of lethality, sublethality and teratogenicity in zebrafish development. In the present study, at concentrations of 50.0 and 100.0 µM, all zebrafish embryos exposed to Diuron, DCA and DCPMU died. observed the onset of malformations (lack of pigmentation and edemas) in zebrafish embryos exposed to diuron only at concentrations of 4 and 8 mg/L. Zaluski et al. (2022) exposed zebrafish embryos to diuron at concentrations of 1 and 1.5 mg/L. The authors reported malformations such as increased the ocular distance after 7 days post-fertilization (dpf). Data demonstrate that diuron has EC_{50} values of approximately 4 mg/L (Velki et al., 2017), which corroborate the results presented here since we did not observe alterations in

embryos and larvae exposed to Diuron to the highest concentration that occurred survival (10.0 µM or 2.33 mg/L). However, it is already well known that Diuron residues are found in the aquatic environment in many different concentrations, which raises environmental concerns (Masiá et al., 2015).

When exposed to DCA at concentrations of 10.0 µM (1.62 mg/L), early life stages of zebrafish had alterations included edemas and the presence of skeletal deformities. Scheil et al. (2009) demonstrated edemas resulting from DCA exposure during 96 h in embryos and larvae of zebrafish. The edemas found were close to the concentrations we studied (1mg/L). In another study evaluating DCA on an aquatic experimental model rare minnow (*Gobiocypris rarus*) in the embryonic and larval phase, it was possible to detect malformations at 72 hpf at low concentrations of 1,426 mg/L and 0.187 mg/L, respectively (Zhu et al., 2013). These results show that the models are sensitive to low concentrations of DCA, a pollutant of environmental concern. Indicating the importance of evaluating contaminants, such as DCA at different concentrations and in different experimental models. Specifically, regarding the zebrafish aquatic model and DCA, published data can have a large variation. For Voelker et al. (2007) the LOEC for survival and sublethal effects in 48 h zebrafish embryos are equivalent to 2.01 mg/L. On the other hand, according Von Hellfeld et al. (2020) the DCA, acute and sublethal toxicity data from embryo tests with zebrafish range from EC_{10}: 1.200 mg/L, EC_{50}: 1.900 mg/l, LC_{10}: 1.600 mg/L and LC_{50}: 2.400 mg /L. As for Schiwy et al. (2020), the LC_{50} of DCA is 2.7 mg/L at 96 hpf. Such results highlight the importance of studying DCA regarding its real toxicity for this experimental model. Since in the present study malformations were seen arising from the action of DCA at 10.0 µM (1.62 mg/L) at 48hpf of exposure. And Scheil et al, (2009) detected malformations in zebrafish larvae exposed to DCA at 1 mg/L.

Malformations consistent with edema were possible to be observed in zebrafish embryos exposed to the DCPMU metabolite. Data on embryotoxicity in zebrafish exposed to DCPMU do not exist, nor are EC and LC values established for the DCPMU metabolite. According to the literature, the action of DCPMU has only been shown to be toxic in adults. Nile tilapia fish (*Oreochromis niloticus)* females exposed to DCPMU had alterations in reproduction with estrogenic effects potentially mediated through enhanced estradiol biosynthesis and accelerated the ovarian

development (Pereira et al., 2015). In another study, alterations in biochemical parameters were demonstrated, as a decrease in the activity of the enzyme Glutathione S-transferase (GST) and oxidative stress, such as an increase in lipid peroxidation (LP) in tissues of Nile tilapia fish exposed to DCPMU. Furthermore, the study points out that DCPMU is the main compound causing changes in fish exposure in an integrated analysis of biomarkers, highlighting the importance of analyzes with this compound (Felício et al., 2018). One of the main alterations found in embryos and larvae exposed to DCA and DCPMU at 10 µM was the presence of edemas. Studies related that edema may be caused by kidney failure, circulatory failure, ionic imbalance, and permeability defects (Wiegand et al., 2023). Therefore, we suggest that the toxicity of DCA and DCPMU during the early stages of zebrafish development and at the concentrations studied potentially impacts the organism's osmoregulatory system. Another important point is that, in general, studies that evaluate DCA and DCPMU, in addition to diuron, indicate that as harmful as the parent molecule is, the metabolites of Diuron, DCA and DCPMU are as toxic as diuron or sometimes even more damage, as seen in studies by (Scheil et al., 2009; Zhu et al., 2013; Pereira et al., 2015; Felício et al., 2018). In the current context, there is a need to evaluate the DCPMU metabolite regarding its effects on fish early life stages and in different contractions and different species.

The Oxygen Consumption Rate (OCR) assay is an essential tool for real-time analysis of mitochondrial function, measuring basal respiration and the impact of mitochondrial modulators (Gnaiger, 2020). Studies suggest that chemical compounds have influenced changes observed during OCR analysis (Jönsson et al., 2007). These changes imply potential modifications to OCR capabilities when exposed to such compounds, potentially impacting the maximum capacity of CTE (Obeidat et al., 2018). These changes imply potential modifications to OCR capabilities when exposed to such compounds, potentially impacting the maximum capacity of CTE (Obeidat et al., 2018).

Previous research has pointed out that some compounds, such as Triclosan, a commonly used antimicrobial agent, can act as uncouplers of mitochondria in Zebrafish embryos (Shim et al., 2016). Additionally, previous research has found that Diuron is linked to mitotoxicity in various experimental models, including human cancer cells (Mohammed et al., 2020). Metabolomic analysis

of mitochondria isolated from urothelial cells of male Wistar rats exposed to 10-100 µM Diuron and metabolites, suggesting mitochondrial dysfunction as a potential molecular initiating event (MIE) of Diuron toxicity (Lima et al., 2022).

To find out whether diuron and its metabolites DCA and DCPMU cause neurotoxicity in the embryo larval phase of the zebrafish model, we evaluated whether the enzyme acetylcholinesterase (AChE). It is established in some studies that the Diuron herbicide can cause neurological alterations in experimental models (Lima et al., 2022). The present study showed an inhibition of AChE activity in larvae exposed to DCA (10.0 µM), suggesting a neurotoxic effect of DCA for zebrafish early life stages. Lima et al. (2022) found that Diuron, DCA and DCPMU induce dopaminergic neurotoxicity in larvae of the model organism *Caenorhabditis elegans*, showing the importance of analyzes at the neurotoxic level for these pollutants as DCA.

The findings suggest that Diuron and its metabolites can cause significant DNA damage, including single or double breaks in phosphodiester bonds, alkaline-labile sites, and/or cross-links (Collins, 2004). Despite not being classified as genotoxic by American and European regulatory agencies (US EPA, 2003; EFSA, 2005), the Mutatox™ test conducted on bacteria has identified Diuron as a suspected genotoxic substance (Canna-Michaelidou et al., 1996). In addition, MCF-7 human cancer cells exposed to concentrations of 0.1-200 µM for 48 hours exhibited primary DNA lesions, indicating potential genotoxicity (Huovinen et al., 2015).

Studies conducted on adult zebrafish exposed to 4.29 nmol/L of Diuron for 14 and 21 days revealed DNA damage in liver and sperm cells. The comet assay detected the damage, indicating that the herbicide's genotoxicity is dependent on the exposure time (Bony et al., 2010). Furthermore, research on trochophore oyster larvae has suggested that Diuron's genotoxicity (0.00858 - 2.145 nM) may be responsible for embryotoxicity in this organism. Behrens et al. (2016) found that exposure concentration of DCA (0.01234 - 3.0863 nM 6 hpf) and DCPMU (0.00912 - 2.282 nM 6 hpf) proportionally increased the intensity of the comet tail in oyster larvae.

The genotoxic effect of Diuron, DCA, and DCPMU may be attributed to their positive dipole moments (3.099, 1.741, and 2.530, respectively), which suggest an electrochemical affinity with DNA (Williams and Maher, 2003). Indirect genotoxicity can occur through mediation by reactive

oxygen species (ROS) (Cadet and Richard Wagner, 2013), indicating that ROS may be responsible for the DNA damage caused by Diuron and its metabolites (Behrens et al., 2016).

The comet assay reveals DNA fragmentation, which is indicative of DNA repair mechanisms (Zheng et al., 2023). At a concentration of 1.0 µM of Diuron and DCA, the percentage of damaged cells with a medium degree of damage is lower than at a concentration of 0.5 µM for both substances. This suggests that repair mechanisms are intensified at the higher concentration. However, the concentration of 5.0 µM resulted in a higher percentage of damaged cells with a medium degree of damage compared to the previous concentration. This suggests a possible exhaustion of the repair system's capacity (Zheng et al., 2023).

Therefore, this study supports previous studies and suggests that Diuron and its metabolites have genotoxic potential, resulting in significant DNA damage at the concentrations studied. However, the fragments are reparable since no micronuclei were identified, which are chromosomal fragments that indicate mutagenic effects.

The study found no significant difference in the presence of micronuclei in zebrafish erythrocytes between adult zebrafish exposures and those exposed to Diuron, which is classified as non-mutagenic by the U.S. EPA and EFSA regulatory agencies (US EPA, 2003; EFSA, 2005). However, previous research has shown that the concentration and duration of exposure may affect micronucleus formation, as observed in adult zebrafish exposed to Diuron (Bony et al., 2010). Diuron has been theorized to be aneugenic and may impact mitotic chromosome segregation (Bony et al., 2010). Other studies have also confirmed the mutagenic nature of Diuron in various organisms such as oysters, and fish (Michael, 2018).

The cause of nuclear abnormalities (NAs) varies based on the shape of the nuclei observed after exposure. Bubbles or buds may indicate genotoxic damage, while notched nuclei may suggest cytotoxicity (Bolognesi and Cirillo, 2014; Canedo et al., 2021). Diuron, DCA, and DCPMU have been found to exhibit cytotoxic effects in various contexts (Huovinen et al., 2015; Mohammed et al., 2020). Lobed nuclei, extensions that indicate cytotoxicity, differ morphologically from bubbles and buds. Nuclear constriction and kidney-shaped nuclei, classified as nuclear pleomorphisms, may result from alterations in mitotic spindle fibers. NAs in erythrocytes have been associated with

genotoxic, cytotoxic, and epigenetic mechanisms (Bolognesi and Cirillo, 2014; Canedo et al., 2021). The tested substances can affect epigenetic factors, disrupt the microtubule of the metaphase spindle, cause cytogenetic effects, and alter DNA methylation and histone proteins (Ren et al., 2017). In this context, DCA disrupts the cell cycle by interfering with the anaphase complex and the expression of mitosis-specific genes (Sawle et al., 2010).

Herein, we evaluated histologically the toxicity of Diuron, DCA and DCPMU in adult zebrafish tissues. The liver, spleen and brain organs were analyzed. These analyzes were carried out, because histological alterations have been suggested as useful biomarkers of environmental contamination (Shah and Parveen, 2022). Despite this biotransformation process from Diuron to DCA and DCPMU, we did not find any significant effects assessing the acute effects on the zebrafish liver, spleen and brain for 96 hours. No studies were found in the literature that describe the potential effects of these pollutes in these organs of adult zebrafish during this exposure period. On the other hand, although not significant we observe mild presence of structural change, vacuolization and hyaline droplets were seen inside hepatocytes of zebrafish at higher concentrations (10.0 µM) of DCA and DCPMU. It is already established that the herbicide Diuron causes alterations in the liver of rats (da Silva Simões et al., 2017). In fish, Kamarudin et al. (2019), demonstrated histopathological changes in the liver of adult medaka fish (*Oryzias javanicus*) exposed to diuron at concentrations of 500 µg/L and 1000 µg/L. And in a recent study with fish, it was shown that DCA promotes fatty liver in zebrafish larvae exposed to concentrations of 1 - 10 µM/L (Park et al., 2020).

Due to the continuous use of herbicide Diuron, the metabolization of DCA and DCPMU and consequent discharging in the environment, there is concern about the possible damage that these products may cause to the aquatic ecosystem and as a consequence for human health. In summary, results suggest that Diuron, DCA and DCPMU exert toxic effects on zebrafish at different life stages. The study model revealed to be sensitive on endpoints studied, presenting alterations after exposure to herbicides at concentrations in the range of environmental concentrations. DCA and DCPMU appear to have a stronger effect than diuron on embryotoxicity. At biochemical level, the DCA affected the AChE activity suggesting impairments in neurotransmission. On the other hand, diuron causes DNA damage and nuclear abnormalities in zebrafish, such as DCA and DCPMU. These

results suggest that diuron and its degradation products, DCA and DCPMU, may be substances of concern due to their potential adverse effects on zebrafish.

The results of this study highlight the importance of analyses with target and non-target organisms to build the weight of evidence for environmental and human health. Practically, the results highlight the importance of toxicological assessment of pesticide metabolites considering their persistence in the environment. Furthermore, these findings are relevant in the regulatory field and may contribute to the re-evaluation and regulation of Diuron by the competent authorities. Finally, this study highlights the growing importance of the zebrafish as a model organism in the field of environmental toxicology.

It is hoped that this research will contribute to the understanding of the potential adverse effects and mechanisms of action of Diuron and its metabolites, providing important information to support the proposal for the safe use of the herbicide diuron.

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CERTIFICADO Nº 1304/2019-CEUA

Certificamos que o projeto intitulado: **"Efeitos agudos e crônicos do diuron e seus metábolitos sob Zebrafish: Toxicidade Bioquímicos e reprodução."**, conduzido pela Pesquisadora: **Bianca Camargo Penteado Sales –** Orientadora: Profa. Dra. Lillian Cristina Pereira de Andrade, registrada com o **nº 1304/2019**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, sub para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 08 de outubro de 2008, do Decreto n. 6. as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela Comissão de Ética no Uso de A de Botucatu, em reunião extraordinária de 07 de agosto de 2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	28 de fevereiro de 2023
Espécie/Linhagem/Raça	Zebrafish
Nº de animais	168
Idade/Peso	Adultos (criação) – aprox. 1 gr
Sexo	Machos e Fêmeas
Origem	Biotério Unipex

Sara Rosa Stanley Sampaio
Secretária da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Profa. Associada Bert
Presidente da Comissão de
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