UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

MARIA EDUARDA DE SOUZA MAIA

AVALIAÇÃO DA TOXICIDADE DOS HERBICIDAS DIURON, GLIFOSATO, ATRAZINA E SUAS MISTURAS EM EMBRIÕES E LARVAS DE PEIXE-ZEBRA (*Danio rerio*)

FORTALEZA
2023

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FORTALEZA
2023

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

M187a Maia, Maria Eduarda de Souza.
Análise da toxicidade dos herbicidas diuron, glifosato, atrazina e suas misturas em embriões e larvas de peixe-zebra (Danio rerio) / Maria Eduarda de Souza Maia. – 2023.
69 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2023.
Orientação: Prof. Dr. Davi Felipe Farias.
Coorientação: Prof. Dr. Luis Fernando Marques dos Santos.

1. Espécies reativas de oxigênio. 2. Estresse oxidativo. 3. Mistura de contaminantes. 4. Pesticidas. 5. Poluição aquática. I. Título.

CDD 572

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Aprovado em: 04/08/2023.

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AGRADECIMENTOS

À UFC (Universidade Federal do Ceará) e à UFPB (Universidade Federal da Paraíba) por terem possibilitado minha formação acadêmica.

Ao programa de pós-graduação em Bioquímica (PPGB) e a todo o seu corpo docente pelo apoio e orientação ao longo dessa jornada.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) por seu apoio e contribuição para o desenvolvimento deste trabalho.

Ao professor Davi Farias, que além de ser meu orientador, foi um amigo constante, contribuindo significativamente para o meu crescimento profissional e pessoal.

Ao professor Luís Fernando, pela valiosa ajuda e orientação no processo de realização deste projeto.

Aos meus colegas de laboratório, Rafael Xavier, Matheus Alves, Leonardo Vieira, Juliana Costa e aos demais estudantes do Labrisco, por todo companheirismo e amizade durante o processo.

A Carlos, cuja inspiração, apoio constante e amor foram essenciais para minha trajetória.

Aos meus pais, Eduardo e Giselly, que sempre me incentivaram e nunca mediram esforços para me ajudar a alcançar meus objetivos.

À minha família, incluindo meus tios e primos, que desempenharam um papel significativo no meu crescimento pessoal. Seu apoio e encorajamento ao longo dos anos foram essenciais para minha jornada acadêmica.

Aos meus amigos, em especial Monalisa e Yasmim, cuja presença e apoio constante têm sido inestimáveis ao longo dessa jornada.

RESUMO

A agricultura convencional tem-se mostrado altamente produtiva e, assim, atendido a uma demanda por alimentos cada vez maior. Apesar das vantagens aparentes, esse modelo agrícola é dependente do uso de agrotóxicos, incluindo os herbicidas, que são o grupo de defensivos agrícolas mais utilizado no Brasil. Dentre estes, glifosato, atrazina e diuron têm sido comercializados em grandes volumes para aplicação em lavouras convencionais e transgênicas. Uma preocupação que acompanha o uso em larga escala destes herbicidas são os seus efeitos tóxicos em organismos não alvo, com destaque para o desequilíbrio de espécies reativas de oxigênio (ERO's) associado a danos celulares. Além disso, a maior parte do que se sabe sobre a toxicidade dessas substâncias é oriunda de estudos com elas isoladas, afastando-se do que normalmente é encontrado no ambiente que é a presença de misturas de contaminantes. Dado o exposto, o objetivo deste trabalho é avaliar a toxicidade aguda dos herbicidas glifosato, atrazina e diuron, isoladamente e em misturas, em embriões e larvas de peixe-zebra (*Danio rerio*).Os resultados revelaram uma relevante toxicidade aguda para o diuron e atrazina, com valores de CL_{50} de 9,6 mg/L e 53.57 mg/L, respectivamente, para peixes-zebra de 96 horas de idade. Por outro lado, nenhum efeito foi observado para o glifosato isoladamente na concentração máxima testada (100 mg/L). A mistura binária de diuron e atrazina apresentou um efeito sinérgico, resultando nos menores valores de CL_{50} medidos, 2,96 mg/L e 16,46 mg/L, respectivamente, entre todas as combinações realizadas. Ao contrário da ausência de efeitos quando isolado, o glifosato aumentou a toxicidade individual do diuron e atrazina, resultando em uma diminuição nos valores de CL_{50} para 5,2 mg/L e 33,03 mg/L, respectivamente, embora não tenha sido considerado sinérgico. Todos os biomarcadores analisados (AChE, LDH, GST, CAT e GPx) mostraram alterações significativas. Além disso, foi observado aumento na produção de ERO's nas larvas expostas aos herbicidas isolados e à mistura de atrazina e diuron. Esses resultados mostraram que os herbicidas podem se tornar mais tóxicos quando presentes em misturas e que o mecanismo de toxicidade, tanto em sua forma isolada quanto em misturas, está pelo menos parcialmente associado ao estresse oxidativo.

Palavras-chave: espécies reativas de oxigênio; estresse oxidativo; mistura de contaminantes; pesticidas; e poluição aquática.

LISTA DE ABREVIATURAS E SIGLAS

ACHE	Acetilcolinesterase
CAT	Catalase
DCA	3,4-dicloroanilina
DCMU	3-(3,4-diclorofenil)-1,1-dimetilureia
DDT	Diclorodifeniltricloroetano
EPSPs	5-enolpiruvil-chiquimato-3-fosfato-sintase
EROS	Espécies Reativas de Oxigênio
GPX	Glutationa Peroxidase
GST	Glutationa S-Transferase
Hpf	Hora pós fertilização
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
LDH	Lactato desidrogenase
NAD	Nicotinamida adenina dinucleotídeo
NADH	Nicotinamida adenina dinucleotídeo (forma reduzida)
OMS	Organização Mundial da Saúde
OPAS	Organização Pan-Americana da Saúde
PARA	Programa de Ação para o Meio Ambiente
SISAGUA	Sistema de Informação de Vigilância da Qualidade da Água para Consumo Humano
USEPA	United States Environmental Protection Agency

SUMÁRIO

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Pesticidas

A expansão das práticas agrícolas, resultante do aumento populacional crescente e da implementação da Revolução Verde, gerou a necessidade de incorporar novos elementos para suprir a demanda por alimentos e obter um rendimento agronômico mais eficiente. Esses elementos englobam a adoção de novas variedades de culturas, a aplicação de práticas de manejo mais avançadas e, principalmente a utilização massiva de pesticidas (LOPEZ *et al*., 2018; JARDIM, 2009).

De acordo com a Lei Federal no 7.802, em seu Artigo 2, Inciso I, os pesticidas, também conhecidos como agrotóxicos se definem como:

> "Produtos e os componentes de processos físicos, químicos ou biológicos destinados ao uso no setor de produção, armazenamento e beneficiamento de produtos agrícolas, nas pastagens, na proteção de florestas nativas ou implantadas e de outros ecossistemas e também em ambientes urbano, hídricos e industriais, cuja finalidade seja alterar a composição da flora e da fauna, a fim de preservá-la da ação danosa de seres vivos considerados nocivos, bem como substâncias e produtos empregados como desfolhantes, dessecantes, estimuladores e inibidores do crescimento"

Portanto, o termo agrotóxico é comumente utilizado para se referir a produtos químicos agrícolas, como bactericidas, fungicidas, herbicidas, inseticidas ou raticidas, que têm como objetivo controlar pragas, como ervas daninhas e insetos (JAYARAJ, MEGHA, SREEDEV, 2016). Esses produtos apresentam diferentes classificações, baseadas em critérios como sua composição química, o alvo específico a ser combatido, bem como aspectos relacionados à toxicidade (OPAS/OMS, 1996).

Em relação à classe química, a maioria dos agrotóxicos podem ser classificados como organoclorados, organofosforados, piretroides e carbamato (GEORGIADIS *et al*., 2018). Os organoclorados são compostos orgânicos sintéticos que possuem o elemento cloro em suas moléculas, sendo uma classe muito utilizada em meados do século XX, com destaque para o DDT (MENDES *et al*., 2019). Eles atuam no sistema nervoso central dos insetos, interferindo na transmissão dos impulsos nervosos. Os piretroides, são derivados sintéticos da piretrina e tem como mecanismo de ação o bloqueio de canais de cálcio resultando na paralisação do inseto (PAVANI, 2016; OLIVEIRA et al., 2015). Os organofosforados e carbamatos atuam inibindo a ação da enzima acetilcolinesterase, que leva ao acúmulo de acetilcolina nas

sinapses nervosas. Isto resulta em superestimulação do sistema nervoso dos insetos, levando à paralisia e morte (PAVANI, 2016).

Os agrotóxicos podem ser classificados em mais de 15 categorias, com destaque para os inseticidas, fungicidas e herbicidas. Os fungicidas são compostos químicos utilizados para controlar e prevenir o crescimento de fungos que podem causar doenças em plantas, reduzindo assim a produtividade. Da mesma forma, os inseticidas são utilizados para eliminar ou controlar insetos que representam uma ameaça às plantações e à saúde humana (YADAV e DEVI, 2017). Por sua vez, os herbicidas são produtos químicos amplamente utilizados na agricultura para eliminar as ervas daninhas que competem com as culturas cultivadas. Eles agem inibindo a atividade de enzimas ou proteínas essenciais nas células das plantas daninhas, o que leva à interrupção de processos vitais e ao impedimento do seu desenvolvimento (EMBRAPA, 2008).

Em relação à toxicidade, os agrotóxicos podem ser classificados em: Classe I -extremamente tóxica (faixa vermelha), classe II - altamente tóxica (faixa amarela), classe III - medianamente tóxica (faixa azul) e classe IV - pouco tóxica (faixa verde). Essa classificação se baseia em pesquisas que avaliam os efeitos tóxicos causados pelos químicos (EMBRAPA, 2010).

Ademais, eles também podem ser classificados em relação ao potencial de periculosidade ambiental como: Classe I - Produto altamente perigoso ao meio ambiente; Classe II - Produto muito perigoso ao meio ambiente; Classe III - Produto perigoso ao meio ambiente; e, Classe IV - Produto pouco perigoso ao meio ambiente. Essa classificação leva em conta fatores como a toxicidade dos agrotóxicos, sua persistência no ambiente, seu potencial de bioacumulação e os efeitos adversos na fauna, flora e recursos hídricos (IBAMA, 2016).

No ano de 2021, foi registrado um expressivo volume de comercialização de agrotóxicos em território brasileiro, totalizando mais de 600 mil toneladas. Dentre os agrotóxicos mais vendidos, merecem destaque os herbicidas glifosato (N-(fosfonometil)glicina), com um volume superior a 200 mil toneladas, a atrazina (2-cloro-4-etilamino-6-isopropilamino-s-triazina), com mais de 33 mil toneladas, e o diuron (3-(3,4-diclorofenil)-1,1-dimetiluréia ou DCMU), com mais de 6 mil toneladas (IBAMA, 2017).

Apesar do aumento da produtividade agrícola, a crescente utilização dos agroquímicos, além de ser maléfica para a saúde humana, pode contaminar os compartimentos do ecossistema como a água e o solo, causando problemas ambientais

(BHAT *et al*., 2015; ISLAM, 2017). Em vista disso, estudos relataram que apenas 0,1% dos pesticidas utilizados exercem efeito nos organismos alvo, acarretando um grande acúmulo de resíduos no ambiente (ISLAM, 2017).

Os resíduos dos agrotóxicos podem atingir as águas superficiais através do escoamento das plantas tratadas e do solo (SYAFRUDIN *et al.*, 2021). Apesar dos mesmos passarem por vias de degradação ambiental, os resíduos mais estáveis podem permanecer no meio ambiente. Consequentemente, isso poderá acarretar efeitos deletérios diretos para organismos não-alvos como peixes, invertebrados e plantas aquáticas (AKTAR *et al*., 2009).

Além da degradação do ecossistema, o contato com os agrotóxicos através de alimentos e da água potável, pode causar efeitos adversos à saúde humana. Um terço dos alimentos consumidos cotidianamente pelos brasileiros estão contaminados com agrotóxicos, segundo análise de amostras coletadas em 26 estados brasileiros realizada pelo Programa de Análise de Resíduos de Agrotóxicos em Alimentos (PARA) da ANVISA (ANVISA, 2011).

Outro dado alarmante, é referente aos agrotóxicos encontrados na água potável. Para auxiliar no acompanhamento dessas informações é utilizado o Siságua (Sistema de Informação de Vigilância da Qualidade da Água para Consumo Humano), que compila os dados referentes às análises de resíduos pelas empresas de abastecimento de água. Com a análise desses dados, foi possível encontrar os 27 agrotóxicos monitorados na água potável de 25% dos municípios brasileiros entre 2014 e 2017 (ARANHA, 2019).

Estes dados chamam atenção, visto que a exposição direta ou indireta a determinados agrotóxicos pode acarretar o aumento de doenças relacionadas à reprodução, cardiovasculares e vários tipos de câncer (ALAVANJA *et al*., 2003; SAMSUDDIN *et al*., 2015; SYAFRUDIN *et al*., 2021).

1.2 Glifosato

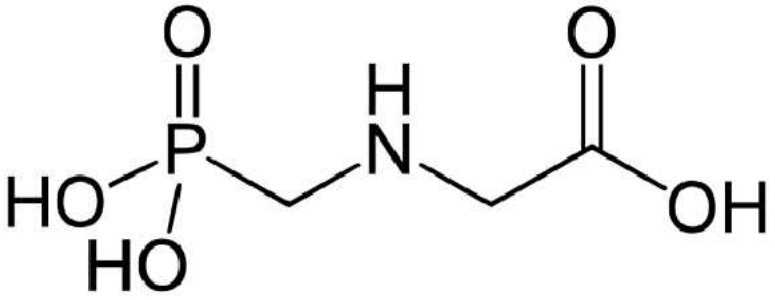
O glifosato (Figura 1) é o ingrediente ativo de uma gama de herbicidas comerciais para controle não seletivo de plantas daninhas (BAER; MARCEL, 2014). Este foi o princípio ativo mais vendido do Brasil entre os anos de 2014 e 2018, representando 35,5% do total das vendas de agrotóxicos o que equivale a 943.626,43 toneladas (IBAMA, 2020).

O seu mecanismo de ação consiste em inibir a enzima 5-enolpiruvil-chiquimato-3-fosfato-sintase (EPSPs), que atua na via do chiquimato. Esta via bioquímica está envolvida na síntese de aminoácidos aromáticos como tirosina, triptofano e fenilalanina, compostos importantes em diversos aspectos essenciais da planta como a produção de hormônios e flavonóides (MARCHI, 2008; ROSS; CHILD,1996)

O glifosato e os herbicidas baseados em glifosato têm sido alvo de extensas investigações nos últimos anos, culminando com a alteração da classificação quanto ao risco de carcinogenicidade em humanos (PORTIER *et al*., 2016). Além disso, devido ao seu extenso uso durante os últimos anos, houve um aumento do registro dos seus resíduos nos compartimentos aquáticos. Trabalhos já reportaram uma concentração em riachos e córregos pode variar entre 0,03 e 303,0 µg/L, dependendo do local de amostragem (ANNETT; HABIBI; HONTELA, 2014). Na Espanha, foram analisadas 140 amostras de água subterrânea, onde foi possível detectar a presença do glifosato em 41% das amostras (SANCHIS *et a*l., 2012).

Com isso, alguns estudos foram feitos para observar a toxicidade em organismos aquáticos não-alvos. Segundo Rodrigues (2016), com a exposição ao glifosato foi possível observar toxicidade no desenvolvimento larval do microcrustáceo *Artemia salina*, além de retardar o desenvolvimento embrionário do ouriço-do-mar mediterrâneo *Sphaerechinus granularis* (MARC *et al*., 2015). Também foram observados múltiplos efeitos tóxicos no estágio embrionário e larval de várias espécies de peixes. Em peixes dourados (*Carassius auratus*), o glifosato em níveis moderadamente baixos perturbou o metabolismo de vários tecidos, além de levar à superprodução de ROS e estresse oxidativo (Li et. al., 2017). Também foi visto o aumento das espécies reativas de oxigênio (ERO`s) no peixe zebra (*Danio rerio*), além de apresentar toxicidade aos sistemas reprodutor, cardiovascular e locomotor (WEBSTER, 2014; ROY, 2016).

Figura 1: Estrutura do glifosato



Fonte: Colaciti, 2020

1.3 Diuron

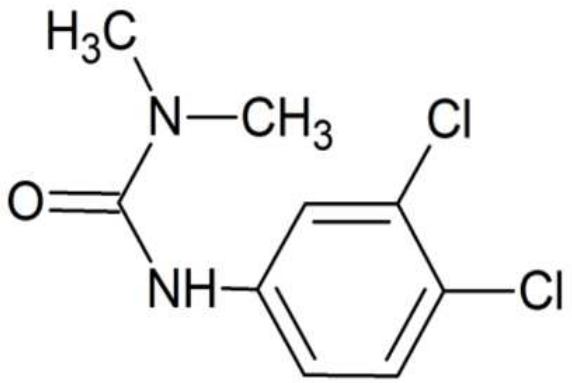
O diuron, também conhecido como DCMU, é um herbicida da classe das fenilureias (Figura 2). Mundialmente utilizado há mais de 40 anos, o diuron é eficiente no controle de uma ampla variedade de ervas daninhas (CABRERA *et al*., 2010). Em relação ao seu

mecanismo de ação, ele atua inibindo a fotossíntese, mais especificamente bloqueando a transferência de elétrons no nível do fotossistema II (ANTONIOU *et a*l., 2014),

Devido a sua meia-vida longa no solo de mais de 90 dias e potente ação herbicida (considerado o mais potente de sua classe), o diuron tem sido alvo de muitos estudos sobre seus efeitos nocivos ao meio ambiente (ROCHA *et al*., 2013; HOLMES, 2014). Estudos têm detectado à presença do diuron em ambientes marinhos costeiros. Na França, o diuron foi detectado em 28% das amostras de rios do sistema de bacias nacionais (IFEN, 1998), resultando um alerta a comunidade científica sobre os possíveis riscos ecotoxicológicos à biota marinha (HAYNES *et al*., 2000; JONES *et al*., 2003).

Alguns estudos já relataram o efeito tóxico do diuron em espécies aquáticas. De Lorenzo (2012) relatou a toxicidade do diuron em diversos organismos aquáticos, como bivalves (*Crassostrea virginica* e *Mytilus edulis*), crustáceos (*Palaemonetes pugio*) e urocordados (*Ciona intestinalis*). Além disso, também foi possível observar efeitos tóxicos em espécies de peixes, como o peixe zebra com uma CL_{50} de 6,5 mg/L, além de apresentar efeitos não letais como edema de saco vitelínico, edema de pericárdio e malformações na coluna (VELKI *et al*., 2017).

Figura 2: Estrutura do diuron



Fonte: Lopes e colaboradores, 2021

1.4 Atrazina

A atrazina (Figura 3) é um herbicida da classe das triazinas, que consiste em um grupo que representa cerca de 30% da produção mundial de pesticidas. Este herbicida é amplamente utilizado para controle e erradicação de ervas daninhas em culturas de milho, cana de açúcar, entres outras plantas dicotiledôneas (CANEVAROLI *et al*, 2021). A atrazina tem ação direta na membrana do cloroplasto, local onde se desenrola a fase luminosa da fotossíntese. Mais precisamente, ela interfere no transporte de elétrons, resultando em uma diminuição da

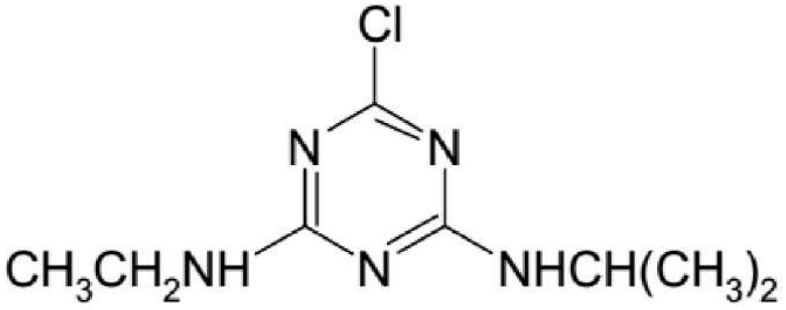
capacidade das ervas daninhas em converter a energia luminosa em energia química. Tal efeito afeta negativamente o crescimento e desenvolvimento dessas plantas indesejadas (COUTINHO *et al*., 2005).

Devido ao seu amplo uso e à sua degradação lenta, com uma meia-vida de 13-260 dias, a atrazina tornou-se uma preocupação ambiental em muitos países (USEPA, 2006). Estudos têm detectado a presença desse herbicida em águas doces no Brasil, com concentrações variando de 0,0027 a 10,4 µg/L em águas superficiais e de 0,001 a 42,77 µg/L em águas subterrâneas (DIAS *et al*., 2018; CANEVAROLI *et al.*, 2021). Em um estudo realizado na bacia do rio Yamaska em Québec, no Canadá, a atrazina foi monitorada em cinco rios que drenam áreas agrícolas, e foram observadas concentrações que variaram de 0,01 a 26,9 µg/L (MUIR *et al*., 1978).

A atrazina tem sido amplamente estudada devido à sua toxicidade em animais aquáticos. Estudos revelaram que a exposição à atrazina pode causar efeitos adversos no peixe-zebra, como malformações e edemas (Gorge, 1990). Além disso, foi observada a toxicidade da atrazina para o peixe guppy (*Poecilia reticulata*), com um valor de CL_{50} relatado de 4,3 mg/L (Solomon *et al*., 2013).

Ademais, estudos indicam que a atrazina pode afetar o sistema endócrino de mamíferos e peixes, interferindo na regulação hormonal e perturbando a função normal do sistema endócrino (Wirbisky e Freeman, 2015).

Figura 3: Estrutura química da atrazina



Fonte: Canevaroli e colaboradores (2021)

1.5 Estresse oxidativo induzido por pesticidas

Os efeitos dos agrotóxicos podem ser bioquímicos, fisiológicos e morfológicos, causando mudanças em nível molecular, celular ou tecidual. Apesar dos mecanismos de ação associados ao efeito tóxico dos herbicidas não serem bem compreendidos, pesquisas

anteriores sobre os mesmos sugerem que a maioria dos agrotóxicos induzem estresse oxidativo (SULE; CONDON; GOMES, 2022).

O estresse oxidativo é causado pelo desbalanço da formação de ERO's e a capacidade de um sistema biológico de desintoxicar esses produtos reativos (LI; JIA; TRUSH, 2016). São compostos que consistem em subprodutos de processos metabólicos celulares normais necessários para gerar energia para os processos vitais. Porém, quando ocorre uma superprodução, decorrente de fatores ambientais ou à exposição a xenobióticos, podem acarretar danificação de moléculas biologicamente relevantes, como DNA, proteínas, carboidratos e lipídios (LOBO *et al*., 2010). Com isso, causando peroxidação lipídica, oxidação de proteínas e modulação da expressão gênica (SLANINOVA, 2009), além de disfunção celular e apoptose (MELE *et al*., 2006; LI; JIA; TRUSH, 2016).

Alguns estudos relatam correlação entre a formação de ERO's e exposição a agrotóxicos. Estudos em torno do metabólito do diuron, DCA (3,4-dicloroanilina), sugerem que a geração de ERO's e a depleção dos antioxidantes induzida por esta anilina pode causar estresse oxidativo e peroxidação lipídica no fígado de carpa cruciana (*C. carassius*) (LI *et al.* 2003). Sendo este efeito, uma das causas para as lesões encontradas no fígado (SLANINOVA, 2009). Resultados similares foram observados na tilápia do Nilo (*Oreochromis niloticus*), onde foi visto que o diuron e seus metabólitos prejudicaram a resposta fisiológica relacionada à biotransformação e atividade antioxidante (FELICIO *et al*., 2018).

Outros herbicidas que já foram relatados como compostos que induzem o estresse oxidativo são a atrazina e o glifosato. Yang e colaboradores (2021) relataram a imunotoxicidade e aumento das ERO`s em crustáceos da espécie *Procambarus clarkii*. Resultados semelhantes foram vistos no animal modelo *Caenorhabditis elegans*, onde também foi possível observar o aumento de ERO`s quando exposto ao glifosato (BAILEY *et al*., 2018).

1.6 Misturas de pesticidas

A maioria dos estudos em torno da toxicidade de agrotóxicos são feitos utilizando apenas os princípios ativos de forma isolada. Isto diverge da realidade, visto que as formulações comerciais de agrotóxicos são frequentemente aplicadas às culturas combinadas a outros agrotóxicos, como forma de melhorar o rendimento das colheitas (PHYU *et al*., 2001).

Neste contexto, mesmo que um agrotóxico isolado não tenha efeito sobre a saúde humana ou sobre os organismos não alvo no ambiente, a ação conjunta dos constituintes da

mistura pode influenciar a absorção, distribuição e o metabolismo dos agrotóxicos, bem como sua interação com os organismos não-alvo e o ambiente. Consequentemente, isto pode levar um ingrediente ativo individual a produzir maior (sinergismo) ou menor toxicidade (antagonismo) ao organismo em relação ao que pode ocorrer quando esse químico está sozinho (PHYU *et al*., 2001; MANSANO *et al*., 2020).

Apesar da escassez de estudos sobre toxicidade de misturas de agrotóxicos, alguns trabalhos já relataram o aumento dos efeitos tóxicos das misturas quando comparado com a toxicidade individual. Wang (2017) observou o efeito de 7 agrotóxicos e suas respectivas misturas binárias, ternárias e quaternárias em embriões de peixe-zebra, mostrando que quase 50% das formulações apresentaram efeitos sinérgicos.

Marins e colaboradores (2020), conduziram um estudo para investigar os efeitos tóxicos da exposição a uma mistura de pesticidas compostos por imidaclopride e o propoxur em concentrações ambientais sobre o peixe da espécie *Rhamdia quelen.* Os resultados mostraram que a mistura desses pesticidas em concentrações ambientais inibiu a atividade da acetilcolinesterase no cérebro e induziu dano oxidativo em todos os tecidos analisados.

1.7 Biomarcadores de estresse oxidativo e estresse metabólico

Como já citado, o estresse oxidativo ocorre quando há o desbalanço entre a produção de ERO`s e sua neutralização, sendo uma resposta comum a organismos expostos a substâncias tóxicas ou condições ambientais adversas (Halliwell *et al*., 2007). Com isso, alguns biomarcadores como: Glutationa – S- transferase (GST), glutationa peroxidase (GPx) e catalase (CAT), são utilizados para mensurar os danos oxidativos causados (HO *et al*., 2013).

A GST é uma família de enzimas que desempenham um papel fundamental no metabolismo e desintoxicação de uma ampla variedade de xenobióticos, incluindo os produtos químicos ambientais (Gertsch, 2007). Consistem em uma medida de defesa antioxidante, pois catalisam a conjugação de glutationa reduzida (GSH) com esses compostos, tornando-os mais solúveis em água e prontos para serem eliminados do organismo (SHEEHAN *et al*., 2001). Portanto, quando o organismo entra em contato com substâncias que acarretam o estresse oxidativo, é possível observar um aumento da expressão dessa enzima (POUR *et al*., 2014).

A GPx pertence a uma família de enzimas oxidorredutases e desempenha um papel essencial na redução do peróxido de hidrogênio em água e oxigênio, resultando na eliminação de radicais livres (Fanuchi, 2014). Da mesma forma, a CAT é outra enzima antioxidante que desempenha um papel crucial na prevenção do dano oxidativo. Esta enzima catalisa a

conversão de peróxido de hidrogênio em água e oxigênio, contribuindo para a remoção de ERO`s e protegendo as células contra o estresse oxidativo (Liu; Kokare, 2017).

A lactato desidrogenase (LDH) desempenha um papel crucial no metabolismo anaeróbio, catalisando a conversão reversível do piruvato em lactato, com a redução de NAD+ a NADH e vice-versa (FARHARA; LAPPIN, 2023). Essa enzima é considerada um biomarcador relevante de estresse metabólico, uma vez que está presente na maioria dos tecidos e é liberada em casos de dano tecidual (DYZOEM *et al*., 2014).

1.8 Biomarcadores de neurotoxicidade

Os biomarcadores de neurotoxicidade são componentes que podem ser medidos e avaliados para identificar e quantificar os efeitos adversos de substâncias tóxicas, como pesticidas, no sistema nervoso (AMORIM, 2003)

A acetilcolinesterase (AChE) é um biomarcador pertencente à família das enzimas colinesterases responsáveis pela hidrólise da acetilcolina, resultando na regulação dos disparos nervosos nas terminações nervosas. Sua função é essencial para o funcionamento adequado do sistema nervoso central e periférico (LIONETTO *et al*., 2013).

No entanto, a atividade dessa enzima pode ser inibida por certos produtos químicos e pesticidas, resultando no acúmulo de acetilcolina. Esse acúmulo tem consequências prejudiciais para o funcionamento normal do sistema nervoso periférico e central. Portanto, a mensuração da atividade da AChE é de grande importância em organismos expostos a compostos tóxicos, fornecendo informações valiosas sobre o impacto dessas substâncias no sistema nervoso (SILVA, 2010).

1.8 Utilização do peixe-zebra (*D. rerio*) para estudos de toxicidade

Os testes de toxicidade convencionais ainda dependem em grande parte do uso de animais, como roedores, em virtude de sua semelhança com os seres humanos (KRATCHMAN *et al*., 2018). No entanto, devido ao manejo trabalhoso, alto custo, questões éticas associadas e a crescente ênfase ao princípio dos 3Rs (redução, substituição e refinamento do inglês “Reduction”, “Replacement” e “Refinement”, respectivamente), os pesquisadores estão buscando métodos alternativos de baixo custo e alto rendimento, como modelos *in silico* e animais não convencionais (HONGMAO *et al*., 2011; FLECKNELL, 2011).

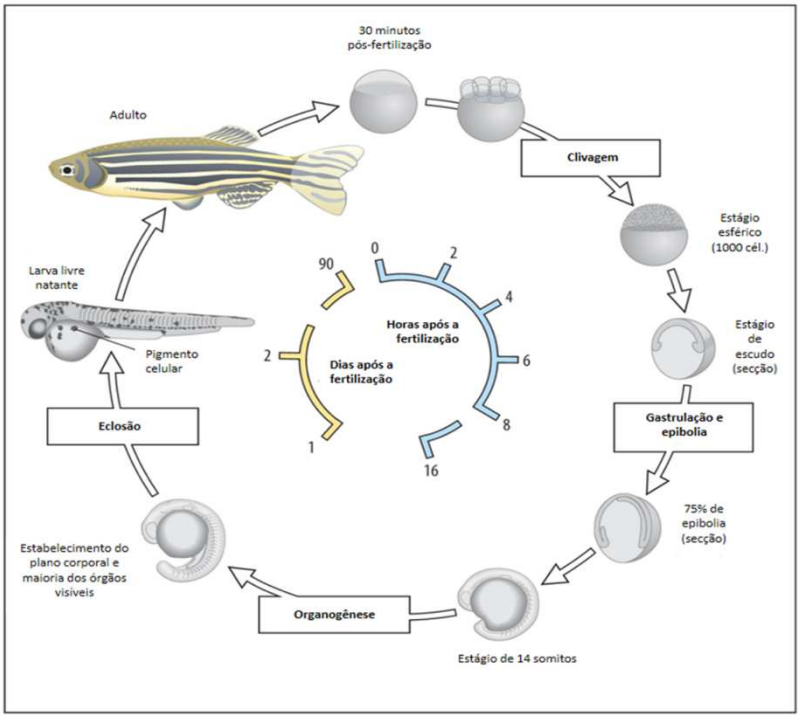
O *D. rerio* (Figura 4), mais conhecido como peixe-zebra, é um peixe tropical de pequeno porte e de água doce, que pertence à família Cyprinidae. Mesma família da carpa

comum (*Cyprinus carpio*) e do peixinho-dourado (*C. auratus*). O corpo do peixe-zebra tem formato fusiforme e comprimido lateralmente, possuindo listras de cores alternadas e com uma boca oblíqua terminal voltada para cima (GERLACH, 2008). A reprodução ocorre de forma externa, produzindo proles numerosas. Além disso, possui um ciclo de vida curto, precisando apenas de 48 a 72 horas para evoluir do estado de ovo para larva, se tornando adulto aos 3 meses de vida e tendo a capacidade de sobreviver por cerca de 3 anos (Figura 5) (SILVEIRA *et al*., 2011; MARTINS, 2017). É um organismo que tem se destacado como um sistema modelo eficiente para a avaliação de efeitos tóxicos de compostos químicos e medicamentos, em virtude de suas características biológicas (GERLACH, 2008).

Figura 4- Peixe-zebra (*Danio rerio*) em sua fase adulta.

Fonte: MTI news (2022)

Figura 5- Ciclo de desenvolvimento do peixe-zebra (*D. rerio*).



Fonte: Adaptado de D'Costa e Shepherd (2009).

O peixe-zebra tem se destacado como um organismo preferencial em várias áreas, especialmente na Toxicologia, devido a características importantes, tais como seu tamanho pequeno, desenvolvimento rápido, tempo de geração curto, facilidade de manejo, dimorfismo sexual, genoma totalmente sequenciado e disponível em bancos de dados públicos, além de uma alta homologia genética com os humanos de aproximadamente 70% (HOWE *et al*., 2003; LAWRENCE, 2007).

Além disso, os embriões e larvas de peixe-zebra (Figura 6a e 6b) estão sendo amplamente utilizados para triagem de toxicidade de compostos e novos medicamentos, sendo muito aceito para ensaios alternativos para avaliar a toxicidade aguda em vertebrados (CASSAR *et al*., 2020). Isso ocorre em decorrência de suas vantagens, tais como alta sensibilidade a compostos químicos e rápido desenvolvimento dos sistemas renal, hepático e cardiovascular (FUKUSHIMA *et al*., 2020; CASSAR *et al*., 2020).

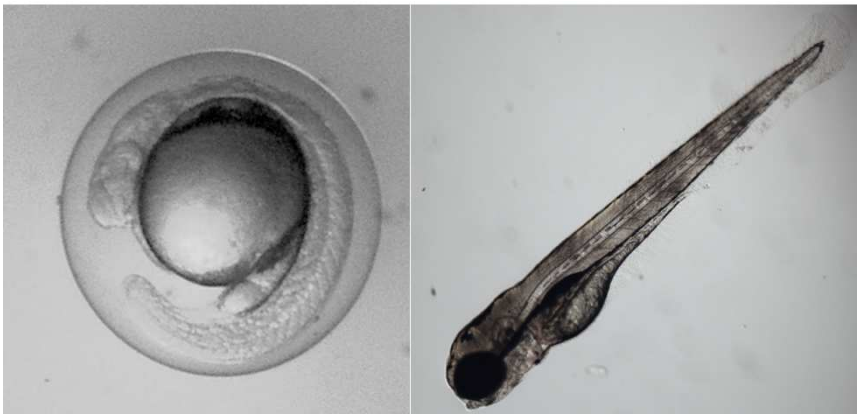
Outra vantagem é a transparência dos embriões, que desempenham um papel fundamental nos testes de toxicidade. Essa característica permite a fácil visualização de mudanças fenotípicas e malformações durante a exposição aos compostos em estudo (MARTINS *et al*., 2021). Um exemplo notável é o teste de toxicidade aguda em embriões de peixe-zebra (FET), estabelecido como o teste nº 236 pela Organização para Cooperação e Desenvolvimento Econômico (OCDE) em 2013. Esse teste tem como objetivo avaliar os efeitos da exposição a determinados compostos químicos durante o estágio inicial de

desenvolvimento dos peixes. A transparência dos embriões de peixe-zebra desempenha um papel crucial nesse teste, permitindo através de uma observação direta e detalhada dos efeitos adversos, sejam eles letais ou subletais, causados pelas substâncias em análise (HILL *et al*., 2005).

Com isso, muitas substâncias, como herbicidas, são testadas em embriões e larvas de peixe-zebra. Martins e colaboradores (2021) observaram a hepatotoxicidade do 2,4-D em embriões e larvas de peixe-zebra, revelando a toxicidade do composto ao fígado do peixe. Resultados similares foram observados em outros modelos como camundongos, além de estudos *in vitro*, utilizando hepatócitos (TAYEB *et al*., 2013; DAKHAKHNI *et al*.,2015). Velki e colaboradores (2017), também observaram toxicidade moderada do diuron e dianizon nos estágios iniciais do peixe-zebra, além de mudança comportamental.

O peixe-zebra também pode ser utilizado para analisar mecanismos de ação de toxicidade. Blahová e colaboradores (2013) constataram um aumento na produção de ERO`s e estresse oxidativo em larvas expostas à atrazina. Da mesma forma, Lanzarin e colaboradores (2021) observaram resultados semelhantes em embriões e larvas expostos a concentrações ambientalmente relevantes de uma formulação comercial de glifosato.

Figura 6- Embrião (à esquerda) e larva (à direita) de peixe-zebra



Fonte: Autoral (2023)

2 HIPÓTESE

- As misturas binárias e terciária dos herbicidas testados são mais tóxicas do que os herbicidas isolados aos embriões e larvas de peixe-zebra, visto que que a interação entre diferentes substâncias químicas pode potencializar os efeitos negativos e prejudicar ainda mais o desenvolvimento e a sobrevivência desses organismos aquáticos.
- Os mecanismos de toxicidade dos herbicidas em embriões e larvas envolvem, pelo menos em parte, a produção aumentada de espécies reativas de oxigênio e, consequentemente, estresse oxidativo. Isto se explica pelo fato de que os herbicidas podem perturbar os sistemas de defesa antioxidante das células, levando a um desequilíbrio na produção ERO's. Esse desequilíbrio resulta em danos celulares, disfunção e possível morte celular, contribuindo para os efeitos adversos observados nos embriões e larvas expostos aos herbicidas

3 OBJETIVOS

3.1 Objetivos gerais

Avaliar os efeitos dos herbicidas diuron, glifosato, atrazina de forma isolada e de suas misturas binárias e terciária no sistema modelo peixe-zebra (*D. rerio*), com foco na toxicidade durante seus estágios iniciais de vida e análise dos mecanismos de estresse oxidativo.

3.2 Objetivos específicos

- Avaliar a toxicidade aguda da atrazina, glifosato e diuron isolados em embriões e larvas de peixe-zebra;
- Analisar a toxicidade aguda das misturas binárias e terciária dos herbicidas em embriões e larvas de peixe-zebra;
- Compreender preliminarmente os mecanismos de estresse oxidativo em embriões e larvas expostos dos herbicidas isolados e das misturas.

4 MANUSCRITO

Os resultados obtidos neste trabalho foram submetidos para publicação no periódico científico *Science of the Total Environemnt* (https://www.sciencedirect.com/journal/science-of-the-total-environment), fator de impacto (JCR 2022) 9,8, Qualis A1.

"Acute Toxicity and Oxidative Stress Induced by Glyphosate, Atrazine, and Diuron Herbicide Mixtures on Zebrafish Embryos and Larvae

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Highlights

- Diuron was the most toxic individually.
- Glyphosate alone did not show any toxic effects.
- Atrazine and diuron mixture showed toxic synergy.
- Glyphosate increased the toxicity of atrazine and diuron.
- All biomarkers of oxidative stress were altered.
- There was an increase in the production of ROS.

Abstract

This study aimed to assess the acute toxicity of the herbicides glyphosate, atrazine, and diuron individually and in binary and ternary mixtures on zebrafish embryos and larvae, exploring their mechanisms of oxidative stress. The results revealed relevant acute toxicity for diuron and atrazine, with LC_{50} values of 9.6 mg/L and 53.57 mg/L for 96-hour-old zebrafish, respectively. On the other hand, no effects were observed for glyphosate alone at the maximum tested concentration (100 mg/L). The binary mixture of diuron and atrazine exhibited a synergistic effect, resulting in the lowest measured LC_{50} values, 2.96 mg/L and 16.46 mg/L, respectively, among all combinations performed. In contrast to its lack of effects alone, glyphosate increased the individual toxicity of diuron and atrazine since a decrease in the LC_{50} values to 5.2 mg/L and 33.03 mg/L, respectively, although it was not considered synergistic. All analyzed biomarkers (AChE, LDH, GST, CAT, and GPx) showed significant alterations. Moreover, increased ROS production was observed in the larvae exposed to the individual herbicides and the mixture composed of atrazine and diuron. These findings showed that herbicides can become more toxic when present in mixtures and that their mechanism of toxicity, both in their isolated form and in mixtures, is at least partially associated with oxidative stress.

Keywords: Emerging contaminants, pesticide mixture, ROS, synergistic effect, water pollution.

1 Introduction

The expansion of agriculture worldwide in response to the increasing demand for food has been accompanied by the continuous adoption of new agricultural practices and the growing use of specialized inputs. Among the most widely used products are pesticides, with a particular emphasis on synthetic herbicides. These agrichemicals play a crucial role in weed eradication in crops, thereby enhancing productivity (SHARMA *et al*., 2019).

Atrazine, glyphosate, and diuron are extensively used herbicides both in Brazil and worldwide (IBAMA, 2017). Their mechanisms of action involve the inhibition of essential biochemical pathways in target weeds: atrazine and diuron inhibit photosynthesis, and glyphosate interferes with the production of amino acids essential for plant growth (ROSS; CHILD, 1996; COUTINHO *et al*., 2005; ANTONIOU *et al*., 2014). Although they contribute to increased agricultural productivity, the intensive use of these compounds has led to environmental contamination, as evidenced by the presence of their residues in various ecosystem compartments (ISLAM, 2017).

The presence of pesticides in the environment represents an important problem for non-target species due to the toxic effects induced by these compounds. One of these effects includes the induction of increased production of reactive oxygen species (ROS), which are unstable and highly reactive molecules capable of triggering oxidative stress (LOBO *et al*., 2010). Numerous studies have already observed elevated ROS levels and oxidative stress in animals exposed to pesticides (BLAHOVÁ *et al*., 2013; WANG *et al*., 2015). This process is associated with cellular damage and the development of diseases such neurological diseases such as Alzheimer's, cardiovascular diseases, and various types of cancer. (PIZZINO *et al*., 2017; SULE; CONDON; GOMES, 2022).

Despite the considerable number of studies on the toxicity of herbicides in non-target organisms, most of them have been conducted using these herbicides individually. However, this contrasts with the reality of their combined usage with other chemical substances in crop management practices (PHYU *et al*., 2001). This is reflected in environmental samples analysis (e.g., water, soil, etc.), which increasingly detect pesticide mixtures in complex matrices (TANG; MANG, 2021).

As a result, several studies have been conducted to investigate the toxicity of pesticide mixtures in aquatic organisms. For instance, Wang *et al*. (2017) observed an increased toxicity of imidacloprid when exposed in combination with atrazine using zebrafish (*Danio rerio*) embryos and larvae as the target organism. Similarly, Fan *et al*. (2021) also observed an

increased toxicity of chlorpyrifos when mixed with carbendazim in experiments conducted on the same target organism as the previous study.

The zebrafish (*D. rerio*) has emerged as a promising model system in ecotoxicological studies of pesticides (VIEIRA *et al*., 2020; GONÇALVES *et al*., 2020; VIEIRA *et al*., 2022). In addition to its technical and biological attributes (e.g., ease of handling, availability in large numbers) (CHAHARDEHI *et al*., 2020), the use of zebrafish embryos and larvae allows for a wide range of toxicity endpoints, from molecular alterations (e.g., changes in key gene expression) to morphophysiological changes (e.g., developmental toxicity) (GONÇALVES *et al*., 2020). This enables preliminary understanding of the mechanisms by which toxicants contribute to pathological conditions. For instance, oxidative stress, associated with neurodegenerative diseases, liver disorders, and various types of cancer, has been observed in exposure tests of zebrafish early life stages to synthetic herbicides, using specific biomarkers. These include alterations in components of the endogenous antioxidant system, oxidation byproducts of biomolecules, and the use of transgenic fish (GONÇALVES *et al*., 2020; SILVA *et al*., 2022).

Based on the considerations, the aim of this study was to evaluate the acute toxicity of the herbicides diuron, glyphosate, and atrazine, as well as their binary and ternary mixtures, in zebrafish embryos and larvae. Additionally, we assessed the toxicological mechanisms of these compounds and their mixtures, with a focus on the oxidative stress process. For that, acute toxicity tests were conducted using the herbicides individually and in their respective combinations. Enzymatic activity tests and fluorescence analyses were performed to investigate mechanisms related to oxidative stress and the detection of ROS.

2 Materials and Methods

2.1 Chemical reagents

The herbicides glyphosate (CAS 1071-83-6, Code 45521, 98% purity), atrazine (CAS 1912-24-9, 45330, 98% purity), and diuron (CAS 330-54-1, 45463, 98% purity) were obtained from Merck Sigma-Aldrich® (Barueri, Brazil) . The solvents dimethyl sulfoxide (DMSO) and acetone were obtained from Labsynth (Diadema, Brazil). Atrazine and diuron were dissolved in pure acetone and DMSO, respectively, to prepare stock solutions of 75 mg/mL. For atrazine, the stock solution was further diluted to the test concentrations of 30, 40, 50, 60, 70, and 80 mg/L, while diuron was diluted to the test concentrations of 1.25, 2.5, 5, 10, and 20 mg/L. Glyphosate was diluted in E3 embryo medium (5 mM NaCl, 0.17 mM

KCl, 0.33 mM MgSO4, 0.33 mM CaCl, and 0.005% methylene blue) to obtain the test concentrations of 12.5, 25, 50, and 100 mg/L. The selected test concentrations were based on previous studies reported in the specialized literature.

2.2 Zebrafish embryos and larvae

Zebrafish embryos of the wild-type AB strain, at approximately 1 hour post-fertilization (hpf), were provided by UniPOM (Unidade de Produção de Organismos Modelo Não Convencionais), Department of Molecular Biology, Federal University of Paraíba (João Pessoa, Brazil). The parental fish were maintained in a closed circulation system with water quality control kept by physical, chemical, and biological filtration, as well as UV-C light sterilization, in a room with controlled temperature (26 ± 1 °C) and a 14:10-hour light-dark photoperiod. The fish were monitored daily for health and received *ad libitum* feeding three times a day with granulated food (ColorBits, Tetra, Germany), organic spirulina (Fazenda Tamanduá, Patos, Brazil), and artemia nauplii (Artemia Salina do RN, Natal, Brazil).

To obtain embryos, males, and females (in a 2:1 ratio) were placed in a breeding tank (1.7 L Slope breeding tank, Techniplast, Italy) at 5 p.m. on the day before the experiment. The next day, at 9 a.m. - 1 hour after the lights in the facility were turned on - the embryos were collected and transferred to E3 embryo medium. Subsequently, the embryos were selected under an inverted light microscope (Televal 31, Zeiss) at 50x magnification. Embryos with normal morphology and cleavage patterns were selected for the study. Batches of embryos with low viability (< 90%), including unfertilized or predominantly malformed embryos, were excluded. Dead and surplus embryos and larvae were euthanized with an overdose of clove oil (1.5 mL/L) and subsequently frozen for later incineration.

All protocols involving zebrafish in this study were approved by the Ethics Committee for Animal Use of the Federal University of Paraíba under protocol number 5901141020.

2.3 Acute toxicity of individual herbicides

The exposure of zebrafish embryos to herbicides was performed following the guidelines of the FET test no. 236 from the OECD (2013), with modifications suggested by Muniz *et al*. (2021). The test was conducted in a 96-well plate, where 20 embryos (1 embryo/well) at up to 3 hpf were individually exposed to different nominal concentrations of atrazine (30, 40, 50, 60, 70, and 80 mg/L), diuron (1.25, 2.5, 5, 10, and 20 mg/L), and glyphosate (12.5, 25, 50, and 100 mg/L) for 96 hours. Three additional plates were prepared,

one with embryos exposed to E3 medium (negative control) and the others with embryos exposed to 0.1% DMSO or 0.1% acetone (solvent controls).

At 24-hour intervals of exposure, the embryos were analyzed for the presence of the following lethality signs: (a) coagulation of the embryo; (b) non-detachment of the tail ; (c) lack of somite formation; and (d) lack of heartbeat. Additionally, the presence of non-lethal effects (*e.g.,*, delayed hatching, yolk sac edema, pericardial edema, head edema, and blood coagulation) was also assessed. The data obtained were used to determine: (1) survival rate % = (number of live organisms/total number of organisms) x 100, per tested concentration and exposure time; (2) cumulative lethal and non-lethal effects % = (number of affected organisms/total number of organisms) x 100, per tested concentration over 96 h ; (3) percentage of organisms affected by non-lethal effects % = (number of affected organisms/number of live organisms) x 100, per tested concentration over 96 h ; and (4) median lethal concentration (LC_{50}) calculated through *probit* regression analyses (Finney, 1971).

The exposures conducted during the study were performed under static conditions without the renewal of the test sample or the E3 medium due to the long half-lives of the herbicides used. Previous studies have indicated estimated half-lives exceeding 40 days for glyphosate, 105 days for atrazine, and 43 days for diuron (EPA, 2001; MERCURIO *et al*., 2014; QU *et al*., 2020). Observations were made under an inverted light microscope (50 x magnification), and recorded using a digital camera (Moticam 5+, Motic, Canada). Three independent biological replicates were performed for each tested concentration.

2.4 Acute toxicity of equitoxic herbicide mixtures

To investigate the acute toxicity of equitoxic herbicide mixtures, the methodology described by Wang *et al*. (2017) was followed. Under the same conditions described in section 2.3, the test was conducted by exposing embryos to serial dilutions of the LC_{50} of each herbicide while maintaining a fixed constant equitoxic ratio (1:1 or 1:1:1) - the same effect is expected from each individual substance. Six dilutions (with a serial dilution factor of 2) were tested for each herbicide. The following combinations were tested: atrazine + diuron; atrazine + glyphosate; diuron + glyphosate; atrazine + diuron + glyphosate. To assess complete concentration-response relationships, the total concentration of each mixture was altered while keeping the aforementioned ratios constant in the mixtures. The tests were repeated three times for each concentration. Table 1 shows the scheme for formulating the equitoxic binary and tertiary herbicide mixtures.

Table 1. Scheme for formulation of binary and tertiary mixtures.

Binary and ternary mixture	Atrazine (mg/L)	Diuron (mg/L)	Glyphosate (mg/L)
ATZ+DIU ÷ 16ϖ	3.34	0.60	-*
ATZ+DIU ÷ 8	6.69	1.20	
ATZ+DIU ÷ 4	13.39	2.40	
ATZ+DIU ÷ 2	26.78	4.80	
ATZ+DIU x1	53.57	9.60	
ATZ+DIU x2	107.14	19.20	
ATZ+GLY ÷ 16	3.34	-*	6.25
ATZ+GLY ÷ 8	6.69		12.50
ATZ+GLY ÷ 4	13.39		25
ATZ+GLY ÷ 2	26.78		50
ATZ+GLY x1	53.57		100
ATZ+GLY x2	107.14		200
DIU+GLY ÷ 16	-*	0.60	6.25
DIU+GLY ÷ 8		1.20	12.50
DIU+GLY ÷ 4		2.40	25
DIU+GLY ÷ 2		4.80	50
DIU+GLY x1		9.60	100
DIU+GLY x2		19.20	200
ATZ+DIU+GLY ÷ 16	3.34	0.60	6.25
ATZ+DIU+GLY ÷ 8	6.69	1.20	12.50
ATZ+DIU+GLY ÷ 4	13.39	2.40	25
ATZ+DIU+GLY ÷ 2	26.78	4.80	50
ATZ+DIU+GLY x1	53.57	9.60	100
ATZ+DIU+GLY x2	107.14	19.20	200

*Not used in the mixture.

ϖ: Individual LC_{50} divided by a dilution factor

2.5 Individual toxicity of herbicides and binary and tertiary mixtures

The acute toxicities of the herbicides and their mixtures were evaluated using *probit* analysis (FINNEY, 1971). The significance level of mean separation ($p < 0.05$) was determined based on the absence of overlap between the 95% confidence limits of the two LC_{50} values. The additivity index (AI) was used to investigate the interactions of the substances based on the LC_{50} values obtained individually and, in their mixtures, (MARKING, 1985). This method defines an AI for the combined effects of a chemical mixture. The biological activity (S) of the tested compounds, generically referred to as A and B, was determined by the equation as follows: S = (Am/Ai) + (Bm/Bi), where A and B represent the test samples; i corresponds to the individual LC_{50} value for A or B; m corresponds to the LC_{50} value for the mixture of A and B; S corresponds to the sum of biological activity. The AI was determined by calculating the values of S using the following equations: AI = 1/S-1 when $S < 1.0$, and AI = 1-S when $S \geq 1.0$.

The interactions of the substances were classified into categories based on the IA values as follows: antagonistic effect ($IA \leq -0.2$), additive ($-0.2 < AI \leq 0.25$), or synergistic ($IA > 0.25$). A higher value of IA implies greater synergy of the substances (SU *et al*., 2016). Synergy was considered significant if the 95% confidence interval of the IA value was greater than 0.

2.6 Biomarker enzymes

The acute toxicity test was repeated under the same conditions described above, but at this time the embryos were independently exposed to three sublethal concentrations (concentrations that did not cause mortality) of the individual herbicides and mixtures (Table 2). At the end of the exposure period (96 h), the larvae were rapidly frozen and stored at -20 °C until the time of analysis.

Groups of larvae from each of the three concentrations of individual compounds and mixtures, a negative control group (exposed only to E3 medium), and solvent control groups (0.1% DMSO and 0.1% acetone) were homogenized with cold distilled water at a ratio of 1 larva to 20 µL of solvent. The homogenates were centrifuged at 2,500-3,000 x g for 10 min at 4 °C, and the resulting supernatants were used for the measurement of soluble protein content and biomarker enzyme activity. The activities of the enzymes acetylcholinesterase (AChE), glutathione S-transferase (GST), catalase (CAT), glutathione peroxidase (Gpx), and lactate dehydrogenase (LDH) were measured. As no lethal or sublethal effects were observed at the

highest concentration tested of glyphosate, enzymatic quantification was not performed for this compound.

The quantification of soluble protein in the supernatant was determined using the photometric method developed by Bradford (1976). Initially, a standard curve was constructed using bovine serum albumin, and the absorbance of the samples was read at a wavelength of 595 nm using the Multiskan GO microplate reader (Thermo Fisher Scientific). The analyses were performed in quadruplicate for each sample.

The procedures for determining the activities of AChE, LDH, and GST were performed as described by Domingues *et al*. (2010). The activity of GPx was measured according to the procedure described by Massarsky *et al*. (2017), while catalase activity (CAT) was determined according to Clairborne (1985). All the methodologies are based on photometric quantification of products formed by specific reactions catalyzed by each of the aforementioned enzymes. The assays were performed in quadruplicate for each test.

Table 2. Concentrations of the individual herbicides and mixtures.

Compound	Concentration (mg/L)
Atrazine	7,5
	15
	30
Diuron	1,25
	2,5
	5
ATZ+DIU ÷ 32$^{\varpi}$	1.67 + 0.30$^{\Sigma}$
ATZ+DIU ÷16	3.34 + 0,60
ATZ+DIU ÷ 8	6.69 + 1,20
ATZ+GLY ÷ 16	3.34 + 6.25
ATZ+GLY ÷ 8	6.69 + 12,5
ATZ+GLY ÷ 4	13.39 + 25
DIU+GLY ÷ 16	0.60 + 6.25
DIU+GLY ÷ 8	1,20 + 12,5
DIU+GLY ÷ 4	2.40 + 25
ATZ+DIU+GLY ÷ 32	1.67 + 0.30 + 3.12
ATZ+DIU+GLY ÷ 16	3.34 + 0.60 + 6.25
ATZ+DIU+GLY ÷ 8	6.69 + 1.2 + 12.5

ϖ: Individual LC_{50} divided by a dilution factor

Σ: Concentrations corresponding to the compounds presented in the same order as the first column

2.7 Production of ROS

To determine the intracellular production of ROS in response to exposure to each herbicide or mixture, a fluorescent probe sensitive to oxygen, 2,7-dichlorodihydrofluorescein diacetate (H2DCFH-DA), was used following the methodology described by Thorstensen *et al*. (2014).

For this assay, a concentration was chosen for each individual herbicide and its respective mixtures. Embryos with < 6 hpf were exposed to the concentrations under conditions similar to those described in section 2.3, with some modifications. After 96 h of exposure, newly hatched larvae were arranged in groups of 5 larvae per treatment and transferred to a new plate.

The optimization of the protocol established by Thorstensen *et al*. (2014) resulted in the use of 1 µM of H2DCFDA instead of the 5 µM probe as described by those authors. On the day of the assay, the larvae were removed from the concentrations containing the individual herbicides and their respective mixtures and transferred to wells with E3 medium. Twelve groups with five larvae each were formed: (i) control without probe and hydrogen peroxide, (ii) positive control with hydrogen peroxide at a concentration of 1 mM and H2DCFDA at 1 µM, (iii) three solvent controls (0.1% acetone, 0.1% DMSO, and 0.1% acetone + 0.1% DMSO) with H2DCFDA at 1 µM, (iv) two groups exposed to a concentration of 5 mg/L and 30 mg/L of diuron and atrazine, respectively, with H2DCFDA at 1 µM, and (v) four groups with larvae exposed to mixtures of the herbicides (ATZ+DIU ÷ 8, ATZ+GLY ÷ 4, DIU+GLY ÷ 4, and ATZ+DIU+GLY ÷ 8) with H2DCFDA at 1 µM.

After the preparation of the plates with the different groups, hydrogen peroxide was added to the positive control, and the plate was incubated for 1 h at 26 °C. When the time was completed, H2DCFDA was added to the solutions of the different treatments, and the plate was incubated for an additional 1 h at 26 °C in a dark chamber to prevent degradation of the probe.

Subsequently, the larvae from each treatment were transferred to glass slides using a disposable pipette and prepared for observation with glycerol solution. The production of ROS was analyzed using fluorescence microscopy (excitation at 488 nm) (BX41 Microscope, Olympus America Inc., Canada). Photographs were taken with the Olympus Q-Color 5 ™ camera (Olympus America Inc., Canada). The mean fluorescence intensity (MFI) was calculated using the free software ImageJ by analyzing parameters related to the average

amount of green-scaled pixels in relation to the selected area. It quantifies the amount of brightness present in each selected area in pixels, resulting in fluorescence intensity.

2.8 Statistical analysis

The data were statistically analyzed using appropriate software available on the Internet. The results were presented as mean ± standard deviation. Differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) to compare the means of the different treatments. GraphPad Prism software was used for the statistical analysis.

3 Results and Discussion

3.1 Lethal and sublethal effects of individual herbicides

The results demonstrated different degrees of toxicity among the individual herbicides. Diuron exhibited the highest toxicity to *D. rerio* embryos and larvae, with coagulation of the egg being the only lethal endpoint, resulting in a LC_{50} (96h) of 9.6 mg/L (Table 3). It was observed that egg coagulation occurred with increasing concentration, reaching nearly 100% at a concentration of 20 mg/L (Figure 1A). Additionally, cumulative sublethal effects (96h) appeared between the concentrations of 5 mg/L and 10 mg/L. The observed sublethal effects included pericardial edema, yolk sac edema, delayed hatching, and blood coagulation, with the first three being more prevalent (Figure 2A and 2B).

Regarding atrazine, it was less toxic than diuron, with a LC_{50} of 53.57 mg/L (96 h) (Table 3) and exhibited egg coagulation as the only lethal effect (Figure 3A). Sublethal cumulative effects (96 h) were also observed at concentrations of 40, 50, 60, and 70 mg/L (Figure 3B), including delayed hatching, yolk sac edema, pericardial edema, and blood congestion, with the first two being more prevalent (Figure 4A and 4B).

Literature reports on the toxic effect of diuron in aquatic organisms are consistent with the findings of this study. Velki *et al*. (2017) investigated the acute toxic effects of diuron on zebrafish embryos and larvae, revealing an LC_{50} value of 6.37 mg/L (96 h) for diuron. Mhadhbi and Beiras (2012) also studied the toxic effect of diuron and other six compounds on the early development stages of *Psetta maxima* fish. They calculated an LC_{50} value of 7.8 mg/L (96 h) for diuron, providing additional evidence of its toxicity in different fish species. These studies are consistent with the results obtained in the present study, demonstrating a similarity in observed lethality.

Available data in the literature reveal different LC_{50} values for early stages of zebrafish exposed to atrazine. Wiegand *et al*. (1998) investigated the toxicity of atrazine in the early stages of zebrafish. The results of this study revealed an LC_{50} value of 36.8 mg/L and identified biochemical alterations resulting from atrazine exposure. In a subsequent study, Zaluski *et al*. (2022) also analyzed the toxicity of atrazine in zebrafish embryos and larvae. In this study, a mortality rate of over 55% was observed at a concentration of 5 mg/L. It is important to note that discrepancies in LC_{50} values and toxicity found in the literature compared to the results of this study may be influenced by various factors, including variable laboratory conditions such as water quality and the composition and purity of the tested substance (MARTINS *et al*., 2021). For example, in the study conducted by Zaluski *et al*. (2022), commercial formulations of atrazine were used in the assays. Additionally, sensitivity to the substance can vary among different strains of zebrafish. These discrepancies can be attributed to the considerable heterogeneity present within this species, even in controlled laboratory environments. Therefore, it is essential to consider these factors when interpreting the results (PARICHY, 2015; MARTINS *et al*., 2021).

Regarding sublethal effects, similar phenotypic changes were observed for both compounds. Among the observed sublethal effects, the occurrence of pericardial and yolk sac edema was prevalent, corroborating previous studies that also reported these malformations in response to exposure to the mentioned compounds (BLAHOVA *et al*., 2020; GORGE; NAGEL, 1990). Such edemas are often observed in early stages of zebrafish life when exposed to toxic substances, reflecting the sensitivity of these organisms during the initial stages of development (LI *et al*., 2017; SANT and TIMME-LARAGY, 2018; MARTINS *et al*., 2021).

In addition to these effects, delayed hatching was also observed in embryos exposed to both herbicides. Zaluski *et al*. (2022) and Blahova *et al*. (2020) also observed delayed hatching in larvae exposed to diuron and atrazine, respectively. Delayed hatching represents a crucial event in fish development and can result from behavioral, biochemical, and even morphological changes, which may be consequences of the edemas observed at the tested concentrations (LI *et al*., 2017).

Regarding glyphosate, no signs of toxicity or sublethal effects were observed at the maximum tested concentration of 100 mg/L, as established by the employed methodology. Consequently, this value was adopted for subsequent tests involving substance mixtures. These results are consistent with previous studies that did not find isolated glyphosate toxicity in zebrafish embryos and larvae (RODRIGUES *et al.*, 2019). However, it is worth mentioning

that research involving commercial formulations of the pesticide has shown toxicity in the early stages of zebrafish development (LU *et al*., 2022; LANZARIN *et al*., 2019). This adverse effect can be attributed to the surfactant POEA (Polyoxyethyleneamine) used in commercial glyphosate formulations (RODRIGUES *et al*., 2019).

Table 3. Individual and combined acute toxicity of diuron, atrazine, and glyphosate with equitoxic ratios for *D. rerio* larvae.

LC_{50} (95% CI) mg/L^{-1} (96 h)			
Atrazine	**Diuron**	**Glyphosate**	**AI$^{\delta}$**
53.57 (49.26-57.64)	-	-	-
-	9.6 (8.49-10.77)	-	-
-	-	100$^{\pi}$	-
16.469 (12.073- 22.467)	2.967 (2.231- 3.946)	-*	0,7
-*	5.291 (4.049- 6.913)	55.112 (42.171- 72.023)	-0,1
33.037 (25.018- 43.627)	-*	61.539 (46.961- 80.641)	-0,22
16.837 (12.307- 22.802)	2.9 (2.191-3.891)	30.418 (22.001- 40.635)	0,1

*Not used in the mixture.

δ: AI (Additive Index) is determined based on the biological activity (S) of test compounds A and B using the equation: S = (Am/Ai) + (Bm/Bi). It involves individual LC_{50} values for A and B (i) and the LC_{50} value for the mixture (m). AI is then calculated from S, with different equations applied depending on the S value: AI = 1/S-1 when S < 1.0, and AI = 1-S when S ≥ 1.0.

π: Absence of LC_{50} values; Concentration employed represents the highest tested value

Figure 1. Overview of the exposure of zebrafish embryos and larvae to increasing concentrations of the herbicide diuron for 96 h. (A) Mortality rate at different concentrations at the end of the exposure. (B) Cumulative lethal and sublethal effects after 96 h. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test, with the asterisk (*:$p < 0.01$; **:$p < 0.001$) indicating significant difference compared to the control.

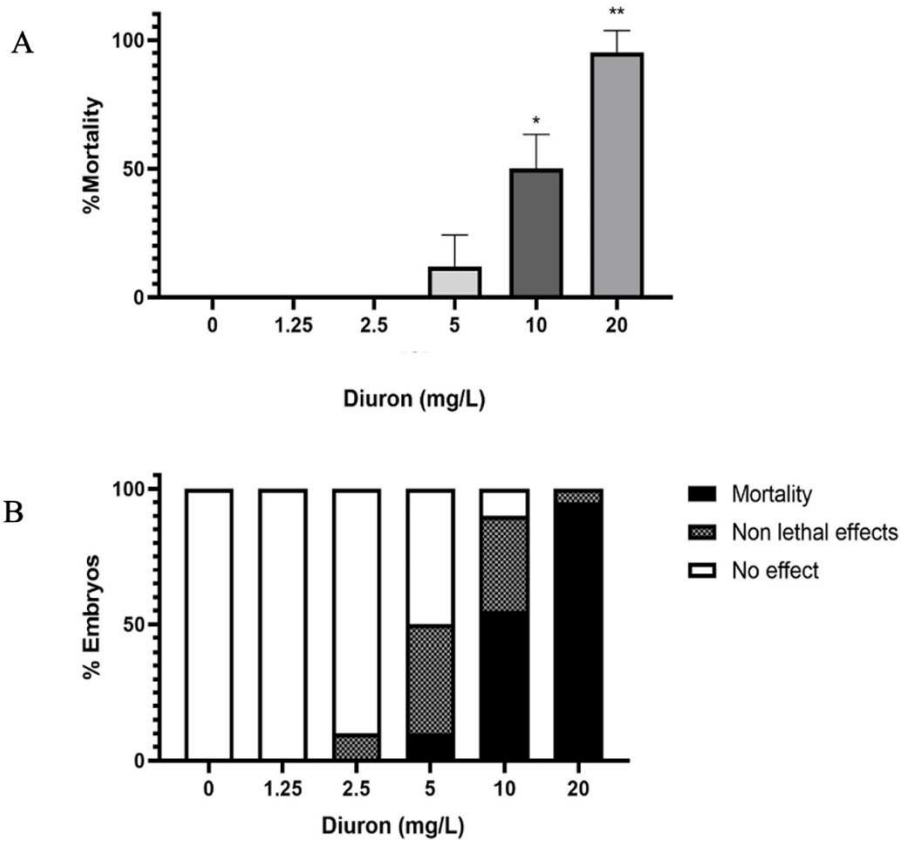


Figure 2. Cumulative non-lethal effects in zebrafish embryos exposed to increasing concentrations of diuron for 96 h. (A) Non-lethal effects observed between intermediate concentrations of 2.5 to 10 mg/L. (B) Quantification of cumulative non-lethal effects at different concentrations of diuron. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test, with the asterisk (*:$p < 0.05$; **: $p < 0.01$***: $p < 0.001$) indicating significant difference compared to the control.

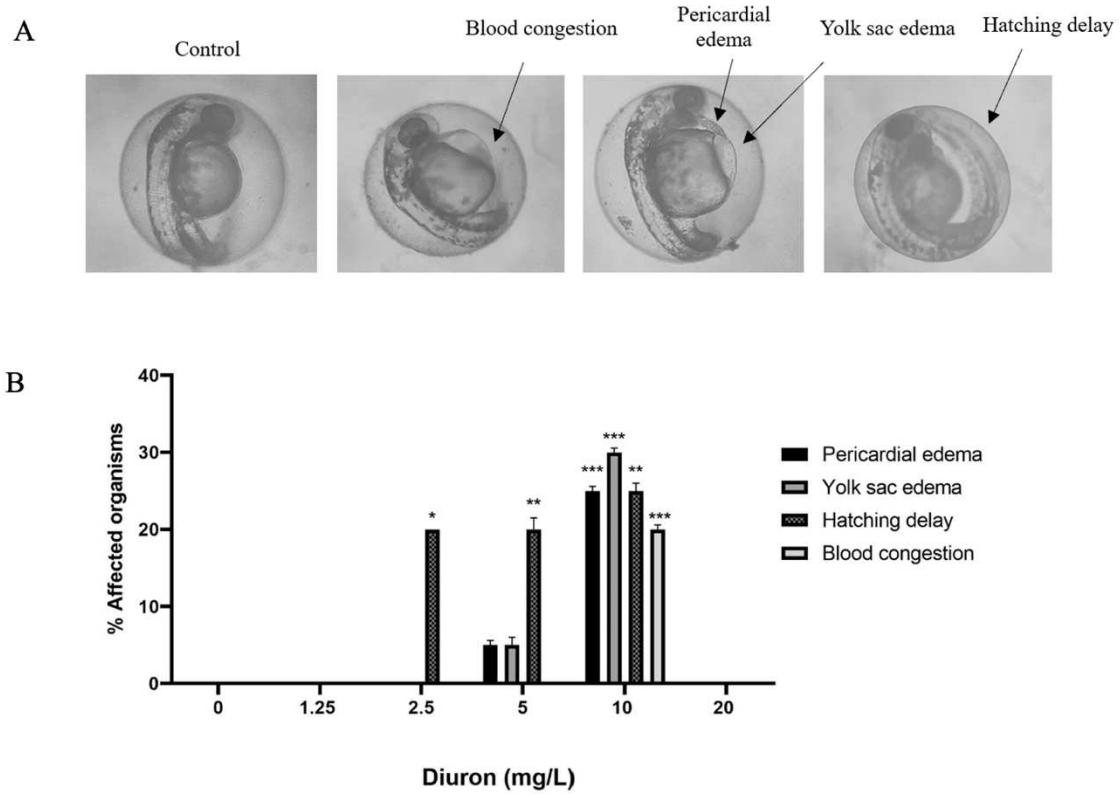


Figure 3. Overview of exposure of zebrafish embryos and larvae to increasing concentrations of atrazine for 96 h. (A) Mortality rate at different concentrations at the end of the exposure. (B) Cumulative lethal and non-lethal effects at different concentrations after 96 h. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test, with the asterisk (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) indicating significant difference compared to the control.

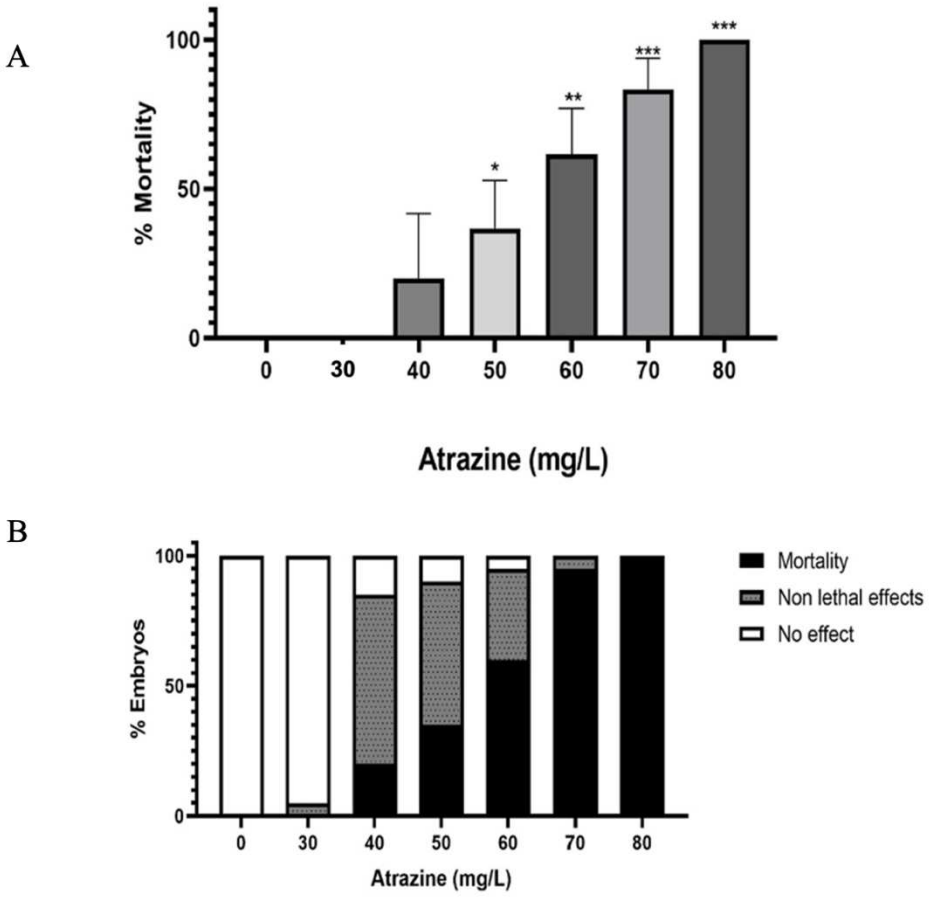
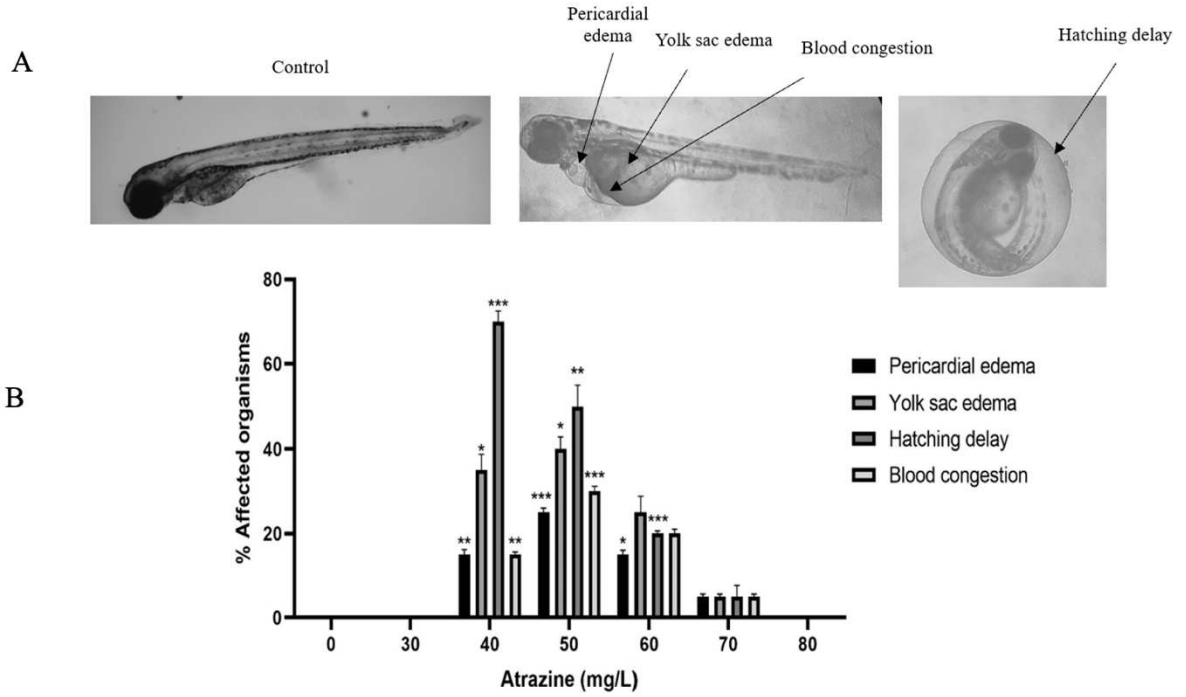


Figure 4. Non-lethal effects at different concentrations of atrazine after 96 h. (A) Non-lethal effects observed between intermediate concentrations of 40 and 70 mg/L. (B) Quantification of cumulative non-lethal effects caused by different concentrations of atrazine. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test, with the asterisk (*: $p < 0.05$; **: $p < 0.01$; ***:$p < 0.001$) indicating significant difference compared to the control**.**



3.2 Lethal and non-lethal effects of the mixtures

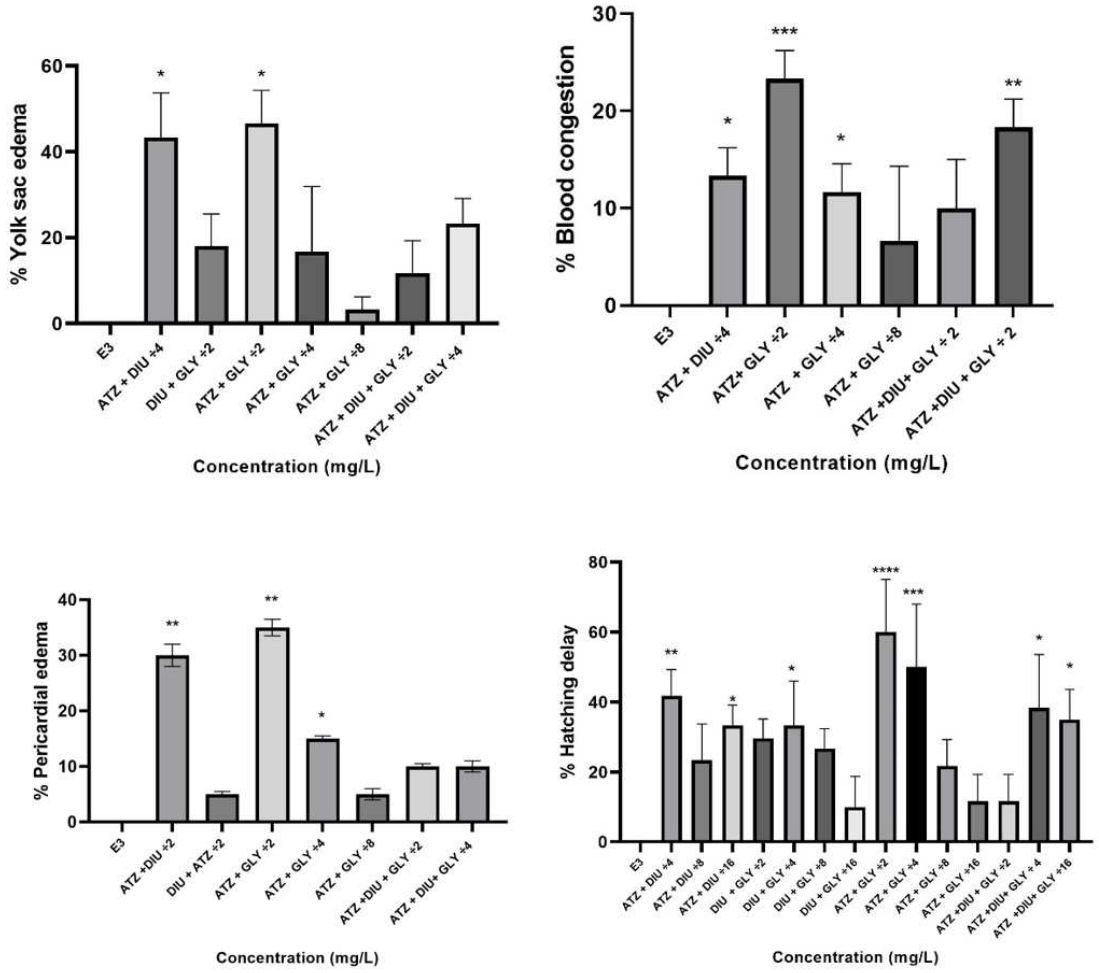
The exposure was performed using serial dilutions of each substance in zebrafish embryos and larvae, maintaining a constant fixed equitoxic ratio based on the individually obtained LC_{50} values for atrazine (53.57 mg/L) and diuron (9.6 mg/L), while the tested maximum concentration (100 mg/L) was used for glyphosate.

To analyze the interactions between the pesticides, the Additive Index (AI) was employed, based on the LC_{50} values obtained for the individual pesticides and their mixtures. The binary mixture of atrazine and diuron exhibited the highest calculated AI value, indicating synergy as the index was greater than zero, with an AI value of 0.7 (Table 3).

On the other hand, the AI value for the mixture of diuron and glyphosate, as well as for the combination of all three compounds, was -0.1 and 0.1, respectively, indicating an additive effect. Conversely, the mixture of atrazine and glyphosate was considered antagonistic, with an AI value of -0.22.

Similarly, to what was observed with the individual compounds, the mixtures also revealed non-lethal effects, including pericardial edema, yolk sac edema, delayed hatching, and blood congestion. It was observed that even at lower concentrations than those tested individually, non-lethal effects emerged when evaluated in combination (Figure 5). For example, the ATZ+DIU ÷4 combination, composed of 2.4 and 13.4 mg/L of diuron and atrazine, respectively, exhibited over 40% occurrence of yolk sac edema, while these effects were not observed at the same concentrations for the individual substances.

Figure 5. Cumulative non-lethal effects at different concentrations after 96 h . DIU: Diuron; ATZ: Atrazine; GLY: Glyphosate. Concentration values represent serial dilutions of the LC_{50} of individual substances. Concentrations were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. The asterisk (*:p< 0.05; **:p < 0.01***:p < 0.001) indicates a significant difference compared to the control group.



Although there is a lack of comprehensive studies on the toxicity of pesticide combinations, current knowledge indicates that increased (synergism) or decreased (antagonism) toxicity occurs due to interactions between pesticides, which can result in the inhibition or induction of enzymes responsible for chemical metabolism. Pesticides undergo metabolism processes in which different enzymes are involved. These enzymes can be negatively affected or stimulated by other chemical compounds. When multiple chemicals are absorbed, this can result in changes in activation and detoxification rates of pesticides, which in turn can cause alterations in the toxicity of the specific compound in question (THOMPSON, 1996; RODNEY *et al*., 2013).

A relevant example of the influence of mixtures on chemical metabolism is atrazine. Synergism of this substance with other pesticides, such as chlorpyrifos and phoxin, resulting in increased toxicity in zebrafish larval stages, has been observed (PEREZ *et al*., WANG *et al*., 2017). It is proposed that this effect is mediated by atrazine's involvement in the overstimulation and induction of cytochrome P450 (CYP450) isoenzymes when combined with organophosphate pesticides. This interaction promotes the formation of more toxic metabolites, contributing to the increased toxicity observed (EGAAS *et al*., 1993; KAO *et al*., 1995; WANG *et al*., 2017).

Thus, a possible explanation for the synergy and additive effect observed between diuron and atrazine in this study lies in the production of diuron's metabolite, 3,4-dichloroaniline (DCA), as a result of atrazine-induced cytochrome P450 (CYP450) induction. The activation of CYP450 by atrazine may increase the enzymatic activity involved in diuron metabolism, resulting in the formation of higher amounts of DCA. The presence of DCA is particularly relevant, as previous studies have documented its high toxicity in zebrafish embryos and larvae (SCHEIL *et al*., 2018; VIEIRA *et al*., 2020). Additionally, it is worth noting that the observed antagonism with glyphosate can be explained by the low toxicity of glyphosate and its metabolite (aminomethylphosphonic acid - AMPA) in the studied organism (RODRIGUES *et al*., 2019; IVANTSOVA *et al*., 2022).

In addition to the influence on enzymatic processes, it is important to highlight that interactions between these compounds can impact other crucial processes such as bioavailability, adsorption, distribution, binding to the target site, and excretion (MANSANO *et al*., 2017; CEDEERGREEN *et al*., 2017).

3.3 Enzymatic activity

To analyze the potential mechanisms of toxicity of the individual compounds and mixtures, enzymatic biomarker analyses were performed. Table 4 and 5 shows the activity of the enzymatic biomarkers (CAT, LDH, GPx, ACHE, and GST) analyzed after 96 hours of exposure to three sublethal concentrations of the individual compounds and their mixtures. The values of GST demonstrated a significant increase in all concentrations of diuron alone compared to the control group. In the presence of atrazine, an increase in GST activity was also observed at the two lower concentrations (Table 3).

As shown in Table 4, GST activity was also altered in the sublethal concentrations of the tested mixtures. All concentrations of the ATZ+GLY, DIU+GLY, and ATZ+GLY+DIU combinations resulted in a significant increase in GST activity compared to the control group. The ATZ+DIU combination also showed an increase in GST activity, but only at the two lower concentrations.

Regarding catalase and LDH, all concentrations of diuron and atrazine increased the activity of these enzymes (Table 4). Similarly, all concentrations of all mixture compositions increased the activity of these biomarkers compared to the control.

For GPx, all concentrations of diuron affected this biomarker. On the other hand, atrazine only showed an alteration at the lower concentration (Table 4). Furthermore, all concentrations of the analyzed mixtures also significantly impacted this biomarker (Table 5).

Regarding AChE, an alteration in the biomarker was reported only at one concentration of atrazine and diuron (Table 4). In the case of the mixtures, both the ATZ+DIU+GLY and ATZ+GLY combinations exhibited alterations in all concentrations tested. However, in the remaining combinations (ATZ+GLY and DIU+GLY), changes were only observed at a single concentration (Table 5).

According to the results obtained in this study, significant alterations were observed in all enzymes related to the production of ROS and, consequently, oxidative stress. One notable enzyme is GST, which plays a crucial role in cellular detoxification processes. GST catalyzes the conjugation of glutathione to electrophilic compounds, especially xenobiotic derivatives, through biotransformation reactions. Therefore, the increased activity of this enzyme, as observed in this study, has been widely used as an indicator of stress (QUINTANEIRO *et al*., 2017; TSUCHIDA, 2022).

CAT and GPx constitute a primary antioxidant defense system that plays a key role in total defense mechanisms and strategies in biological systems. Their function is to neutralize hydrogen peroxide (H_2O_2) through different modes, converting it into water (IGHODARO;

AKYLOIE, 2017). Therefore, the increase in catalase and GPx activity, as observed in this work, can be considered an adaptive response aimed at accelerating the degradation of H_2O_2 caused by these compounds. This response is essential to reduce the accumulation of ROS and minimize oxidative damage to cells (JIN *et al*., 2010; MARTINS *et al*., 2021).

In addition, alterations in LDH enzymatic activity were observed in larvae exposed to all herbicides and their mixtures. LDH is widely distributed in the body and plays an essential role in anaerobic metabolism. Therefore, any modification in LDH activity may indicate disturbances in energy metabolism, suggesting a possible reduction in oxygen availability in tissues or the occurrence of tissue injuries (MUNIZ *et al*., 2021; MARTINS *et al*., 2021). In the context of this study, the increased activity of the enzyme is possibly related to oxidative stress, as in this state, the integrity of the cell membrane can be compromised, leading to an increase in extracellular enzyme activity (JOVANOVIC *et al*., 2010).

In contrast to the aforementioned enzymes, AChE plays a crucial role in the nervous system and is responsible for the degradation of the neurotransmitter acetylcholine. The results of this study, especially from the mixtures, suggest a reduction in AChE activity. Inhibition of this enzyme by agrochemicals can lead to the accumulation of acetylcholine, resulting in adverse effects or even death in aquatic organisms (AL-SAWAFI *et al*., 2013; WANG *et al*., 2015).

Similarly, to the present study, previous investigations have reported alterations in enzymatic activities related to oxidative stress in aquatic organisms exposed to the mentioned herbicides. Studies involving zebrafish embryos and larvae exposed to the mentioned herbicides (SANTOS; MARTINEZ, 2012; CHROMCOVÁ *et al*., 2013; QUINTANEIRO *et al*., 2017;) have reported modifications in GST, GPx, and CAT enzymes. Furthermore, alterations in LDH and AChE enzymes have also been observed in response to herbicide exposure (AKHTAR *et al*., 2021; BORDIN *et al*., 2023). These enzymatic alterations signal that the mechanism of toxicity of these pesticides, at least in part, is associated with the establishment of oxidative stress in zebrafish.

Table 4. Enzymatic activity in zebrafish larvae exposed to increasing concentrations of diuron and atrazine individually and to controls for 96 h. DIU: diuron; ATZ: atrazine; GLY: glyphosate; CAT: catalase; GST: Glutathione S transferase; GPx: glutathione peroxidase; LDH: lactate dehydrogenase; AChE: acetylcholinesterase; Control: E3 embryonic medium negative control; Acetone: 0.1% acetone solvent control; DMSO: 0.1% dimethyl sulfoxide solvent control. All data are mean ± standard deviation. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, with an asterisk (*) indicating significant difference ($p < 0.05$) compared to the control.

Treatment	Concentration	Biomarkers				
		CAT	GST	GPx	LDH	AChE
Control	E3 medium	6.06 ± 0.07	27.90 ± 0.14	4.11 ± 0.20	173.00 ± 1.68	39.05 ± 1.97
Acetone	0.1%	16.01 ± 6.40*	33.80 ± 5.20	4.02 ± 0.20	203.02 ± 6.60*	43.41 ± 2.27
DMSO	0.1%	6.22 ± 0.30	37.88 ± 0.70*	4.90 ± 0.70	176.50 ± 12.30	34.49 ± 4.27
DIU	1.25 mg/L	13.04 ± 0.50*	45.5 ± 0.51*	6.60 ± 0.45*	208.40 ± 1.00*	41.18 ± 1.80
	2.5 mg/L	9.75 ± 0.50*	46.05 ± 0.41*	7.00 ± 0.60*	190.16 ± 14.00*	36.83 ± 0.30
	5 mg/L	7.69 ± 0.50	61.71 ± 0.90*	10.55 ±1.60*	196.00 ± 6.85*	53.27 ± 4.30*
ATZ	7.5 mg/L	24.55 ± 2.23*	40.87 ± 6.50*	7.25 ± 0.87*	312. 57 ± 2.25*	49.60 ± 3.90*
	15 mg/L	21.56 ± 3.75*	37.97 ± 0.99*	5.35 ± 0.65	247.30 ± 2.86*	36.84 ± 1.86
	30 mg/L	24.55 ± 2.20*	33.16 ± 2.30	4.90 ± 0.43	256.64 ±6.35*	35.15 ± 0.77

Table 5. Enzymatic activity in zebrafish larvae exposed to increasing concentrations of the mixtures and controls for 96 h . DIU: diuron; ATZ: atrazine; GLY: glyphosate; CAT: catalase; GST: Glutathione S transferase; GPx: glutathione peroxidase; LDH: lactate dehydrogenase; AChE: acetylcholinesterase; Control: E3 embryonic medium negative control; Acetone: 0.1% acetone solvent control; DMSO: 0.1% dimethyl sulfoxide solvent control. All data are mean ± standard deviation. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, with an asterisk (*) indicating significant difference ($p < 0.05$) compared to the control.

Treatment	Concentration (mg/L)	Biomarkers				
		CAT	GST	GPx	LDH	AChE
Control	0	19.32 ± 2.45	36.06 ± 0.47	4.11 ± 0.20	135. 22 ± 0.70	62.01 ± 1.35
Acetone +DMSO	0,1 %	40.83 ± 8.60*	37.13 ± 2.20	7.50 ± 0.40*	126.42 ± 0.75*	64.16 ± 0.29
DIU+ATZ ÷ 32$^{\varpi}$	1,60+0.30$^{\Sigma}$	61.23 ± 3.20*	46.19 ± 2.90*	8.20 ± 0.30*	156.50 ± 6.70*	64.70 ± 0.69
DIU+ATZ ÷16	3.34 + 0.60	51.69 ± 3.60*	41.48 ± 1.10*	7.50 ± 0.40*	141.56 ± 5.66 *	72.24 ± 1.28*
DIU+ATZ ÷8	6.69 + 1.20	53.26 ± 5.61*	37.45 ± 2.24	7.30 ± 0.70*	128.16 ± 3.17	71. 33 ± 0.69*
ATZ+GLY ÷ 16	3.34+ 6.25	79.34 ± 5.04*	62.30 ± 0.80*	8.19 ± 0.50*	327.80 ± 2.70*	61.80 ± 1.10
ATZ+GLY ÷ 8	6.69 + 12.50	66.81 ± 10.97*	58.55 ± 1.30*	8.12 ± 0.20*	264.44 ± 3.50*	69.28 ± 2.70*
ATZ+GLY÷ 4	13.39 + 25	84.15 ± 5.35*	49.45 ± 0.90*	7.50 ± 0.47*	242.01 ± 11.50*	63.03 ± 1.02
DIU+GLY ÷ 16	0.60 + 6.25	63.16 ± 15.00*	51.57 ± 1.60*	7.80 ± 0.20*	174.93 ± 12.20*	64.63 ± 0.59
DIU+GLY ÷ 8	1.20 + 12.50	54.46 ± 3.47*	52.88 ± 1.35*	7.80 ± 0.15*	175.22 ± 4.60*	73.57± 1.04*
DIU+GLY ÷ 4	2.40 + 25	48.38 ± 2.45*	51.08 ± 1.00*	8.02 ± 0.50*	207.80 ± 6.30*	58.89 ± 1.49
ATZ+DIU+GLY ÷32	1.67 + 0.30 + 3.12	101.70 ± 9.97*	90.6 ± 11.90*	8.30 ± 0.10*	344. 7 ± 15.00 *	91.50 ± 2.20*
ATZ+DIU+GLY ÷16	3.34 + 0.60 + 6,25	77.31 ±0.95*	77.53 ± 2.23*	8.50 ± 0.30*	336.20 ± 1.96*	99.89 ± 5.20*
ATZ+DIU+GLY÷ 8	6.69 + 1.20+ 12.50	103.93 ± 8.50*	71.63 ± 2.23*	8.90 ± 0.40*	331. 37 ± 7.20*	98.30 ± 0.80*

ϖ: Individual LC_{50} divided by a dilution factor

Σ: Concentrations corresponding to the compounds presented in the same order as the first column

3.4 ROS production in larvae exposed to individual herbicides and mixtures

The compound H2DCFDA is cell-permeable and can diffuse inside cells. Once inside, it is deacetylated by cellular enzymes, resulting in the formation of 2',7'-dichlorodihydrofluorescein (H2DCF). Under conditions where ROS, mainly H_2O_2, are present, H2DCF is readily oxidized, transforming into 2',7'-dichlorofluorescein (DCF). DCF is highly fluorescent, allowing for the detection and quantification of ROS through microscopy (YANG *et al*., 2014). Thus, the induction of oxidative stress by individual herbicides and their mixtures could be analyzed.

In Figure 6, the difference in fluorescence intensity between the negative control group (E3 medium) and the positive control group, which was treated with a powerful oxidant, H_2O_2, is evident. Moreover, regions such as the yolk sac and pericardium exhibited more pronounced fluorescence compared to other regions of the larva's body treated with this oxidant.

When analyzing the arbitrary fluorescence units in Figure 7, a significant difference ($p < 0.05$) in the concentrations of individual pesticides compared to the control group was observed. However, regarding the mixtures, only the ATZ+DIU combination showed a significant difference ($p < 0.05$) compared to the E3 control.

A plausible explanation for this observation lies in the possibility that, in compositions where significant fluorescence was not detected, ROS may have already been eliminated due to the presence of a highly efficient antioxidant system in zebrafish exposed to such compositions (VELKI *et al*., 2017). The higher levels of enzymes such as CAT, GPx, and GST, which are markers of oxidative stress, in compositions where no fluorescence was observed support this explanation.

Figure 6. Reactive oxygen species (ROS) production in 96 hpf larvae exposed to individual herbicides and mixtures for 1 h. Control: E3 embryonic medium negative control; DMSO: 0.1% dimethyl sulfoxide solvent control; ACETONE: 0.1% acetone solvent control; H202: Hydrogen peroxide positive control; DIU: diuron; ATZ: atrazine; GLY: glyphosate; ATZ 30: 30 mg/L atrazine; DIU 5: 5 mg/L diuron; ATZ+DIU ÷8: 6.7 mg/L ATZ + 1.2 mg/L DIU; ATZ+GLY ÷4: 13.5 mg/L ATZ + 25 mg/L GLY; DIU+GLY ÷4: 2.3 mg/L DIU + 25 mg/L GLY; ATZ+DIU+GLY ÷8: 6.7 mg/L ATZ + 1.2 mg/L DIU + 12.5 mg/L GLY. Concentration values represent serial dilutions based on the LC_{50} of the individual substances. All larvae were treated with 1 µM of the fluorescent probe H2DCFDA.

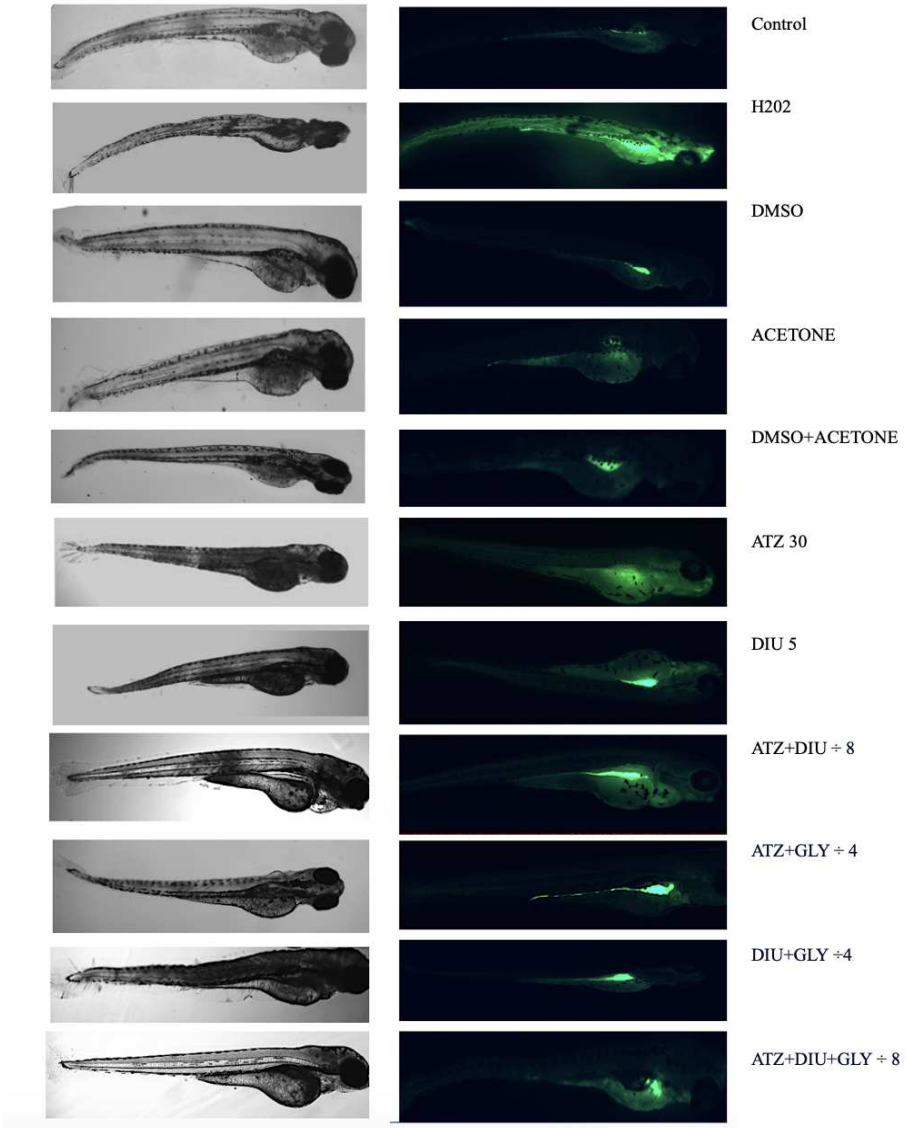
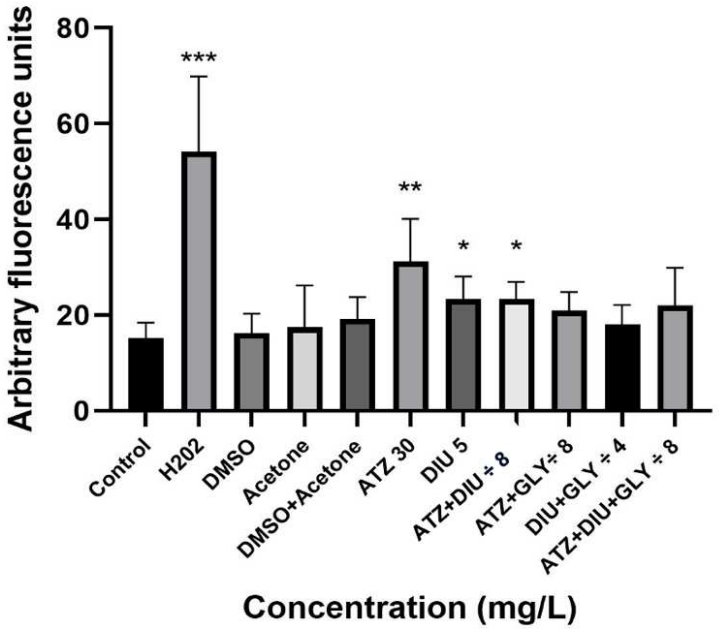


Figure 7. Quantification of average fluorescence intensity in 96 hpf larvae exposed to individual herbicides and mixtures for 1 hour. Control: E3 embryonic medium negative control; DMSO: 0.1% dimethyl sulfoxide solvent control; ACETONE: 0.1% acetone solvent control; H202: Hydrogen peroxide positive control; DIU: diuron; ATZ: atrazine; GLY: glyphosate; ATZ 30: 30 mg/L atrazine; DIU 5: 5 mg/L diuron; ATZ+DIU ÷8: 6.7 mg/L ATZ + 1.2 mg/L DIU; ATZ+GLY ÷4: 13.5 mg/L ATZ + 25 mg/L GLY; DIU+GLY ÷4: 2.3 mg/L DIU + 25 mg/L GLY; ATZ+DIU+GLY ÷8: 6.7 mg/L ATZ + 1.2 mg/L DIU + 12.5 mg/L GLY. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, with an asterisk (*:$p < 0.05$; **:$p < 0.01$***:$p < 0.001$) indicating significant difference compared to the control. All larvae were treated with 1 µM of the fluorescent probe H2DCFDA.



4 Conclusion

The results of this study revealed that individually atrazine and diuron cause a decrease in the survival of zebrafish embryos and larvae, while glyphosate does not have any effect even at high concentrations. Interestingly, a synergistic effect was observed between atrazine and diuron, resulting in higher toxicity to the tested organisms compared to exposures with these herbicides individually. Although glyphosate is not toxic in its isolated form, when present in combination with atrazine and diuron, it can increase their toxicity, although not considered synergistic. The results demonstrated that both individual herbicides and their mixtures induce changes in biomarkers related to oxidative and metabolic stress. This finding was confirmed by the increased production of ROS. Therefore, this study provides information that enhances the understanding of herbicide toxicity and their mixtures, as well as possible toxicity mechanisms, using a vertebrate model system.

Acknowledgments

We thank to Universidade Federal da Paraíba (UFPB, Brazil), Fundação de Apoio à Pesquisa do Estado da Paraíba (FAPESQ, Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) for supporting this research with grants and scholarships.

Author contribution

M.S.M, M.E.S.M, I.C.A.A., R.X.M., T.L.R. and D.F. designed and conducted the literature review and wrote the manuscript. All authors approved the submitted version.

Funding

This research was funded by: Public Call n. 03 Produtividade em Pesquisa PROPESQ/PRPG/UFPB, grant number PVA13245-2020; Public Call Demanda Universal FAPESQ, grant number 3045/2021, and public call FAPESQ/FAPESP 2019.

Data availability

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All listed authors have approved the manuscript before submission, including the names and order of authors.

Competing interests

The authors declare no competing interests.

REFERENCES

AKHTAR, Naveed *et al.* Adverse effects of atrazine on blood parameters, biochemical profile and genotoxicity of snow trout (Schizothorax plagiostomus). **Saudi Journal Of Biological Sciences**, [*s.l.*], v. 28, n. 3, p. 1999-2003, mar. 2021. Disponível em: https://www.sciencedirect.com/science/article/pii/S1319562X21000012. Acesso em: 23 dez. 2022.

AL-SAWAFI, Abeer Ghazie A.; YAN, Yunjun. Alterations of Acetylcholinesterase Activity and Antioxidant Capacity of Zebrafish Brain and Muscle Exposed to Sublethal Level of Cadmium. **International Journal Of Environmental Science And Development**, [*s.l.*], p. 327-330, 2013. Disponível em: https://www.researchgate.net/publication/269839107. Acesso em: 22 jan. 2023.

ANTONIOU, M. *et al.* Practices that Prevent the Formation of Cyanobacterial Blooms in Water Resources and Remove Cyanotoxins During Physical Treatment of Drinking Water. **Comprehensive Water Quality And Purification**, [*s.l.*], p. 173-195, 2014. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/B9780123821829000323?via%3Dihub. Acesso em: 22 fev. 2023.

BLAHOVÁ, Jana *et al.* Oxidative stress responses in zebrafish Danio rerio after subchronic exposure to atrazine. **Food And Chemical Toxicology**, [*s.l.*], v. 61, p. 82-85, nov. 2013. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0278691513001506. Acesso em: 22 fev. 2023.

BORDIN, E. *et al.* Sublethal effects of the herbicides atrazine and glyphosate at environmentally relevant concentrations on South American catfish (*Rhamdia quelen*) embryos. **Environmental Toxicology and Pharmacology**, v. 98, p. 104057, 2023. Disponível em: https://doi.org/10.1016/j.etap.2022.104057. Acesso em: 18 mar. 2022.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, [*s.l.*], v. 72, n. 1-2, p. 248-254, maio 1976. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/0003269776905273. Acesso em: 2 jan. 2023.

CEDERGREEN, Nina *et al.* A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. **Environmental Toxicology and Chemistry**, v. 27, n. 7, p. 1621-1632, 1 jul. 2008. Disponível em: https://pubmed.ncbi.nlm.nih.gov/18271647/. Acesso em: 4 jan. 2023.

CHROMCOVA, Luci. *et al.* The effects of atrazine exposure on early life stages of common carp (Cyprinus carpio). **Neuro endocrinology letters**, v. 34 Suppl 2, p. 95–101, 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24362100/. Acesso em: 2 jan. 2023.

COUTINHO, Cláudia F. B. *et al.* Pesticidas: mecanismo de ação, degradação e toxidez. Pesticidas: **Revista de Ecotoxicologia e Meio Ambiente**, v. 15, 2005. Disponível em: https://revistas.ufpr.br/pesticidas/article/view/4469. Acesso em: 18 fev. 2023.

DOMINGUES, Inês *et al*. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. **Comparative Biochemistry**

And Physiology Part C: Toxicology & Pharmacology, v. 152, n. 3, p. 338-345, set. 2010. Disponível em: https://pubmed.ncbi.nlm.nih.gov/20566315/. Acesso em: 2 abr. 2023.

EGAAS, Eliann *et al*. A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the in vitro phase II atrazine metabolism in some fish, insects, mammals and one plant species. **Comparative Biochemistry And Physiology Part C: Pharmacology, Toxicology and Endocrinology**, v. 106, n. 1, p. 141-149, set. 1993. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/074284139390265M. Acesso em: 22 jan. 2023

FAN, Ruiqi *et al*. Individual and synergistic toxic effects of carbendazim and chlorpyrifos on zebrafish embryonic development. **Chemosphere**, v. 280, p. 130769, out. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34162088/. Acesso em: 7 maio 2023.

FINNEY, D.J. Probit Analysis. **Journal Of Pharmaceutical Sciences**,[*s.l.*], v. 60, n. 9, p. 1432, set. 1971. Disponível em: https://onlinelibrary.wiley.com/doi/10.1002/jps.2600600940. Acesso em: 15 mar. 2023.

GONÇALVES, Íris Flávia Sousa *et al*. Toxicity testing of pesticides in zebrafish—a systematic review on chemicals and associated toxicological endpoints. **Environmental Science And Pollution Research**, [*s.l.*], v. 27, n. 10, p. 10185-10204, 15 fev. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32062774/. Acesso em: 30 maio 2023.

GÖRGE, Gerhard; NAGEL, Roland. Toxicity of lindane, atrazine, and deltamethrin to early life stages of zebrafish (Brachydanio rerio). **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 20, n. 3, p. 246-255, dez. 1990. Disponível em: https://pubmed.ncbi.nlm.nih.gov/1708710. Acesso em: 4 maio 2023.

IBAMA. (2017) Relatórios de comercialização de agrotóxicos.

IGHODARO, O.M.; AKINLOYE, O.A.. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. **Alexandria Journal Of Medicine**, [*s.l.*], v. 54, n. 4, p. 287-293, 1 dez. 2018. Disponível em: https://www.sciencedirect.com/science/article/pii/S2090506817301550. Acesso em: 15 abr. 2022.

ISLAM, Faisal *et al*. Potential impact of the herbicide 2,4-dichlorophenoxyacetic acid on human and ecosystems. **Environment International**, [*s.l.*], v. 111, p. 332-351, fev. 2018. Disponível em: https://pubmed.ncbi.nlm.nih.gov/29203058/. Acesso em: 22 fev. 2022.

IVANTSOVA, Emma *et al*. Developmental and behavioral toxicity assessment of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in zebrafish embryos/larvae. **Environmental Toxicology And Pharmacology**, [*s.l.*], v. 93, p. 103873, jul. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35504511/. Acesso em: 15 abr. 2023

JIN, Yuanxiang *et al*. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (Danio rerio). **Chemosphere**, [*s.l.*], v. 78, n. 7, p. 846-852, fev. 2010. Disponível em: https://pubmed.ncbi.nlm.nih.gov/20036412/. Acesso em: 15 jan. 2023.

JOVANOVIć, Predrag *et al*. Lactate dehydrogenase and oxidative stress activity in primary open-angle glaucoma aqueous humour. **Bosnian Journal Of Basic Medical Sciences**, [*s.l.*], v. 10, n. 1, p. 83-88, 20 fev. 2010. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5596618/. Acesso em: 10 jul. 2023.

KAO, L. M.; WILKINSON, C. F.; BRATTSTEN, L. B.. In‐vivo effects of 2,4‐D and atrazine on cytochrome P‐450 and insecticide toxicity in southern armyworm (Spodoptera eridania) larvae. **Pesticide Science**, [*s.l.*], v. 45, n. 4, p. 331-334, dez. 1995. Wiley.Disponível em: https://onlinelibrary.wiley.com/doi/abs/10.1002/ps.2780450407. Acesso em: 4 dez. 2022.

LANZARIN, Germano *et al*. Inflammatory, Oxidative Stress, and Apoptosis Effects in Zebrafish Larvae after Rapid Exposure to a Commercial Glyphosate Formulation. **Biomedicines**, [*s.l.*], v. 9, n. 12, p. 1784, 27 nov. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34944599/. Acesso em: 15 jan. 2023.

LI, Kang *et al*. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. **Chemosphere**, [*s.l.*], v. 171, p. 40-48, mar. 2017. Elsevier BV. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28002765/. Acesso em: 4 maio 2022

LOBO, V; A PATIL,; A PHATAK,; CHANDRA, N. Free radicals, antioxidants and functional foods: impact on human health. **Pharmacognosy Reviews**, [*s.l.*], v. 4, n. 8, p. 118, 2010. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC3249911/. Acesso em: 15 dez. 2022.

LU, Jian *et al*. Characterization of glyphosate-induced cardiovascular toxicity and apoptosis in zebrafish. **Science Of The Total Environment**, [*s.l.*], v. 851, p. 158308, dez. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/36030873/. Acesso em: 7 jan. 2023.

MANSANO, Adrislaine S *et al*. Individual and mixture toxicity of carbofuran and diuron to the protozoan Paramecium caudatum and the cladoceran Ceriodaphnia silvestrii. **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 201, p. 110829, set. 2020. Disponível em: https://www.sciencedirect.com/science/article/pii/S0147651320306680. Acesso em: 22 maio

]MARKING, L. Toxicity of chemical mixtures. In: Rand, G., Petroceli, S. (Eds.), **Fundamentals of Aquatic Toxicology**. Hemisphere Publishing Corporation, 1985

MARTINS, Rafael Xavier *et al*. Exposure to 2,4-D herbicide induces hepatotoxicity in zebrafish larvae. **Comparative Biochemistry And Physiology Part C: Toxicology & Pharmacology**, [*s.l.*], v. 248, p. 109110, out. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34144256/. Acesso em: 7 abr. 2022.

MASSARSKY, Andrey *et al*. Glutathione and zebrafish: old assays to address a current issue. **Chemosphere**, [*s.l.*], v. 168, p. 707-715, fev. 2017. Disponível em: https://www.sciencedirect.com/science/article/pii/S0045653516315387. Acesso em: 12 mar. 2022.

MERCURIO, Philip *et al*. Glyphosate persistence in seawater. **Marine Pollution Bulletin**, [*s.l.*], v. 85, n. 2, p. 385-390, ago. 2014. Disponível em:

https://www.sciencedirect.com/science/article/pii/S0025326X14000228. Acesso em: 27 mar. 2022.

MHADHBI, Lazhar; BEIRAS, Ricardo. Acute Toxicity of Seven Selected Pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the Marine Fish (Turbot, Psetta maxima). **Water, Air, & Soil Pollution**, [*s.l.*], v. 223, n. 9, p. 5917-5930, 28 set. 2012. Disponível em: https://link.springer.com/article/10.1007/s11270-012-1328-9#citeas. Acesso em: 10 mar. 2022.

MUNIZ, Marta Silva *et al*. Moxidectin toxicity to zebrafish embryos: bioaccumulation and biomarker responses. **Environmental Pollution**, [*s.l.*], v. 283, p. 117096, ago. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/33866217/. Acesso em: 30 abr. 2023.

OECD. Test No. 236, Fish Embryo Acute Toxicity (FET) Test. **OECD Guidelines for the Testing of Chemicals**, Section 2. Paris: OECD Publishing, 2013. Disponível em: https://doi.org/10.1787/9789264203709-en. Acesso em: 10 mar. 2025.

PARICHY, David M. Advancing biology through a deeper understanding of zebrafish ecology and evolution. **Elife**, [*s.l.*], v. 4, p. 1-10, 25 mar. 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/25807087/. Acesso em: 4 jan. 2022.

PÉREZ, Joanne *et al*. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. **Environmental Science And Pollution Research**, [*s.l.*], v. 20, n. 7, p. 4671-4680, 5 jan. 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/23288680/. Acesso em: 9 jan. 2023

PIZZINO, Gabriele *et al*. Oxidative Stress: harms and benefits for human health. **Oxidative Medicine And Cellular Longevity**, [*s.l.*], v. 2017, n. 1, p. 10-20, jan. 2017. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5551541/. Acesso em: 15 fev. 2023.

PHYU, Y.L. *et al*. A comparison of mixture toxicity assessment: examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to ceriodaphnia cf. dubia. **Chemosphere**, [*s.l.*], v. 85, n. 10, p. 1568-1573, nov. 2011. Disponível em: https://pubmed.ncbi.nlm.nih.gov/21925699/. Acesso em: 23 dez. 2021.

QU, Mengjie *et al*. Fate of atrazine and its relationship with environmental factors in distinctly different lake sediments associated with hydrophytes. **Environmental Pollution**, [*s.l.*], v. 256, p. 113371, jan. 2020. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0269749119322705. Acesso em: 4 jan. 2023.

QUINTANEIRO, C. *et al*. Endocrine and physiological effects of linuron and S-metolachlor in zebrafish developing embryos. **Science Of The Total Environment**, [*s.l.*], v. 586, p. 390-400, maio 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28209406/. Acesso em: 15 abr. 2021.

RODNEY, Sara *et al*. Estimating the Toxicity of Pesticide Mixtures to Aquatic Organisms: a review. **Human And Ecological Risk Assessment**: An International Journal, [*s.l.*], v. 19, n. 6, p. 1557-1575, 30 maio 2013. Disponível em:

https://www.tandfonline.com/doi/full/10.1080/10807039.2012.723180. Acesso em: 16 dez. 2022

RODRIGUES, Laís de Brito *et al*. Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms. **Mutation Research/Genetic Toxicology And Environmental Mutagenesis**, [*s.l.*], v. 842, p. 94-101, jun. 2019. Disponível em: https://pubmed.ncbi.nlm.nih.gov/31255230/. Acesso em: 4 jan. 2023

ROSS, M. Herbicide mode-of-action summary.**Weed science**, 1996. Disponível em: https://extension.purdue.edu/extmedia/WS/WS-23-W.html

SANT, Karilyn E.; TIMME-LARAGY, Alicia R.. Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo. **Current Environmental Health Reports**, [*s.l.*], v. 5, n. 1, p. 125-133, 7 fev. 2018. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5876134/. Acesso em: 15 jun. 2023.

SANTOS, Thais G.; MARTINEZ, Cláudia B.R.. Atrazine promotes biochemical changes and DNA damage in a Neotropical fish species. **Chemosphere**, [*s.l.*], v. 89, n. 9, p. 1118-1125, nov. 2012. Disponível em: https://pubmed.ncbi.nlm.nih.gov/22739540/. Acesso em: 23 mar. 2022

SCHEIL, Volker *et al*. Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (Danio rerio) embryos and larvae. **Ecotoxicology**, [*s.l.*], v. 18, n. 3, p. 355-363, 18 dez. 2008. Springer Science and Business Media LLC. Disponível em: https://pubmed.ncbi.nlm.nih.gov/19093198/. Acesso em: 25 nov. 2023.

SHARMA, Anket *et al*. Worldwide pesticide usage and its impacts on ecosystem. **Sn Applied Sciences**, [*s.l.*], v. 1, n. 11, p. 327-345, 21 out. 2019. Disponível em: https://link.springer.com/article/10.1007/s42452-019-1485-1. Acesso em: 29 ago. 2022.

SILVA, Rafaella *et al*. Transgenic zebrafish (Danio rerio) as an emerging model system in ecotoxicology and toxicology: historical review, recent advances, and trends. **Science Of The Total Environment**, [*s.l.*], v. 848, p. 157665, nov. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35907527/. Acesso em: 18 maio 2022.

SULE, Rasheed O.; CONDON, Liam; GOMES, Aldrin V.. A Common Feature of Pesticides: oxidative stress–the role of oxidative stress in pesticide-induced toxicity. **Oxidative Medicine And Cellular Longevity**, [*s.l.*], v. 2022, p. 1-31, 19 jan. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35096268/. Acesso em: 12 abr. 2022

TANG, Fiona H.M.; MAGGI, Federico. Pesticide mixtures in soil: a global outlook. **Environmental Research Letters**, [*s.l.*], p. 20-30, 12 fev. 2021. Disponível em: https://iopscience.iop.org/article/10.1088/1748-9326/abe5d6. Acesso em: 29 mar. 2023.

THOMPSON, Helen M. Interactions between pesticides; a review of reported effects and their implications for wildlife risk assessment. **Ecotoxicology**, [*s.l.*], v. 5, n. 2, p. 59-81, abr. 1996. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24193572/. Acesso em: 6 out. 2022
.

THORSTENSEN, Kristine. Pesticides induce oxidative stress in zebrafish embryo. 2014. 72 f. **Dissertação (Mestrado em Biomedicina)** – University of Bergen, Bergen, 2014. Disponível em: https://bora.uib.no/bora-xmlui/handle/1956/11678. Acesso em: 14 mar. 2023.

TSUCHIDA, Shigeki; SATO, Kiyomi. Glutathione Transferases and Cancer. **Critical Reviews In Biochemistry And Molecular Biology**, [*s.l.*], v. 27, n. 4-5, p. 337-384, jan. 1992. Disponível em: https://pubmed.ncbi.nlm.nih.gov/1521461/. Acesso em: 11 fev. 2023.

VELKI, Mirna *et al*. Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity. **Chemosphere**, [*s.l.*], v. 180, p. 65-76, ago. 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28391154/. Acesso em: 16 dez. 2022.

VIEIRA, Leonardo R *et al*. Proteomics analysis of zebrafish larvae exposed to 3,4‐dichloroaniline using the fish embryo acute toxicity test. **Environmental Toxicology**, [*s.l.*], v. 35, n. 8, p. 849-860, 14 mar. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32170993/. Acesso em: 10 mar. 2023.

VIEIRA, Leonardo R *et al.* Supporting the Next Generation of Risk Assessment in Toxicology: the design of aops based on the alternative model zebrafish. **Zebrafish Model For Biomedical Research**, [*s.l.*], p. 381-399, 2022. Springer Nature Singapore. Disponível em: https://link.springer.com/chapter/10.1007/978-981-16-5217-2_17. Acesso em: 19 abr. 2023.

WANG, Hao *et al*. Effects of Atrazine on the Development of Neural System of Zebrafish,Danio rerio. **Biomed Research International**, [*s.l.*], v. 2015, p. 1-10, 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/26114119/. Acesso em: 28 jul. 2022.

WANG, Yanhua *et al*. Single and joint toxic effects of five selected pesticides on the early life stages of zebrafish (Denio rerio). **Chemosphere**, [*s.l.*], v. 170, p. 61-67, mar. 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/27974272/. Acesso em: 09 set. 2022

WIEGAND, C *et al*. Uptake, Toxicity, and Effects on Detoxication Enzymes of Atrazine and Trifluoroacetate in Embryos of Zebrafish. **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 45, n. 2, p. 122-131, fev. 2000. Disponível em: https://pubmed.ncbi.nlm.nih.gov/10648131/. Acesso em: 15 maio 2023.

YANG, Chendong *et al*. Analysis of Hypoxia-Induced Metabolic Reprogramming. **Methods In Enzymology**, [*s.l.*] p. 425-455, 2014. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24862279/. Acesso em: 05 mar. 2023.

ZALUSKI, Amanda B *et al*. Atrazine and Diuron Effects on Survival, Embryo Development, and Behavior in Larvae and Adult Zebrafish. **Frontiers In Pharmacology**, [*s.l.*], v. 13, p. 34-45, 4 abr. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35444550/. Acesso em: 14 mar. 2023.

5 CONSIDERAÇÕES GERAIS

Este estudo revelou que a exposição aos herbicidas atrazina e diuron resultou em redução da taxa de sobrevivência e afetou o desenvolvimento dos estágios iniciais do peixe-zebra, evidenciado pelo aumento da mortalidade e ocorrência de efeitos não letais, como edemas de pericárdio e saco. Em contraste, o glifosato não demonstrou efeitos não letais ou mortalidade. Porém, foi observado um aumento na toxicidade quando a atrazina e o diuron foram utilizados em conjunto, indicando uma sinergia entre esses herbicidas. Análises bioquímicas revelaram alterações significativas nas enzimas relacionadas ao estresse oxidativo e dano tecidual, fornecendo informações fundamentais sobre possíveis mecanismos de toxicidade. Adicionalmente, foram realizadas análises de fluorescência para monitorar a produção de ERO's nas larvas expostas tanto aos herbicidas isoladamente como em suas misturas. Verificou-se um aumento significativo da fluorescência nas larvas expostas aos compostos que exibiram atividades enzimáticas reduzidas, em particular nas enzimas antioxidantes, em comparação com os demais compostos. Com base nos resultados obtidos, é possível inferir que o mecanismo de toxicidade está, pelo menos em parte, associado ao estresse oxidativo

Portanto, este estudo contribui para o avanço do conhecimento dos efeitos tóxicos decorrentes da exposição a misturas de agrotóxicos, bem como para a compreensão dos possíveis mecanismos de ação envolvidos. Essas descobertas são de grande importância para uma possível reavaliação dos limites permitidos em termos de impactos ambientais.

REFERÊNCIAS

AMORIM, Leiliane Coelho André. O uso dos biomarcadores na avaliação da exposição ocupacional a substâncias químicas. **Revista Brasileira de Medicina do Trabalho**, v. 1, n. 2, p. 124-132, 2003. Disponível em: https://cdn.publisher.gn1.link/rbmt.org.br/pdf/v1n2a05.pdf. Acesso em: 15 mai. 2023.

AKTAR, Md Wasim; SENGUPTA, Dwaipayan; CHOWDHURY, Ashim. Impact of pesticides use in agriculture: their benefits and hazards. **Interdisciplinary Toxicology**, v. 2, n. 1, p. 1-12, mar. 2009. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC2984095/. Acesso em: 18 fev. 2023.

AKHTAR, Naveed *et al.* Adverse effects of atrazine on blood parameters, biochemical profile and genotoxicity of snow trout (Schizothorax plagiostomus). **Saudi Journal Of Biological Sciences**, [*s.l.*], v. 28, n. 3, p. 1999-2003, mar. 2021. Disponível em: https://www.sciencedirect.com/science/article/pii/S1319562X21000012. Acesso em: 23 dez. 2022.

AL-SAWAFI, Abeer Ghazie A.; YAN, Yunjun. Bioconcentration and Antioxidant Status Responses in Zebrafish (Danio Rerio) Under Atrazine Exposure. **International Journal Of Chemical Engineering And Applications**, [*s.l.*] , p. 204-208, 2013. Disponível em: https://www.researchgate.net/publication/269800895. Acesso em: 22 fev. 2023.

AL-SAWAFI, Abeer Ghazie A.; YAN, Yunjun. Alterations of Acetylcholinesterase Activity and Antioxidant Capacity of Zebrafish Brain and Muscle Exposed to Sublethal Level of Cadmium. **International Journal Of Environmental Science And Development**, [*s.l.*], p. 327-330, 2013. Disponível em: https://www.researchgate.net/publication/269839107. Acesso em: 22 jan. 2023.

ANTONIOU, M. *et al.* Practices that Prevent the Formation of Cyanobacterial Blooms in Water Resources and Remove Cyanotoxins During Physical Treatment of Drinking Water. **Comprehensive Water Quality And Purification**, [*s.l.*], p. 173-195, 2014. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/B9780123821829000323?via%3Dihub. Acesso em: 22 fev. 2023.

ARANHA, Ana.; ROCHA, Luana. **"Coquetel" com 27 agrotóxicos foi achado na água de 1 em cada 4 municípios**. 2019. Disponível em: https://apublica.org/2019/04/coquetel-com-27-agrotoxicos-foi-achado-na-agua-de-1-em-cada-4-municipios-consulte-o-seu/. Acesso em: 18 fev. 2023.

BAER, K. N.; MARCEL, B. J. Glyphosate. In: **Encyclopedia of Toxicology**, v. 2, p. 767–769, 2014. Disponível em: https://ipga.us/wp-content/uploads/Glyphosate-Journal-of-toxicology.pdf. Acesso: 18 fev. 2023.

BLAHOVÁ, Jana *et al.* Oxidative stress responses in zebrafish Danio rerio after subchronic exposure to atrazine. **Food And Chemical Toxicology**, [*s.l.*], v. 61, p. 82-85, nov. 2013. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0278691513001506. Acesso em: 22 fev. 2023.

BORDIN, E. *et al.* Sublethal effects of the herbicides atrazine and glyphosate at environmentally relevant concentrations on South American catfish (*Rhamdia quelen*) embryos. **Environmental Toxicology and Pharmacology**, v. 98, p. 104057, 2023. Disponível em: https://doi.org/10.1016/j.etap.2022.104057. Acesso em: 18 mar. 2022.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, [*s.l.*], v. 72, n. 1-2, p. 248-254, maio 1976. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/0003269776905273. Acesso em: 2 jan. 2023.

CANEVAROLI, Miriam Ruiz *et al.* Remoção de herbicida atrazina por meio de filtros de carvão ativado granular associados com microrganismos no tratamento de água para abastecimento. **Engenharia Sanitária e Ambiental**, v. 26, n. 2, p. 263-272, abr. 2021. Disponível em:https://www.scielo.br/j/esa/a/BLyRw9TpV8h6gZyqyqrJWzr/abstract/?lang=pt. Acesso em: 2 dez. 2022.

CASSAR, Steven *et al.* Use of Zebrafish in Drug Discovery Toxicology. **Chemical Research in Toxicology**, v. 33, n. 1, p. 95-118, 18 out. 2019. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC7162671/. Acesso em: 4 abr. 2023.

CEDERGREEN, Nina *et al.* A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. **Environmental Toxicology and Chemistry**, v. 27, n. 7, p. 1621-1632, 1 jul. 2008. Disponível em: https://pubmed.ncbi.nlm.nih.gov/18271647/. Acesso em: 4 jan. 2023.

CHAHARDEHI, Amir Modarresi *et al.* Zebrafish as a Successful Animal Model for Screening Toxicity of Medicinal Plants. **Plants**, v. 9, n. 10, p. 1345, 12 out. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/33053800/. Acesso em: 2 jan. 2023.

CHROMCOVA, Luci. *et al.* The effects of atrazine exposure on early life stages of common carp (Cyprinus carpio). **Neuro endocrinology letters**, v. 34 Suppl 2, p. 95–101, 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24362100/. Acesso em: 2 jan. 2023.

CLAIRBONE, A. Catalase activity. In: GREENWALD, R. (ed.). *CRC* **handbook of methods in oxygen radical research**. Boca Raton: CRC Press, 1985. p. 283–284.

COUTINHO, Cláudia F. B. *et al.* Pesticidas: mecanismo de ação, degradação e toxidez. Pesticidas: **Revista de Ecotoxicologia e Meio Ambiente**, v. 15, 2005. Disponível em: https://revistas.ufpr.br/pesticidas/article/view/4469. Acesso em: 18 fev. 2023.

DELORENZO, Marie E.; FULTON, Michael H. Comparative risk assessment of permethrin, chlorothalonil, and diuron to coastal aquatic species. **Marine Pollution Bulletin**, [*s.l.*], v. 64, n. 7, p. 1291-1299, jul. 2012. Disponível em: https://pubmed.ncbi.nlm.nih.gov/22682878/. Acesso em: 22 fev. 2023.

DIAS, Ágata Cristina Lima *et al*. Ocorrência de atrazina em águas no Brasil e remoção no tratamento da água: revisão bibliográfica. **Revista Internacional de Ciências**, v. 8, n. 2, p. 149-168, 4 dez. 2018. Disponível em: https://www.e-publicacoes.uerj.br/index.php/ric/article/view/34202. Acesso em: 23 fev. 2023.

DOMINGUES, Inês *et al*. Biomarkers as a tool to assess effects of chromium (VI): comparison of responses in zebrafish early life stages and adults. **Comparative Biochemistry And Physiology Part C: Toxicology & Pharmacology**, v. 152, n. 3, p. 338-345, set. 2010. Disponível em: https://pubmed.ncbi.nlm.nih.gov/20566315/. Acesso em: 2 abr. 2023.

EGAAS, Eliann *et al*. A comparative study of effects of atrazine on xenobiotic metabolizing enzymes in fish and insect, and of the in vitro phase II atrazine metabolism in some fish, insects, mammals and one plant species. **Comparative Biochemistry And Physiology Part C: Pharmacology, Toxicology and Endocrinology**, v. 106, n. 1, p. 141-149, set. 1993. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/074284139390265M. Acesso em: 22 jan. 2023

FAN, Ruiqi *et al*. Individual and synergistic toxic effects of carbendazim and chlorpyrifos on zebrafish embryonic development. **Chemosphere**, v. 280, p. 130769, out. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34162088/. Acesso em: 7 maio 2023.

FANUCCHI, Michelle V.. Development of Antioxidant and Xenobiotic Metabolizing Enzyme Systems. **The Lung**, [*s.l.*], p. 177-185, 2004. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/B9780123247513500462?via%3Dihub. Acesso em: 7 maio 2023.

FELÍCIO, Andréia Arantes *et al.* Isolated and mixed effects of diuron and its metabolites on biotransformation enzymes and oxidative stress response of Nile tilapia (Oreochromis niloticus). **Ecotoxicology And Environmental Safety**, v. 149, p. 248-256, mar. 2018. Disponível em: https://pubmed.ncbi.nlm.nih.gov/29248837/. Acesso em: 7 jan. 2023.

FINNEY, D.J. Probit Analysis. **Journal Of Pharmaceutical Sciences**, [*s.l.*], v. 60, n. 9, p. 1432, set. 1971. Disponível em: https://onlinelibrary.wiley.com/doi/10.1002/jps.2600600940. Acesso em: 15 mar. 2023.

FLECKNELL, Paul. Replacement, Reduction, Refinement. **ALTEX - Alternatives to animal experimentation**, [S. l.], v. 19, n. 2, p. 73–78, 2002. Disponível em: https://www.altex.org/index.php/altex/article/view/1106. Acesso em: 18 feb. 2023

GEORGIADIS, Nikolaos *et al*. Pesticides and cardiotoxicity. Where do we stand? **Toxicology And Applied Pharmacology**, [*s.l.*], v. 353, p. 1-14, ago. 2018. Disponível em: https://pubmed.ncbi.nlm.nih.gov/29885332/. Acesso em: 23 maio 2023.

GONÇALVES, Íris Flávia Sousa *et al*. Toxicity testing of pesticides in zebrafish—a systematic review on chemicals and associated toxicological endpoints. **Environmental Science And Pollution Research**, [*s.l.*], v. 27, n. 10, p. 10185-10204, 15 fev. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32062774/. Acesso em: 30 maio 2023.

GÖRGE, Gerhard; NAGEL, Roland. Toxicity of lindane, atrazine, and deltamethrin to early life stages of zebrafish (Brachydanio rerio). **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 20, n. 3, p. 246-255, dez. 1990. Disponível em: https://pubmed.ncbi.nlm.nih.gov/1708710. Acesso em: 4 maio 2023.

HALLIWELL, B. Biochemistry of oxidative stress. **Biochemical Society Transactions**, Disponível em: https://pubmed.ncbi.nlm.nih.gov/17956298/. Acesso em: 15 fev. 2023

HAYNES, David *et al*. The impact of the herbicide Diuron on photosynthesis in three species of tropical seagrass. **Marine Pollution Bulletin**, [*s.l.*], v. 41, n. 7-12, p. 288-293, 2000. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0025326X00001272. Acesso em: 18 maio 2023.

HILL, Adrian J. *et al*. Zebrafish as a model vertebrate for investigating chemical toxicity. **Toxicological Sciences**, [*s.l.*], v. 86, n. 1, p. 6-19, 9 fev. 2005. Disponível em: https://pubmed.ncbi.nlm.nih.gov/15703261/. Acesso em: 23 jan. 2023

HO, Edwin *et al*. Biological markers of oxidative stress: applications to cardiovascular research and practice. **Redox Biology**, [*s.l.*], v. 1, n. 1, p. 483-491, 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24251116/. Acesso em: 22 dez. 2022.

HOLMES, Glen. Australia's pesticide environmental risk assessment failure: the case of diuron and sugarcane. **Marine Pollution Bulletin**, [*s.l.*], v. 88, n. 1-2, p. 7-13, nov. 2014. Disponível em: https://pubmed.ncbi.nlm.nih.gov/25152182/. Acesso em: 13 mar. 2023.

IBAMA. Relatórios de comercialização de agrotóxicos. [*s.l.*.: s.n.], 2017

IBAMA. Relatórios de comercialização de agrotóxicos. [*s.l.*.: s.n.], 2019

IFEN. Etudes et Travaux N 19: Les pesticides dans les eaux: collecte et traitement de donnéns. [*s.l.*: s.n.], 1998

IGHODARO, O.M.; AKINLOYE, O.A.. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. **Alexandria Journal Of Medicine**, [*s.l.*], v. 54, n. 4, p. 287-293, 1 dez. 2018. Disponível em: https://www.sciencedirect.com/science/article/pii/S2090506817301550. Acesso em: 15 abr. 2022.

ISLAM, Faisal *et al*. Potential impact of the herbicide 2,4-dichlorophenoxyacetic acid on human and ecosystems. **Environment International**, [*s.l.*], v. 111, p. 332-351, fev. 2018. Disponível em: https://pubmed.ncbi.nlm.nih.gov/29203058/. Acesso em: 22 fev. 2022.

IVANTSOVA, Emma *et al*. Developmental and behavioral toxicity assessment of glyphosate and its main metabolite aminomethylphosphonic acid (AMPA) in zebrafish embryos/larvae. **Environmental Toxicology And Pharmacology**, [*s.l.*], v. 93, p. 103873, jul. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35504511/. Acesso em: 15 abr. 2023

JAYARAJ, Ravindran *et al*. Review Article. Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. **Interdisciplinary Toxicology**, [*s.l.*], v. 9, n. 3-4, p. 90-100, 1 dez. 2016. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28652852/. Acesso em: 7 abr. 2023.

JIN, Yuanxiang *et al*. Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (Danio rerio). **Chemosphere**, [*s.l.*], v. 78, n. 7, p. 846-852, fev. 2010. Disponível em: https://pubmed.ncbi.nlm.nih.gov/20036412/. Acesso em: 15 jan. 2023.

JONES, Rj *et al*. Effects of herbicides diuron and atrazine on corals of the Great Barrier Reef, Australia. **Marine Ecology Progress Series**, [*s.l.*], v. 251, p. 153-167, 2003. Disponível em: https://espace.library.uq.edu.au/view/UQ:65824. Acesso em: 7 jan. 2023.

JOVANOVIć, Predrag *et al*. Lactate dehydrogenase and oxidative stress activity in primary open-angle glaucoma aqueous humour. **Bosnian Journal Of Basic Medical Sciences**, [*s.l.*], v. 10, n. 1, p. 83-88, 20 fev. 2010. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5596618/. Acesso em: 10 jul. 2023.

KANG, Min-Cheol *et al*. Protective effect of marine algae phlorotannins against AAPH-induced oxidative stress in zebrafish embryo. **Food Chemistry**, [*s.l.*], v. 138, n. 2-3, p. 950-955, 15 jun. 2013. Disponível em: https://www.sciencedirect.com/science/article/pii/S0308814612016068. Acesso em: 4 maio 2022.

KAO, L. M.; WILKINSON, C. F.; BRATTSTEN, L. B.. In‐vivo effects of 2,4‐D and atrazine on cytochrome P‐450 and insecticide toxicity in southern armyworm (Spodoptera eridania) larvae. **Pesticide Science**, [*s.l.*], v. 45, n. 4, p. 331-334, dez. 1995. Wiley.Disponível em: https://onlinelibrary.wiley.com/doi/abs/10.1002/ps.2780450407. Acesso em: 4 dez. 2022.

KRATCHMAN, Jessica; WANG, Bing; GRAY, George. Which is most sensitive? Assessing responses of mice and rats in toxicity bioassays. **Journal Of Toxicology And Environmental Health, Part A**, [*s.l.*], v. 81, n. 7, p. 173-183, 6 fev. 2018. Informa UK Limited.Disponível em: https://pubmed.ncbi.nlm.nih.gov/29405849/. Acesso em: 2 abr. 2023.

LANZARIN, Germano *et al*. Dose-dependent effects of a glyphosate commercial formulation – Roundup® UltraMax - on the early zebrafish embryogenesis. **Chemosphere**, [*s.l.*], v. 223, p. 514-522, maio 2019. Elsevier BV. Disponível em: https://pubmed.ncbi.nlm.nih.gov/30784758/. Acesso em: 7 jan. 2022.

LANZARIN, Germano *et al*. Inflammatory, Oxidative Stress, and Apoptosis Effects in Zebrafish Larvae after Rapid Exposure to a Commercial Glyphosate Formulation. Biomedicines, [*s.l.*], v. 9, n. 12, p. 1784, 27 nov. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34944599/. Acesso em: 15 jan. 2023.

LAWRENCE, Christian. The husbandry of zebrafish (Danio rerio): a review. **Aquaculture**, [*s.l.*], v. 269, n. 1-4, p. 1-20, set. 2007. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0044848607004012. Acesso em: 22 fev. 2023.

LI, Kang *et al*. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. **Chemosphere**, [*s.l.*], v. 171, p. 40-48, mar. 2017. Elsevier BV. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28002765/. Acesso em: 4 maio 2022

LI, Ming-Hui *et al*. Metabolic profiling of goldfish (Carassius auratis) after long-term glyphosate-based herbicide exposure. **Aquatic Toxicology**, [*s.l.*], v. 188, p. 159-169, jul. 2017. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0166445X17301315. Acesso em: 2 abr. 2023.

LI, Wei-Min *et al*. 3,4-Dichloroaniline-induced oxidative stress in liver of crucian carp (Carassius auratus). **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 56, n. 2, p. 251-255, out. 2003. Disponível em: https://pubmed.ncbi.nlm.nih.gov/12927556/. Acesso em: 1 maio 2023.

LIONETTO, Maria Giulia *et al*. Acetylcholinesterase as a Biomarker in Environmental and Occupational Medicine: new insights and future perspectives. **Biomed Research International**, [*s.l.*], v. 2013, p. 1-8, 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/23936791/. Acesso em: 3 jul. 2022.

LOBO, V; A PATIL,; A PHATAK,; CHANDRA, N. Free radicals, antioxidants and functional foods: impact on human health. **Pharmacognosy Reviews**, [*s.l.*], v. 4, n. 8, p. 118, 2010. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC3249911/. Acesso em: 15 dez. 2022.

LU, Jian *et al*. Characterization of glyphosate-induced cardiovascular toxicity and apoptosis in zebrafish. **Science Of The Total Environment**, [*s.l.*], v. 851, p. 158308, dez. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/36030873/. Acesso em: 7 jan. 2023.

MANSANO, Adrislaine S *et al*. Individual and mixture toxicity of carbofuran and diuron to the protozoan Paramecium caudatum and the cladoceran Ceriodaphnia silvestrii. **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 201, p. 110829, set. 2020. Disponível em: https://www.sciencedirect.com/science/article/pii/S0147651320306680. Acesso em: 22 maio

MARC, Julie *et al***.** A glyphosate-based pesticide impinges on transcription. **Toxicology And Applied Pharmacology**, [*s.l.*], v. 203, n. 1, p. 1-8, fev. 2005. Disponível em: https://pubmed.ncbi.nlm.nih.gov/15694458/. Acesso em: 2 jan. 2023.

MARCHI, G et al. Herbicidas: mecanismos de ação e uso. **Embrapa Cerrados-Documentos**, 2008. Disponível em: https://www.infoteca.cnptia.embrapa.br/bitstream/doc/571939/1/doc227.pdf.

MARINS, Aline Teixeira *et al*. A mixture of pesticides at environmental concentrations induces oxidative stress and cholinergic effects in the neotropical fish Rhamdia quelen. **Ecotoxicology**, [*s.l.*], v. 30, n. 1, p. 164-174, 16 nov. 2020. Disponível em: https://link.springer.com/article/10.1007/s10646-020-02300-6. Acesso em: 23 out. 2021.

MARKING, L. Toxicity of chemical mixtures. In: Rand, G., Petroceli, S. (Eds.), **Fundamentals of Aquatic Toxicology**. Hemisphere Publishing Corporation, 1985

MARTINS, Rafael Xavier *et al*. Exposure to 2,4-D herbicide induces hepatotoxicity in zebrafish larvae. **Comparative Biochemistry And Physiology Part C: Toxicology & Pharmacology**, [*s.l.*], v. 248, p. 109110, out. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34144256/. Acesso em: 7 abr. 2022.

MASSARSKY, Andrey *et al*. Glutathione and zebrafish: old assays to address a current issue. **Chemosphere**, [*s.l.*], v. 168, p. 707-715, fev. 2017. Disponível em: https://www.sciencedirect.com/science/article/pii/S0045653516315387. Acesso em: 12 mar. 2022.

MELE, James *et al*. Characterization of Transgenic Mice That Overexpress Both Copper Zinc Superoxide Dismutase and Catalase. **Antioxidants & Redox Signaling**, [*s.l.*], v. 8, n. 3-4, p. 628-638, mar. 2006. Disponível em: https://pubmed.ncbi.nlm.nih.gov/16677106/. Acesso em: 15 dez. 2021.

MERCURIO, Philip *et al*. Glyphosate persistence in seawater. **Marine Pollution Bulletin**, [*s.l.*], v. 85, n. 2, p. 385-390, ago. 2014. Disponível em: https://www.sciencedirect.com/science/article/pii/S0025326X14000228. Acesso em: 27 mar. 2022.

MHADHBI, Lazhar; BEIRAS, Ricardo. Acute Toxicity of Seven Selected Pesticides (Alachlor, Atrazine, Dieldrin, Diuron, Pirimiphos-Methyl, Chlorpyrifos, Diazinon) to the Marine Fish (Turbot, Psetta maxima). **Water, Air, & Soil Pollution**, [*s.l.*], v. 223, n. 9, p. 5917-5930, 28 set. 2012. Disponível em: https://link.springer.com/article/10.1007/s11270-012-1328-9#citeas. Acesso em: 10 mar. 2022.

MUIR, Derek C. G.; YOO, Jai Y.; BAKER, Bruce E.. Residues of Atrazine and N-deethylated Atrazine in water from five agricultural watersheds in Québec. **Archives Of Environmental Contamination And Toxicology**, [*s.l.*], v. 7, n. 1, p. 221-235, dez. 1978. Disponível em: https://pubmed.ncbi.nlm.nih.gov/677946/. Acesso em: 11 fev. 2023.

MUNIZ, Marta Silva *et al*. Moxidectin toxicity to zebrafish embryos: bioaccumulation and biomarker responses. **Environmental Pollution**, [*s.l.*], v. 283, p. 117096, ago. 2021. Disponível em: https://pubmed.ncbi.nlm.nih.gov/33866217/. Acesso em: 30 abr. 2023.

NA, Yi-Rang *et al*. Protective effects of vitamin E against 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) induced toxicity in zebrafish embryos. **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 72, n. 3, p. 714-719, mar. 2009. Disponível em: https://pubmed.ncbi.nlm.nih.gov/18973944/. Acesso em: 2 jan. 2023.

NESKOVIC, N.K *et al*. Acute and Subacute Toxicity of Atrazine to Carp (Cyprinus carpio L.). **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 25, n. 2, p. 173-182, abr. 1993. Disponível em: https://pubmed.ncbi.nlm.nih.gov/7682501/. Acesso em: 31 mar. 2023.

OECD. Test No. 236, Fish Embryo Acute Toxicity (FET) Test. **OECD Guidelines for the Testing of Chemicals**, Section 2. Paris: OECD Publishing, 2013. Disponível em: https://doi.org/10.1787/9789264203709-en. Acesso em: 10 mar. 2025.

OSTEN, Jaime Rendon-Von; DZUL-CAAMAL, Ricardo. Glyphosate Residues in Groundwater, Drinking Water and Urine of Subsistence Farmers from Intensive Agriculture Localities: a survey in hopelchén, campeche, mexico. **International Journal Of Environmental Research And Public Health**, [*s.l.*], v. 14, n. 6, p. 595, 3 jun. 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28587206/. Acesso em: 3 abr. 2023.

PARICHY, David M. Advancing biology through a deeper understanding of zebrafish ecology and evolution. **Elife**, [*s.l.*], v. 4, p. 1-10, 25 mar. 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/25807087/. Acesso em: 4 jan. 2022.

PÉREZ, Joanne *et al*. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. **Environmental Science And Pollution Research**, [*s.l.*], v. 20, n. 7, p. 4671-4680, 5 jan. 2013. Disponível em: https://pubmed.ncbi.nlm.nih.gov/23288680/. Acesso em: 9 jan. 2023

PIZZINO, Gabriele *et al*. Oxidative Stress: harms and benefits for human health. **Oxidative Medicine And Cellular Longevity**, [*s.l.*], v. 2017, n. 1, p. 10-20, jan. 2017. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5551541/. Acesso em: 15 fev. 2023.

PHYU, Y.L. *et al*. A comparison of mixture toxicity assessment: examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to ceriodaphnia cf. dubia. **Chemosphere**, [*s.l.*], v. 85, n. 10, p. 1568-1573, nov. 2011. Disponível em: https://pubmed.ncbi.nlm.nih.gov/21925699/. Acesso em: 23 dez. 2021.

POPP, József; PETő, Károly; NAGY, János. Pesticide productivity and food security. A review. **Agronomy For Sustainable Development**, [*s.l.*], v. 33, n. 1, p. 243-255, 17 out. 2012. Disponível em: https://link.springer.com/article/10.1007/s13593-012-0105-x. Acesso em: 8 set. 2022.

POUR, L. Activity assay of glutathione S-transferase (GSTs) enzyme as a diagnostic biomarker for liver hydatid cyst in vitro. **Iranian Journal of Public Health**, v. 43, n. 7, p. 994, 2014. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC4401064/. Acesso em: 8 set. 2022.

QU, Mengjie *et al*. Fate of atrazine and its relationship with environmental factors in distinctly different lake sediments associated with hydrophytes. **Environmental Pollution**, [*s.l.*], v. 256, p. 113371, jan. 2020. Disponível em: https://www.sciencedirect.com/science/article/abs/pii/S0269749119322705. Acesso em: 4 jan. 2023.

QUINTANEIRO, C. *et al*. Endocrine and physiological effects of linuron and S-metolachlor in zebrafish developing embryos. **Science Of The Total Environment**, [*s.l.*], v. 586, p. 390-400, maio 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28209406/. Acesso em: 15 abr. 2021.

RECKZIEGEL, Patrícia *et al*. Antioxidant protection of gallic acid against toxicity induced by Pb in blood, liver and kidney of rats. **Toxicology Reports**, [*s.l.*], v. 3, p. 351-356, 2016. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28959556/. Acesso em: 17 nov. 2022.

ROCHA, Paulo Roberto Ribeiro *et al*. Meia-vida do diuron em solos com diferentes atributos físicos e químicos. **Ciência Rural**, [*s.l.*], v. 43, n. 11, p. 1961-1966, nov. 2013. Disponível em: https://www.scielo.br/j/cr/a/Bx3Pp7qfPpzmqFbqcyj5ZFH/ Acesso em: 7 fev. 2021.

RODNEY, Sara *et al*. Estimating the Toxicity of Pesticide Mixtures to Aquatic Organisms: a review. **Human And Ecological Risk Assessment**: An International Journal, [*s.l.*], v. 19, n. 6, p. 1557-1575, 30 maio 2013. Disponível em: https://www.tandfonline.com/doi/full/10.1080/10807039.2012.723180. Acesso em: 16 dez. 2022

RODRIGUES, Laís de Brito *et al*. Ecotoxicological assessment of glyphosate-based herbicides: effects on different organisms. **Environmental Toxicology And Chemistry**, [*s.l.*], v. 36, n. 7, p. 1755-1763, 12 ago. 2016. Disponível em: https://pubmed.ncbi.nlm.nih.gov/27517480/. Acesso em: 11 maio 2022

RODRIGUES, Laís de Brito *et al*. Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms. **Mutation Research/Genetic Toxicology And Environmental Mutagenesis**, [*s.l.*], v. 842, p. 94-101, jun. 2019. Disponível em: https://pubmed.ncbi.nlm.nih.gov/31255230/. Acesso em: 4 jan. 2023

ROSS, M. Herbicide mode-of-action summary.**Weed science**, 1996. Disponível em: https://extension.purdue.edu/extmedia/WS/WS-23-W.html

SANCHÍS, Josep *et al*. Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry. **Analytical And Bioanalytical Chemistry**, [*s.l.*], v. 402, n. 7, p. 2335-2345, 20 nov. 2011. Disponível em: https://pubmed.ncbi.nlm.nih.gov/22101424/. Acesso em: 15 abr. 2023.

SANT, Karilyn E.; TIMME-LARAGY, Alicia R.. Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo. **Current Environmental Health Reports**, [*s.l.*], v. 5, n. 1, p. 125-133, 7 fev. 2018. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5876134/. Acesso em: 15 jun. 2023.

SANTOS, Thais G.; MARTINEZ, Cláudia B.R.. Atrazine promotes biochemical changes and DNA damage in a Neotropical fish species. **Chemosphere**, [*s.l.*], v. 89, n. 9, p. 1118-1125, nov. 2012. Disponível em: https://pubmed.ncbi.nlm.nih.gov/22739540/. Acesso em: 23 mar. 2022

SCHEIL, Volker *et al*. Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (Danio rerio) embryos and larvae. **Ecotoxicology**, [*s.l.*], v. 18, n. 3, p. 355-363, 18 dez. 2008. Springer Science and Business Media LLC. Disponível em: https://pubmed.ncbi.nlm.nih.gov/19093198/. Acesso em: 25 nov. 2023.

SHARMA, Anket *et al*. Worldwide pesticide usage and its impacts on ecosystem. **Sn Applied Sciences**, [*s.l.*], v. 1, n. 11, p. 327-345, 21 out. 2019. Disponível em: https://link.springer.com/article/10.1007/s42452-019-1485-1. Acesso em: 29 ago. 2022.

SHEEHAN, David *et al*. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. **Biochemical Journal**, [*s.l.*], v. 360, n. 1, p. 1-16, 8 nov. 2001. Portland Press Ltd. Disponível em: https://pubmed.ncbi.nlm.nih.gov/11695986/. Acesso em: 24 out. 2022

SILVA, Rafaella *et al*. Transgenic zebrafish (Danio rerio) as an emerging model system in ecotoxicology and toxicology: historical review, recent advances, and trends. **Science Of The Total Environment**, [*s.l.*], v. 848, p. 157665, nov. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35907527/. Acesso em: 18 maio 2022.

SLANINOVA, A. *et al*. A review: Oxidative stress in fish induced by pesticides. **Neuro Endocrinology Letters**, v. 30 Suppl 1, p. 2–12, 21 dez. 2009. Disponível em: https://pubmed.ncbi.nlm.nih.gov/20027135/. Acesso em: 18 maio 2022.

SOLOMON, Keith R *et al*. Ecological risk assessment of atrazine in North American surface waters. **Environmental Toxicology And Chemistry**, [*s.l.*], v. 32, n. 1, p. 10-11, 1 jan. 2013. Disponível em: https://academic.oup.com/etc/article/32/1/10/7736652. Acesso em: 23 mar. 2022

SULE, Rasheed O.; CONDON, Liam; GOMES, Aldrin V.. A Common Feature of Pesticides: oxidative stress–the role of oxidative stress in pesticide-induced toxicity. **Oxidative Medicine And Cellular Longevity**, [*s.l.*], v. 2022, p. 1-31, 19 jan. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35096268/. Acesso em: 12 abr. 2022

SUN, Hongmao *et al*. Paradigm Shift in Toxicity Testing and Modeling. **The Aaps Journal**, [*s.l.*], v. 14, n. 3, p. 473-480, 20 abr. 2012. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC3385826/. Acesso em: 22 maio 2022.

SZYMANSKA, Renata; POSPISIL, Pavel; KRUK, Jerzy. Plant-Derived Antioxidants in Disease Prevention. **Oxidative Medicine And Cellular Longevity**, [*s.l.*], v. 2016, n. 1, p. 30-40, jan. 2016. Disponível em: https://pmc.ncbi.nlm.nih.gov/articles/PMC5209612/. Acesso em: 4 dez. 2022.

TANG, Fiona H.M.; MAGGI, Federico. Pesticide mixtures in soil: a global outlook. **Environmental Research Letters**, [*s.l.*], p. 20-30, 12 fev. 2021. Disponível em: https://iopscience.iop.org/article/10.1088/1748-9326/abe5d6. Acesso em: 29 mar. 2023.

TEAME, Tsegay *et al*. The use of zebrafish (Danio rerio) as biomedical models. **Animal Frontiers**, [*s.l.*], v. 9, n. 3, p. 68-77, 25 jun. 2019. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32002264/. Acesso em: 15 jun. 2022

THOMPSON, Helen M. Interactions between pesticides; a review of reported effects and their implications for wildlife risk assessment. **Ecotoxicology**, [*s.l.*], v. 5, n. 2, p. 59-81, abr. 1996. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24193572/. Acesso em: 6 out. 2022
.
THORSTENSEN, Kristine. Pesticides induce oxidative stress in zebrafish embryo. 2014. 72 f. **Dissertação (Mestrado em Biomedicina)** – University of Bergen, Bergen, 2014. Disponível em: https://bora.uib.no/bora-xmlui/handle/1956/11678. Acesso em: 14 mar. 2023.

TSUCHIDA, Shigeki; SATO, Kiyomi. Glutathione Transferases and Cancer. **Critical Reviews In Biochemistry And Molecular Biology**, [*s.l.*], v. 27, n. 4-5, p. 337-384, jan. 1992. Disponível em: https://pubmed.ncbi.nlm.nih.gov/1521461/. Acesso em: 11 fev. 2023.

VELKI, Mirna *et al*. Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity. **Chemosphere**, [*s.l.*], v. 180, p. 65-76, ago. 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28391154/. Acesso em: 16 dez. 2022.

VIEIRA, Leonardo R *et al*. Proteomics analysis of zebrafish larvae exposed to 3,4-dichloroaniline using the fish embryo acute toxicity test. **Environmental Toxicology**,

[*s.l.*], v. 35, n. 8, p. 849-860, 14 mar. 2020. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32170993/. Acesso em: 10 mar. 2023.

VIEIRA, Leonardo R *et al.* Supporting the Next Generation of Risk Assessment in Toxicology: the design of aops based on the alternative model zebrafish. **Zebrafish Model For Biomedical Research**, [*s.l.*], p. 381-399, 2022. Springer Nature Singapore. Disponível em: https://link.springer.com/chapter/10.1007/978-981-16-5217-2_17. Acesso em: 19 abr. 2023.

WANG, Hao *et al*. Effects of Atrazine on the Development of Neural System of Zebrafish,Danio rerio. **Biomed Research International**, [*s.l.*], v. 2015, p. 1-10, 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/26114119/. Acesso em: 28 jul. 2022.

WANG, Yanhua *et al*. Single and joint toxic effects of five selected pesticides on the early life stages of zebrafish (Denio rerio). **Chemosphere**, [*s.l.*], v. 170, p. 61-67, mar. 2017. Disponível em: https://pubmed.ncbi.nlm.nih.gov/27974272/. Acesso em: 09 set. 2022

WIEGAND, C *et al*. Uptake, Toxicity, and Effects on Detoxication Enzymes of Atrazine and Trifluoroacetate in Embryos of Zebrafish. **Ecotoxicology And Environmental Safety**, [*s.l.*], v. 45, n. 2, p. 122-131, fev. 2000. Disponível em: https://pubmed.ncbi.nlm.nih.gov/10648131/. Acesso em: 15 maio 2023.

WIRBISKY, Sara; FREEMAN, Jennifer. Atrazine Exposure and Reproductive Dysfunction through the Hypothalamus-Pituitary-Gonadal (HPG) Axis. **Toxics**, [*s.l.*], v. 3, n. 4, p. 414-450, 2 nov. 2015. Disponível em: https://pubmed.ncbi.nlm.nih.gov/28713818/. Acesso em: 10 mar. 2023.

YADAV, I.; *et al*. Pesticides classification and its impact on human and environment. **Environmental science and engineering**, v. 6, p. 140-158, 2017 Disponível em: https://www.ijcmas.com/8-3-2019/Rajveer%20Kaur,%20et%20al.pdf.Acesso em: 05 mar. 2023.

YANG, Chendong *et al*. Analysis of Hypoxia-Induced Metabolic Reprogramming. **Methods In Enzymology**, [*s.l.*], p. 425-455, 2014. Disponível em: https://pubmed.ncbi.nlm.nih.gov/24862279/. Acesso em: 05 mar. 2023.

ZHANG, Mei *et al*. Antioxidant Properties of Quercetin. **Advances In Experimental Medicine And Biology**, [*s.l.*], p. 283-289, 2011. Disponível em: https://pubmed.ncbi.nlm.nih.gov/21445799/. Acesso em: 11 out. 2022

ZHAO, Chongjun *et al*. Nux Vomica Exposure Triggered Liver Injury and Metabolic Disturbance in Zebrafish Larvae. **Zebrafish**, [*s.l.*], v. 15, n. 6, p. 610-628, dez. 2018. Disponível em: https://pubmed.ncbi.nlm.nih.gov/30277848/. Acesso em: 12 out. 2022.

ZALUSKI, Amanda B *et al*. Atrazine and Diuron Effects on Survival, Embryo Development, and Behavior in Larvae and Adult Zebrafish. **Frontiers In Pharmacology**, [*s.l.*], v. 13, p. 34-45, 4 abr. 2022. Disponível em: https://pubmed.ncbi.nlm.nih.gov/35444550/. Acesso em: 14 mar. 2023.

ANEXO A- CERTIFICADO DA COMISSÃO DE ÉTICA

Universidade Federal da Paraíba

CEUA

Universidade Federal da Paraíba

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Do rio ao mar: perfil ecotoxicológico dos herbicidas 2,4-D, diuron, glifosato e suas misturas em organismos aquáticos", protocolada sob o CEUA nº 5901141020 (ID 001238), sob a responsabilidade de **Luis Fernando Marques dos Santos** *e equipe; Davi Felipe Farias* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA/UFPB) na reunião de 04/12/2020.

We certify that the proposal "From the river to the sea: ecotoxicological profile of the herbicides 2,4-D, diuron, glyphosate and their mixtures in aquatic organisms", utilizing 1768 Fishes (males and females), protocol number CEUA 5901141020 (ID 001238), under the responsibility of **Luis Fernando Marques dos Santos** *and team; Davi Felipe Farias* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Federal University of Paraíba (CEUA/UFPB) in the meeting of 12/04/2020.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 01/2021 a 12/2022 Área: Biologia Celular E Molecular

Origem:	Unidade de Produção de Organismos Modelo Não Convencionais (UniPOM)			
Espécie:	Peixes	sexo: Machos e Fêmeas	idade: 2 a 4 dias	Quantidade: 1768
Linhagem:	Danio rerio		Peso: 2 a 4 g	

João Pessoa, 18 de julho de 2023

Prof. Dr. Luiz Henrique César Vasconcelos
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal da Paraíba

Profa. Dra. Gláucia Veríssimo Faheina Martins
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal da Paraíba