UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Avaliação dos efeitos da exposição aguda aos agrotóxicos 2,4-D e Fipronil em brânquias de *Danio rerio*

Natália Prudêncio Viana

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Dissertação apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais, do Centro de Ciências Biológicas e da Saúde, como parte dos requisitos para obtenção do Título de Mestra em Ecologia e Recursos Naturais.

Orientadora: Marisa Narciso Fernandes

Co-orientadora: Iara da Costa Souza

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Comissão Julgadora:

Profa. Dra. Marisa Narciso Fernandes (UFSCar)

Profa. Dra. Adrislaine da Silva Mansano Dornfeld (UFSCar)

Profa. Dra. Juliane Silberschmidt Freitas (UEMG)

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RESUMO

Os agrotóxicos utilizados nas monoculturas têm como função principal a diminuição de perdas durante o processo de produção. Esses compostos podem afetar um amplo espectro de espécies vegetais e animais, induzindo desde alterações bioquímicas e morfológicas, até à bioconcentração nos tecidos, podendo levar à morte desses organismos. Assim, análises bioquímicas e histológicas de organismos bioindicadores se tornam ferramentas eficazes para a determinação do impacto dessas substâncias. Nesse trabalho, o peixe *Danio rerio*, modelo biológico mundialmente utilizado em ensaios toxicológicos, foi escolhido para avaliar o impacto de concentrações de relevância ambiental de dois agrotóxicos utilizados em monoculturas de cana-de-açúcar no Brasil, fipronil e 2,4-D, testados isoladamente e em mistura. Fipronil e 2,4-D bioconcentraram no corpo de *D. rerio*, apresentando o fator de bioconcentração (BCF) de 1,092 para o fipronil, 1,373 para o 2,4-D isolados; em mistura, o BCF apresentou valores de 1,261 e 1,005 para o inseticida e herbicida, respectivamente. Brânquias de peixes expostos ao fipronil apresentaram diminuição na atividade da catalase e aumentaram quando expostos ao herbicida. Em mistura, houve aumento na atividade da glutationa-S-transferase em amostras de brânquias e do corpo inteiro, além do aumento observado na atividade da acetilcolinesterase em músculo de *D. rerio*. As brânquias dos peixes expostos tiveram alterações histopatológicas e aumento da área fracional das células cloreto. Os resultados obtidos mostram respostas de *D. rerio* quando exposto ao inseticida e herbicida por 96 h, indicando que os agrotóxicos oferecem risco a saúde do peixe em concentrações ambientalmente relevantes.

Palavras-chave: indicadores ambientais; agroquímicos; biomarcadores

.ABSTRACT

The pesticides used in monocultures have the main function of reducing losses during the production process. These compounds can affect a wide spectrum of plant and animal species, inducing from biochemical and morphological changes to bioconcentration in tissues, which can lead to the death of these organisms. Thus, biochemical and histological analyzes of bioindicator organisms become effective tools for determining the impact of these substances. In this work the fish *Danio rerio*, a biological model used worldwide in toxicological tests, was chosen to assess the impact of concentrations of environmental relevance of two pesticides used in sugarcane monocultures in Brazil, fipronil and 2,4-D, tested alone and in mixture. Fipronil and 2,4-D bioconcentrate in the body of *D. rerio*, with a bioconcentration factor (BCF) of 1,092 for fipronil, 1,373 for 2,4-D alone; in mixture, BCF presented values of 1.261 and 1.005 for the insecticide and herbicide, respectively. Gills of fish exposed to fipronil showed a decrease in catalase activity and increased when exposed to the herbicide. In mixture, there was an increase in the activity of glutathione-S-transferase in samples of gills and of the whole body, in addition to the increase observed in the activity of acetylcholinesterase in *D. rerio* muscle. The gills of the exposed fish had histopathological changes and an increase in the fractional area of the chloride cells. The results obtained show responses of *D. rerio* when exposed to the insecticide and herbicide for 96 h, indicating that pesticides pose a risk to fish health in environmentally relevant concentrations.

Keywords: environmental indicators; agrochemicals; biomarkers.

LISTA DE ILUSTRAÇÕES

deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

Figure 5. Scanning electron microscopy (MEV) of the gills of *Danio rerio* from control group and groups exposed to fipronil, 2,4-D and their mixture. A and B: Control group: filament (F) and lamella (L). Note the pavement cell (PVC) shape and surface microridges (arrows). B: Chloride cells (CC) with microvilli (arrows) in the filament epithelium. C: CC having low microvillus (black arrow) and mucus dropped (white arrow). D: PVC (*) and CC having low microvillus in fish exposed to fipronil, E: CC among PVCs cells in the interlamellar epithelium in fish exposed to 2,4-D. Note short and few microvilli among PVC (arrow). F: CC without microvillus and undefined shape at epithelial surface in fish exposed to the mixture fipronil + 2,4-D (arrows).

Figure 6. Chloride cell fractional area (CCFA, A) and density (B) in the gill filaments of *Danio rerio* from control group and groups exposed to fipronil, 2,4-D and their mixture. Bars are the mean ± standard deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

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1. INTRODUÇÃO

1.1. Contaminação de ambientes aquáticos

Os ecossistemas de água doce têm grande importância não só apenas para utilização humana e seu desenvolvimento; é o habitat de espécies de diversas classes. Cerca de 97 % dessas águas são encontradas em lençóis freáticos, neve ou geleira, restando pouco para cursos d'água distribuídos como rios, riachos e lagoas, que estão cada vez mais susceptíveis a sofrerem impacto das atividades antrópicas em decorrência ao aumento no desenvolvimento dos centros urbanos, monocultura e agropecuária (Hettige et al., 2000; Mitchell, 2005; Evans et al., 2019).

O aumento da produção agrícola está ligado com a demanda para produção alimentícia e derivados de plantas, fazendo com que houvesse maior utilização de Organismos Geneticamente Modificados (OGM), agrotóxicos e fertilizantes, com a finalidade de minimizar cada vez mais as perdas durante o processo de desenvolvimento das monoculturas (Elahi et al., 2019). Entretanto, na maioria dos casos não há preocupação com sustentabilidade, principalmente em países subdesenvolvidos ou em desenvolvimento (Moran et al., 2007).

O Brasil é o segundo maior produtor mundial de etanol a partir da cana-de-açúcar e se destaca como um grande consumidor de agrotóxicos (Souza et al., 2019; Andrade et al., 2019). A cana-de-açúcar, uma das principais atividades agrícolas praticadas no país, com produção anual de aproximadamente 600 milhões de toneladas, utiliza uma grande quantidade e variedade de defensivos agrícolas (CONAB, 2017). Segundo o MAPA (Ministério da Agricultura, Pecuária e Abastecimento), 309 formulações comerciais de agrotóxicos classificados como "muito perigoso ao meio ambiente" estão registrados para controle de pragas em monoculturas de cana de açúcar (MAPA, 2020).

Esse elevado uso dos agroquímicos no cultivo da cana-de-açúcar no estado tornou-se uma grande preocupação ambiental, pois é tido como uma das principais ameaças de contaminação para os corpos hídricos. Em muitas áreas de produção de monocultura não se respeita as normas de preservação de mata ciliar no entorno dos cursos d'água (Teresa et al., 2015). Quando se soma à legalização de agrotóxicos e a falta de treinamento para quantidade de aplicações dessas substâncias, pode acabar resultando em impactos em organismos não-alvo, devido ao carreamento desses compostos pela lixiviação e/ou pela técnica de *spray-drift*. (Silva et al., 2013; Sanches et al., 2017).

O risco da contaminação da água por xenobióticos pode atingir diversas espécies não-alvo (Freitas et al., 2019; López-Perea et al., 2019; Sehonova et al., 2018), além de contribuir para a diminuição da qualidade de vida humana (Valdés et al., 2014). Podem acontecer diferentes tipos de contaminação de ambientes aquáticos (Derísio, 1992): (i) industrial, devido a produção de resíduos líquidos, sólidos e/ou gasosos, (ii) urbana, proveniente de aterros sanitários, esgotos e deficiência no tratamento dos resíduos produzidos, (iii) natural, por meio da decomposição animal ou vegetal ou de chuvas que podem levar ao carreamento de partículas sólidas rochosas ou solo, (iv) agrícola/pecuária, devido a utilização de agrotóxicos, fertilizantes ou contaminação através das fezes e urina animais.

1.2. Agrotóxicos de ampla utilização na cana-de-açúcar brasileira: Fipronil e 2,4-D

O fipronil é um inseticida de alta eficácia sintetizado em 1987 e muito utilizado nos últimos anos como uma alternativa para substituição de agrotóxicos da classe dos organofosforados, devido a sua alta eficácia e por ser pouco menos agressivo ao meio ambiente. É facilmente encontrado no comércio devido sua ampla utilização, que vai desde fórmulas para

agricultura, veterinárias (para controle de ectoparasitas) e, em casas, para combater insetos indesejados (Gupta e Anandón, 2018).

A alta eficácia do fipronil se dá pelo modo de ação, que ocorre diretamente nos canais de cloro dos receptores GABA (ácido gama-aminobutírico), onde se ligam e impedem a entrada dos íons de cloro na célula, resultando em uma hiperexcitação neural, aumento de contrações e resultando na morte do organismo-alvo (Gupta e Anandón, 2018). Além disso, os subprodutos formados pela degradação do fipronil (Figura 1) podem ser mais tóxicos e persistentes no ambiente do que sua forma primária (Baird et al., 2013; Gripp et al., 2017), tornando-se um risco muito maior para organismos não-alvo devido, principalmente, à alta lipofilicidade desses compostos, que atravessam com facilidade membranas celulares (Guo et al., 2018).

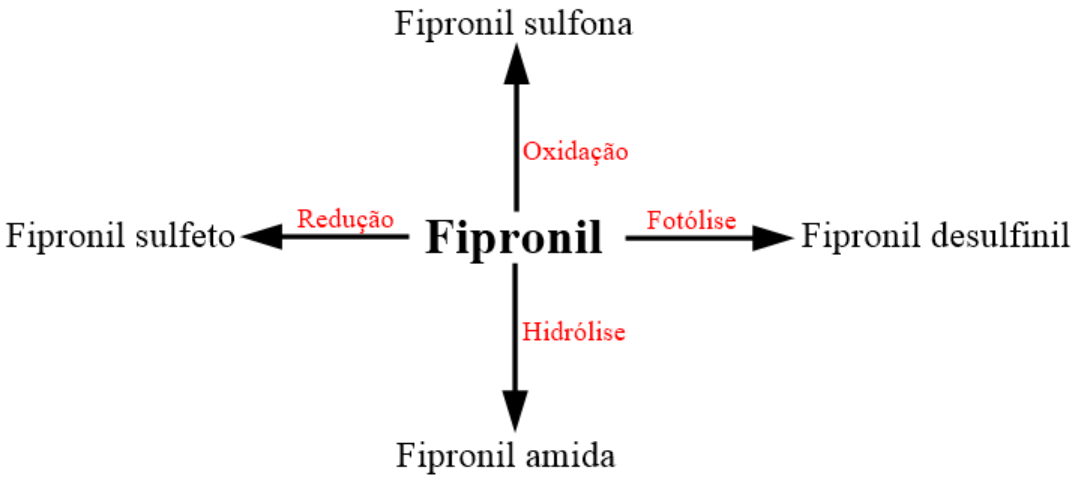

Figura 1. Forma primária do fipronil, modos de degradação e metabólitos resultantes.

O princípio ativo do fipronil possui solubilidade moderada ou baixa em água e tem alta capacidade de ligar-se a partículas do solo (Gripp et al., 2017). Possui rápida degradação e formação de seus metabólitos em água (de 4 a 12 horas), persistindo em solos aeróbicos de 122 a 128 dias (EPA, 1996). Em formulações comerciais são adicionados surfactantes/adjuvantes

que tem como função aumentar a solubilidade do composto, podendo influenciar a biodisponibilidade do composto no meio ambiente e aumentar a toxicidade (Wang et al., 2010; Gripp et al., 2017). A toxicidade desse inseticida em sua forma primária, bem como seus metabólitos, levou a recomendação da regulação de seu uso, segundo a Organização Mundial da Saúde (2000).

A quantidade aceitável da ingestão diária (através de resíduos em itens de consumo) é de 0,2 µg/kg de peso corporal, para humanos. Na União Europeia e China, por exemplo, o fipronil é proibido em monoculturas; por outro lado, é legalizado e muito utilizado no Brasil. Alburquerque et al. (2016) mostra a ocorrência em 54 % das 251 amostras de água analisadas no estado do Rio Grande do Sul (variando de 0,05 a 26,2 µg/L), enquanto que análise divulgada pela CETESB (2018), no estado de São Paulo, apontou a maior concentração registrada: 465 µg/L. Além disso, sabe-se que o inseticida pode ser tóxico a diversos organismos não-alvo como invertebrados (terrestres e aquáticos), peixes (marinhos e de água doce), mamíferos e aves (Tomlin, 2006).

O 2,4-D é uma auxina sintetizada na década de 1940 que atua como um herbicida seletivo, sendo muito utilizado atualmente devido ao baixo custo e alta especificidade em dicotiledôneas, podendo ser utilizado tanto em monoculturas quanto em jardinagem domiciliar, ou seja, é um herbicida de fácil acesso no comércio. Apesar de ter a mesma ação que o hormônio natural auxina sobre o organismo vegetal, o mesmo não consegue degradar o 2,4-D, que possui efeito como herbicida em concentrações acima de 500 mg/L. Em solo, o 2,4-D possui meia-vida de até 10 dias, já em ambientes aquáticos varia de 15 dias em ambientes aeróbicos e 41 a 333 dias em anaeróbicos (EPA, 2005).

Seu modo de ação ocorre de três maneiras na planta: (i) aumentando a quantidade da produção de proteínas, (ii) aumentando a produção de etileno, uma das primeiras respostas

hormonais diante algum estresse e (iii) modificando a plasticidade da parede celular da dicotiledônea. Além disso, o 2,4-D estimula o crescimento descontrolado da planta, levando ao enrolamento do caule, ressecamento das folhas e, consequente, morte (Song, 2014). No Brasil, o relatório da CETESB (2018) quantificou 366,6 µg/L de 2,4-D em cursos d'água no estado de São Paulo e a presença do herbicida foi identificada em 14 % dos 461 pontos de amostragem. O efeito desse herbicida pode ser observado em espécies não-alvo vegetais e animais, tendo influência de variáveis físico-químicas ambientais (WHO, 1989). Estudos mostram alterações histopatológicas e de peroxidação lipídica em lagostim (Benli et al., 2016), alterações bioquímicas e consequente diminuição da taxa respiratória e natatória de girinos (Freitas et al., 2019) e diminuição da taxa de sobrevivência de larvas de *Pimephales promelas* (Dehnert et al., 2018).

Agrotóxicos podem ser detectados simultaneamente em amostras de cursos d'água próximos às monoculturas (CETESB, 2018; Albuquerque et al., 2016), isso se deve a aplicações de diferentes agrotóxicos realizadas ao mesmo tempo ou em curto intervalo, que tem como finalidade o aumento da eficiência da produção e diminuição de espécies consideradas praga. Nesse caso, quando observados ou testados em conjunto, os agrotóxicos estão em "mistura". A mistura desses compostos pode causar a potencialização de efeitos em organismos não-alvo com a ligação das moléculas dos compostos, levando ao aumento ou diminuição da metabolização desses agentes estressores no corpo do organismo (Cedergreen, 2014).

Estudos mostram o efeito sinérgico de pesticidas como abamectina e difeconazol quando aplicados combinados em suas formulações comerciais, em cladocera *Macrothrix flabelliger* (Moreira et al., 2017) e em indivíduos adultos do peixe *Danio rerio* (Sanches et al., 2017). Gottardi et al. (2017) observou efeito sinérgico entre epoxiconazol e α-cipermetrina através da inibição do citrocromo P450 e da diminuição do crescimento de cladocera, *Daphnia magna*. Entretanto, não há informação na literatura do efeito combinado dos dois agroquímicos

intensamente usados em culturas de cana-de-açúcar, o inseticida fipronil (Regent® 800 WG) e o herbicida 2,4-D (DMA® 806 BR) em peixes.

1.3. Peixes como bioindicadores

Bioindicadores são, por definição, espécies que podem apresentar respostas a partir da ocorrência de algum estresse caracterizado como interferência no ambiente que está inserido (Van Gestel e Van Brummelen, 1996). Além disso, organismos tidos como bioindicadores “ideais" precisam apresentar certas características, segundo Li et al. (2010): (i) amplo conhecimento de sua taxonomia, ecologia e fisiologia, (ii) sensibilidade a estressores ambientais; (iii) boa quantidade de n amostral para estabelecer padronização do método utilizado; (iv) espécies adaptadas para experimentos em laboratório (a fim de validar metodologias).

Nesse contexto, respostas biológicas a nível bioquímico, fisiológico e/ou morfológico observadas em um bioindicador após alterações no ambiente ou exposição a algum estressor em potencial, são determinadas como biomarcadores (Peakall, 1994). Essas respostas podem ser mensuradas a nível individual, desde análises dentro do organismo até de seus produtos (fezes, urina, pelos etc), que podem indicar mudança quando comparado a indivíduos que não foram expostos ao estressor (Van der Oost et al., 2003).

Se tratando do ambiente aquático, peixes são excelentes bioindicadores para experimentos em campo (Nimet et al., 2020; Van Dyk et al., 2012) e em laboratório (Chaulet et al., 2019; Huang et al., 2019), pois além da ampla distribuição de espécies (ambientes de água doce, salina e estuários), esses organismos possuem diferentes hábitos alimentares e interações em uma cadeia trófica. Sua importância também se dá pela relevância econômica e utilização para consumo humano, podendo ter boa estimativa de contaminação.

Em peixes, a porta de entrada de xenobióticos se dá principalmente pelas trocas gasosas realizadas pelas brânquias entre água e sangue, onde, ao entrar na corrente sanguínea, o agente estressor passa pelos demais órgãos. Fígado, rins e intestino são órgãos que desempenham papeis importantes no processo de detoxificação e eliminação de substâncias tóxicas ao organismo (Lins et al., 2010). As respostas desencadeadas são analisadas em biomarcadores:

(i) Morfológicos: alterações celulares e estudadas em órgãos como brânquias, fígado e rins através da avaliação de índices histopatológicos (Paulino et al., 2014; Santos et al., 2014) ou contagem de células de muco (Moron et al., 2009);

(ii) Bioquímicos: por enzimas ou moléculas que podem indicar a ocorrência de alterações no organismo como estresse oxidativo, que é uma das primeiras respostas do organismo (Narra et al., 2017; Sinhorin et al., 2014);

(iii) Fisiológicos: alterações em níveis relativos ao balanço hídrico e iônico, taxas respiratórias etc, por meio de estudos feitos sobre a respiração e trocas entre o organismo e ambiente (Cerqueira e Fernandes, 2002).

Além disso, também podem ser realizados estudos que avaliam a taxa de desenvolvimento dos peixes (Li et al., 2017; Schreiber et al., 2009) e transferência maternal de xenobóticos (Xu et al., 2019) após a exposição a algum agente estressor. Assim, estudos com peixes podem mostrar como determinadas substâncias podem induzir efeitos que impeçam a capacidade de forrageamento e reprodução, atingindo esses organismos desde níveis individuais a populacionais.

1.3.1. Danio rerio

Danio rerio, de nome popular “peixe-zebra/*zebrafish*” ou paulistinha, pertence à Família Cyprinidae e é encontrado em habitats naturais da Índia, Bangladesh e Myanmar. O

comprimento total geralmente não ultrapassa 40 mm, possui hábito alimentar onívoro e geralmente é encontrado próximos a culturas irrigadas de arroz (Spence, 2008).

Devido ao total conhecimento de seu sequenciamento genético, *D. rerio* acabou se tornando um modelo biológico para experimentos em laboratório. Além disso, esse peixe tem outras características que facilitaram a constante utilização em experimentos laboratoriais (Teame et al., 2019): (i) fácil adaptação em ambiente de laboratório devido ao pequeno tamanho; (ii) alta taxa de fecundidade, podendo reproduzir durante o ano todo; (iii) tempo geracional rápido (3 a 4 meses); (iv) embriões translúcidos que começam a desenvolver em 24 h, facilitando a visualização de modificações; (v) existência de múltiplas linhagens, possibilitando a realização de estudos de diversos tipos de doenças ou deficiências; (vi) similaridade com o genoma humano.

Sendo assim, a espécie também pode ser utilizada em estudos relacionados com a saúde humana, como doenças metabólicas e cardiovasculares (Gut et al., 2017), obesidade (Oka et al., 2010), diabete mellitus (Zang et al., 2017), entre outros, como também em estudos ecotoxicológicos através de exposições a fungicidas (Tian et al., 2019), inseticidas (Wang et al., 2010), herbicidas (Wang et al., 2017) e outros possíveis agentes estressores (Novelli et al., 2016).

2. OBJETIVOS

O objetivo do presente trabalho foi avaliar a toxicidade das formulações comerciais de fipronil e 2,4-D e mistura de ambos através da quantificação de bioconcentração dos compostos no corpo inteiro de *Danio rerio*, respostas de enzimas antioxidantes e alterações morfológicas em brânquias. Também teve como objetivo avaliar a interação desses agrotóxicos em mistura, observando se ocorreu interações sinérgicas ou antagônicas.

Esta dissertação será apresentada na forma de trabalho científico.

REFERÊNCIAS BIBLIOGRÁFICAS

ALBUQUERQUE, A. F.; RIBEIRO, J. S.; KUMMROW, F.; NOGUEIRA, A. J. A.; MONTAGNER, C. C.; UMBUZEIRO, G. A. Pesticides in Brazilian freshwaters: A critical review. **Environ Sci-Proc Imp**. v. 18, p. 779-787, 2016. https://doi.org/10.1039/C6EM00268D

BAIRD, S.; GARRISON, A.; JONES, J.; BRINGOLF, R.; BLACK, M. Enantioselective toxicity and bioaccumulation of fipronil in fathead minnows (*Pimephales promelas*) following water and sediment exposures. **Environ Toxicol Chem**. v. 32, p. 222-227, 2013. https://doi.org/10.1002/etc.2041

BENLI, A. Ç. K.; ŞAHIN, D.; SARIKAYA, R.; MEMMI, B. K.; DINÇEL, A. S. The sublethal effects of (2,4-Dichlorophenoxy) acetic acid (2,4-D) on narrow-clawed crayfish (*Astacus leptodactylus* Eschscholtz, 1823). **Arh Hig Rada Toksikol**. v. 67, p. 289-296, 2016. https://doi.org/10.1515/aiht-2016-67-2793

CEDERGREEN, N. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. **PLoS ONE**. v. 9, p. e96580, 2014. https:// 10.1371/journal.pone.0096580

CERQUEIRA, C. C. C.; FERNANDES, M. N. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. **Ecotox Environ Safe**. v. 52, p. 83-91, 2002. https://doi.org/10.1002/etc.2041

CETESB (2018). Qualidade das águas interiores no estado de São Paulo, 2017.

CHAULET, F. C.; BARCELLOS, H. H. A.; FIOR, D.; POMPERMAIER, A.; KOAKOSKI, G.; ROSA, J. G. S.; FAGUNDES, M.; BARCELLOS, L. J. G. Glyphosate- and Fipronil-Based Agrochemicals and Their Mixtures Change Zebrafish Behavior. **Arch Environ Con Tox**. v. 77, p. 443-451, 2019. https://doi.org/10.1007/s00244-019-00644-7

CONAB. Companhia Nacional de Abastecimento. Acompanhamento da safra brasileira - cana-de-açúcar. 81p., 2017.

DERÍSIO, J. C. Introdução ao controle de poluição ambiental. São Paulo: CETESB, 1992.

DEHNERT, G. K.; FREITAS, M. B.; DEQUATTRO, Z. A.; BARRY, T.; KARASOV, W. H. Effects of low, subchronic exposure of 2,4-Dichlorophenoxyacetic acid (2,4-D) and commercial 2,4-D formulations on early life stages of fathead minnows (*Pimephales promelas*). **Environ. Toxicol. Chem.** v. 37, p. 2550-2559, 2018. https://doi.org/10.1002/etc.4209

ELAHI, E.; WEIJUN, C.; ZHANG, H.; NAZEER, M. Agricultural intensification and damages to human health in relation to agrochemicals: Application of artificial intelligence. **Land use policy**. v. 83, p. 461-474, 2019. https://doi.org/10.1016/j.landusepol.2019.02.023

ENVIRONMENTAL PROTECTION AGENCY (EPA). **New Pesticide Fact Sheet - Fipronil; EPA 737-F-96-005**; U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, U.S. Government Printing Office Washington, DC, p 1-10, 1996.

ENVIRONMENTAL PROTECTION AGENCY (EPA). **Reregistration Eligibility Decision (RED) 2,4-D; EPA 738-R-05-002;** U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances, Office of Pesticide Programs, U.S. Government Printing Office: Washington, DC, 2005.

EVANS, A. E. V.; MATEO-SAGATA, J.; QADIR, M.; BOELEE, E.; IPPOLITO, A. Agricultural water pollution: key knowledge gaps and research needs. **Curr Opin Environ Sustain**. v. 36, p. 20-27, 2019. https://doi.org/10.1016/j.cosust.2018.10.003

FREITAS, J. S.; GIROTTO, L.; GOULART, B. V.; ALHO, L. O. G.; GEBARA, R. C.; MONTAGNER, C. C.; SCHIESARI, L.; ESPÍNDOLA, E. L. G. Effects of 2,4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. **Ecotox Environ Safe**. v. 182, p. 109446, 2019. https://doi.org/10.1016/j.ecoenv.2019.109446

GRIPP, H. S.; FREITAS, J. S.; ALMEIDA, E. A.; BISINOTI, M. C.; MOREIRA, A. B. Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (*Eupemphix nattereri*: Leiuperidae). **Ecotox Environ Safe**. v. 136, p. 173-179, 2017. https://doi.org/10.1016/j.ecoenv.2016.10.027

GOTTARDI, M.; BIRCH, M. R.; DALHOFF, K.; CEDERGREEN, N. The effects of epoxiconazole and A-cypermethrin on *Daphnia magna* growth, reproduction and offspring size. **Environ. Toxicol. Chem**. v. 36, p. 2155-2166, 2017. https://doi.org/10.1002/etc.3752

GUO, Q.; ZHAO, S.; ZHANG, J.; KAILUN, Q.; DU, Z.; SHAO, B. Determination of fipronil and its metabolites in chicken egg, muscle and cake by a modified QuEChERS method coupled with LC-MS/MS. **Food Addit Contam Part A Chem Anal Control Expo Risk Assess**. v. 35, p. 1543-1552, 2018. https://doi.org/10.1080/19440049.2018.1472395

GUPTA, R. C.; ANANDÓN, A. Fipronil. In: GUPTA, R. **Veterinary Toxicology: Basic and Clinical Principles**. Academic Press, 2018. p. 533-538.

GUT, P.; REISCHAUER, S.; STAINIER, D. Y. R.; ARNAOUT, R. Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. **Physiol. Rev**. v. 97, p. 889-938, 2017. https://doi.org/10.1152/physrev.00038.2016

HETTIGE, H.; MANI, M.; WHEELER, D. Industrial pollution in economic development: the environmental Kuznets curve revisited. **J. Dev. Econ.** v. 62, p. 445-476, 2000. https://doi.org/10.1016/S0304-3878(00)00092-4

HUANG, Q-T.; SHENG, C-W.; JIANG, J.; TANG, T.; JIA, Z-Q.; HAN, Z-J.; ZHAO, C-Q. Interaction of insecticides with heteromeric GABA-gated chloride channels from zebrafish *Danio rerio* (Hamilton). **J. Hazard. Mater**. v. 366, p. 643-650, 2019. https://doi.org/10.1016/j.jhazmat.2018.11.085

ANDRADE JR, M. A. U.; VALIN, H.; SOTERRONI, A. C.; RAMOS, F. M.; HALOG, A. Exploring future scenarios of ethanol demand in Brazil and their land-use implications. v. 134, 2019. https://doi.org/10.1016/j.enpol.2019.110958

LI, K.; WU, J-Q.; JIANG, L-L.; SHEN, L-Z.; LI, J-Y.; HE, Z-H.; WEI, P.; LV, Z.; HE, M-F. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. **Chemosphere**. v. 171, p. 40-48, 2017. https://doi.org/10.1016/j.chemosphere.2016.12.032

LI, L.; ZHENG, B.; LIU, L. Biomonitoring and bioindicators used for river ecosystems: Definitions, approaches and trends. **Procedia Environ. Sci**. v. 2, p. 1510-1524, 2010. https://doi.org/10.1016/j.proenv.2010.10.164

LINS, J. A. P. N.; KIRSCHNIK, P. G.; QUEIROZ, V. S.; CIRIO, S. M. Uso de peixes como biomarcadores para monitoramento ambiental aquático. **Rev. Acad., Ciênc. Agrár Ambient**. v. 8, p. 469-484, 2010. http://doi.org/10.7213/cienciaanimal.v8i4.11018

LÓPEZ-PERA, J. J.; CAMARERO, P. R.; SÁNCHEZ-BARBUDO, I. S.; MATEO, R. Urbanization and cattle density are determinants in the exposure to anticoagulant rodenticides of non-target wildlife. **Environ Pollut**. v. 244, p. 801-808, 2019. https://doi.org/10.1016/j.envpol.2018.10.101

MITCHELL, G. Mapping hazard from urban non-point pollution: a screening model to support sustainable urban drainage planning. **J. Environ. Manage**. v. 74, p. 1-9, 2005.

MOREIRA, R. A.; DAAM, M. A.; VIEIRA, B. H. et al. Toxicity of abamectin and difenoconazole mixtures to a Neotropical cladoceran after simulated run-off and spray drift exposure. Aquat Toxicol. v. 185, p. 58-66, 2017. https://doi.org/10.1016/j.aquatox.2017.02.001

MORAN, D. D.; WACKERNAGEL, M.; KITZES, J. A.; GOLDFINGER, S. H.; BOUTAUD, A. Measuring sustainable development — Nation by nation. **Ecol Econ**. v. 64, p. 470-474, 2008. https://doi.org/10.1016/j.ecolecon.2007.08.017

MORON, S. E.; ANDRADE, C. A.; FERNANDES, M. N. Response of mucous cells of the gills of traíra (*Hoplias malabaricus*) and jeju (*Hoplerythrinus unitaeniatus*) (Teleostei: Erythrinidae) to hypo- and hyper-osmotic ion stress. **Neotrop. Ichthyol**. v. 7, p. 491-498, 2009. https://doi.org/10.1590/S1679-62252009000300017

NARRA, M. R.; RAJENDER, K.; REDDY, R. R.; MURTY, U. S.; BEGUM, G. Insecticides induced stress response and recuperation in fish: Biomarkers in blood and tissues related to oxidative damage. **Chemosphere**. v. 168, p. 350-357, 2017. https://doi.org/10.1016/j.chemosphere.2016.10.066

NIMET, J. NEVES, M. P.; VIANA, N. P.; AMORIM, J. P. A.; DELARIVA, R. L. Histopathological alterations in gills of a fish (*Astyanax bifasciatus*) in neotropical streams: negative effects of riparian forest reduction and presence of pesticides. **Environ Monit Assess**. v. 192, p. 58, 2020. https://doi.org/10.1007/s10661-019-8030-y

NOVELLI, A.; VIEIRA, B. H.; BRAUN, A. S.; MENDES, L. B.; DAAM, M. A.; ESPÍNDOLA, E. L. G. Impact of runoff water from an experimental agricultural field applied with Vertimec® 18EC (abamectin) on the survival, growth and gill morphology of zebrafish juveniles. **Chemosphere**. v. 144, p. 1408-1414, 2016. https://doi.org/10.1016/j.chemosphere.2015.10.004

OKA, T.; NISHIMURA, Y.; ZANG, M.; HIRANO, Y.; SHIMADA, Z.; WANG, N.; UMEMOTO, N.; KUROYANAGI, J.; NISHIMURA, N.; TANAKA, T. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. **BMC Physiol**. v. 10, p. 21, 2010. https://doi.org/10.1186/1472-6793-10-21

PAULINO, M.G.; BENZE, T.P.; SADAUSKAS-HENRIQUE, H.; SAURAGUI, M.M.; FERNANDES, J.B.; FERNANDES, M.N. The impact of organochlorines and metals on wild fish living in a tropical hydroelectric reservoir: bioaccumulation and histopathological biomarkers. **Sci Total Environ**. v. 497-498, p. 293-306, 2014. https://doi.org/10.1016/j.scitotenv.2014.07.122

PEAKALL, D. W. Biomarkers: the way forward in environmental assessment. **Toxicol. Ecotoxicol. News**. v. 1, p. 55-60, 1994.

SANCHES, A. L. M.; VIEIRA, B. H.; REGHINI, M. V. et al. Single and mixture toxicity of abamectin and difenoconazole to adult zebrafish (*Danio rerio*). **Chemosphere**. v. 188, p. 582-587, 2017. https://doi.org/10.1016/j.chemosphere.2017.09.027

SANTOS, D. M. S.; MELO, M. R. S.; MENDES, D. C. S.; ROCHA, I. K. B. S.; SILVA, J. P. L.; CANTANHÊDE, S. M.; MELETTI, P. C. Histological Changes in Gills of Two Fish Species as Indicators of Water Quality in Jansen Lagoon (São Luís, Maranhão State, Brazil). **Int J Env Res Pub He**. v. 11, p. 12927-12937, 2014. https://doi.org/10.3390/ijerph111212927

SCHREIBER, R.; ALTENBURGER, R.; PASCHKE, A.; SCHÜÜRMANN, G.; KÜSTER, E. A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs. **Chemosphere**. v. 77, p. 928-933, 2009. https://doi.org/10.1016/j.chemosphere.2009.08.038

SEHONOVA, P.; SVOBODOVA, Z.; DOLEZELOVA, P.; VOSMEROVA, P.; FAGGIO, C. Effects of waterborne antidepressants on non-target animals living in the aquatic environment: A review. **Sci Total Environ**. v. 631-632, p. 789-794, 2018. https://doi.org/10.1016/j.scitotenv.2018.03.076

SILVA, M. R.; CAMPOS, A. C. E.; BOHM, F. Z. Agrotóxicos e seus impactos sobre ecossistemas aquáticos continentais. **SaBios: Rev. Saúde e Biol**. v. 8, p.46-58, 2013.

SINHORIN, V. D. G.; SINHORIN, A. P.; TEIXEIRA, J. M. S.; MILÉSKI, K. M. L.; HANSEN, P. C.; MOREIRA, P. S. A.; KAWASHITA, N. H.; BAVIERA, A. M.; LORO, V. L. Effects of the acute exposition to glyphosate-based herbicide on oxidative stress parameters and antioxidant responses in a hybrid Amazon fish surubim (*Pseudoplatystoma sp*). **Ecotox Environ Safe**. v. 106, p. 181-187, 2014. https://doi.org/10.1016/j.ecoenv.2014.04.040

SONG, Y. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. **J. Integr. Plant**. v. 56, p. 106-113, 2014. https://doi.org/10.1111/jipb.12131

SOUZA, N. R. D.; FRACAROLLI, J. A.; JUNQUEIRA, T. L.; CHAGAS, M. F.; CARDOSO, T. F.; WATANABE, M. D. B.; CAVALETT, O.; FILHO, S. P. V.; DALE, B. E.; BONOMI, A.; CORTEZ, L. A. B. Sugarcane ethanol and beef cattle integration in Brazil. **Biomass Bioenergy**. v. 120, p. 448-457, 2019. https://doi.org/10.1016/j.biombioe.2018.12.012

SPENCE, R.; GERLACH, G.; LAWRENCE, C.; SMITH, C. The behaviour and ecology of the zebrafish, *Danio rerio*. **Biol. Rev**. v. 83, p. 13-34, 2008. https://doi.org/10.1111/j.1469-185X.2007.00030.x

TEAME, T.; ZHANG, Z.; RAN, C.; ZHANG, H.; YANG, Y.; DING, Q.; XIE, M.; GAO, C.; YE, Y.; DUAN, M.; ZHOU, Z. The use of zebrafish (*Danio rerio*) as biomedical models. **Anim Front**. v. 9, p. 68-77, 2019. https://doi.org/10.1093/af/vfz020

TERESA, F. B.; CASATTI, L.; CIANCIARUSO, M. V. Functional differentiation between fish assemblages from forested and deforested streams. **Neotrop. Ichthyol**. v.13, p. 361-370, 2015. https://doi.org/10.1590/1982-0224-20130229

TIAN, S.; TENG, M.; MENG, Z.; YAN, S.; JIA, M.; LI, R.; LIU, L.; YAN, J.; ZHOU, Z.; ZHU, W. Toxicity effects in zebrafish embryos (*Danio rerio*) induced by prothioconazole. **Environ Pollut**. v. 255, p. 113269, 2019. https://doi.org/10.1016/j.envpol.2019.113269

TOMLIN, C. D. S. The Pesticide Manual, A World Compendium. 14th Edition. British Crop Protection Council: Hampshire, England, 2006; p 462-464.

VALDÉS, M. E.; AMÉ, M. V.; BISTONI, M. A.; WUNDERLIN, D. A. Occurrence and bioaccumulation of pharmaceuticals in a fish species inhabiting the Suquía River basin (Córdoba, Argentina). **Sci Total Environ**. v. 472, p. 389-396, 2014. https://doi.org/10.1016/j.scitotenv.2013.10.124

VAN DER OOST,. R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environ Toxicol and Phar**. v. 13, p. 57-149, 2003. https://doi.org/10.1016/s1382-6689(02)00126-6

VAN DYK, J. C.; COCHRANE, M. J.; WAGENAAR, G. M. Liver histopathology of the sharptooth catfish *Clarias gariepinus* as a biomarker of aquatic pollution. **Chemosphere**. v. 87, p. 301-311, 2012. https://doi.org/10.1016/j.chemosphere.2011.12.002

VAN GESTEL, C. A. M.; VAN BRUMMELEN, T. C. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. **Ecotoxicology**. v. 5, p. 217-225, 1996. https://doi.org/10.1007/BF00118992

WANG, X.; ZHOU, S.; DING, X.; ZHU, G.; GUO, J. Effect of triazophos, fipronil and their mixture on miRNA expression in adult zebrafish. **J. Environ. Sci. Health B**. v. 45, p. 648-657, 2010. https://doi.org/10.1080/03601234.2010.502435

WANG, Y.; YANG, G.; DAI, D.; XU, Z.; CAI, L.; WANG, Q.; YU, Y. Individual and mixture effects of five agricultural pesticides on zebrafish (*Danio rerio*) larvae. **Environ Sci Pollut R**. v. 24, p. 4528-4536, 2017. https://doi.org/10.1007/s11356-016-8205-9

WORLD HEALTH ORGANIZATION. Environmental Health Criteria 84, Environmental Aspects - 2,4-Dichlorophenoxyacetic acid (2,4-D); International Programme on Chemical Safety, World Health Organization: Geneva, Switzerland, 1989. Disponível em: < http://www.inchem.org/documents/ehc/ehc/ehc84.htm>

WORLD HEALTH ORGANIZATION. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. **FAO Plant Production and Protection Paper**, v. 163, 2000. Disponível em: < http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Reports_1991-2006/Report__2000.pdf>

XU, C.; NIU, L.; LIU, J.; SUN, X.; ZHANG, C.; YE, J.; LIU, W. Maternal exposure to fipronil results in sulfone metabolite enrichment and transgenerational toxicity in zebrafish offspring: Indication for an overlooked risk in maternal transfer? **Environ Pollut**. v. 246, p. 876-884, 2019. https://doi.org/10.1016/j.envpol.2018.12.096

ZANG, M.; DI MARTINO, J. S.; BOWMAN, R. L.; CAMPBELL, N. R.; BAKSH, S. C.; SIMON-VERMONT, T.; KIM, I. S.; HALDEMAN, P.; MONDAL, C.; YONG-GONZALES, V. et al. Adipocyte-derived lipids mediate melanoma progression via FATP proteins. **Cancer Discov**. v. 8, p. 1006-1025, 2018. https://doi.org/10.1158/2159-8290.CD-17-1371

Multi-approach evaluation of fipronil and 2,4-D commercial formulations (isolated and in mixture) in the tropical fish (*Danio rerio*).

Natália Prudêncio Viana[a]

[a] Programa de Pós-graduação em Ecologia e Recursos Naturais, Universidade Federal de São Carlos, São Carlos, Rodovia Washington Luiz, km 235, 13565-905 São Carlos, São Paulo, Brazil.

ABSTRACT

The southeastern Brazil is an important agricultural region for its high sugarcane production. The insecticide fipronil and the herbicide 2,4-D are among the most pesticides applied in these crops leading to aquatic contamination. The toxicity of fipronil and 2,4-D, isolated and in mixture of both, in their commercial formulations Regent® 800 WG and DMA® 806 BR respectively, were evaluated to *Danio rerio* by determining the whole-body bioconcentration and the biochemical and morphological biomarkers. After 96 h exposure, the catalase (CAT) and glutathione S-transferase (GST) activity in gills and whole-body, and acetylcholinesterase (AChE) in muscle were analyzed. Gill histopathology were analyzed as well as the chloride and pavement cells at gill surface. Bioconcentration of fipronil (2.69 L kg^{-1}) and 2,4-D (1.73 L/kg) in isolated exposure and fipronil (3.10 L kg^{-1}) and 2,4-D (1.27 L kg^{-1}) in mixture occurred after exposure. The activity of CAT in the gills was reduced in fish exposed to Regent® and increased in those exposed to DMA® and mixture, GST increased in the gills and whole body and AChE increased in the muscle. Gill alterations occurred in all animals exposed to both pesticides, isolated and in mixture; however, the histopathological alteration index (HAI) was below 10 indicating normal organ structure. The chloride cell fractional area in the gill surface increased significantly in fish exposed to mixture of both pesticides and microridges on the pavement cells were reduced. The recommended doses of fipronil and 2,4-D, isolated and mixture for sugarcane crops induced biochemical changes in the gills and

muscle of *D. rerio* and minor histopathology in the gills. However, such effects can trigger other long-term damage, especially in the natural environment where there are a series of natural and anthropogenic variables that can interfere with fish homeostasis.

Key words: Acetylcholinesterase, bioconcentration**,** catalase, chloride cells, glutathione S-transferase, histopathology

Highlights

1. *Danio rerio* was exposed to Fipronil, 2,4-D and mixture at environmental concentration

2. Fipronil and 2,4-D exposure alone and mixture bioconcentrated in whole-body fish

3. Fipronil isolated inhibited the catalase activity in the gills of fish

4. Both pesticides increased AChE activity in muscle and histopatological index in gills.

5. Synergism occurred in catalase activity and chloride cell fractional area in gills

1. INTRODUCTION

The agricultural expansion has been accompanied by the intense use of pesticides to control organisms considered pests in the crops, minimize economic losses, and optimize production. In monoculture crops, the application of high amounts of different agrochemicals simultaneously has been a common practice. It results in increased pesticides levels in the soil and in aquatic ecosystem contamination, which are the final receptors of these compounds (Gottardi et al., 2017). Among agrochemicals, the insecticide fipronil and the herbicide 2,4-D have been intensively applied in sugarcane, soya and other monoculture due to high efficiency in pest control.

The active ingredient fipronil (5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolil)-4-trifluoromethylsulfinilpirazole-3-carbonitrile) is highly toxic having moderate persistence in the environment; it is stable in acidic (pH 5.5) and neutral (pH 7.0) water remained at about 80%, in both conditions, after 100 days (Gunasekara et al., 2007, ANVISA, 2019). Fipronil is used against leaf cutter ants, beetles, termites and in veterinary treatment of ectoparasites. This pesticide acts on the chlorine channels of GABA receptors (gamma-aminobutyric acid) in the central nervous system of organisms inhibiting the Cl- flow into the cell and leading to increasing nerve cell excitation (Gupta and Anadón, 2018). The toxic effect of fipronil have been reported in non-target species in the aerial environment as bees and wasps, terrestrial environment as rats and rabbits and, even in the aquatic environment, in water flea, shrimp, and fish (Gunasekara et al., 2007). In fish, fipronil caused behavior, and biochemical alterations (Wang et al., 2016, Moreira et al., 2021), reduction in immune capability and histological alterations in the gills and liver (El-Murr et al., 2015).

The 2,4-D (2,4-dichlorophenoxyacetic acid) is an extremely toxic selective synthetic herbicide (ANVISA, 2016) whose half-life in water is between 38 and 90 days (EFSA, 2014). Its efficiency, especially in dicotyledons, is due to increasing auxin levels causing accelerated

growth and death. The 2,4-D has been mainly applied in monocultures and home gardening although, its toxicity has already been demonstrated in different organisms, including humans (Islam et al., 2018). In fish, 2,4-D caused genotoxicity in *Cnesterodon decemmaculatus* (Arcaute et al., 2016), changed the development of *D. rerio* embryos (Li et al., 2017) and swimming (swimming speed) in serpae tetra, *Hyphessobrycon eques* (Moreira et al., 2021).

The use of combined pesticides is increasing and there are some concerns about mixing pesticides in agricultural production. Synergy between pesticides can increase toxicity and limit the potential recovery of non-target species as in cladocerans (Gottardi et al., 2017; Moreira et al., 2017) and fish *D. rerio* (Sanches et al., 2017).

In the aquatic environment, bioavailable pesticides may be absorbed by fish through the gills or via ingestion of contaminated food. The gills are considered target organs in fish due to the extent surface area and continuous water contacting the epithelial surface during the breathing process (Fernandes and Moron, 2020). After pesticide absorption, biotransformation/metabolization and excretion occurs in the gills and other organs; however, if absorption exceeds the body's metabolization/elimination capacity, bioconcentration occurs (Van der Oost et al., 2003; Konwick et al., 2006). The metabolization process, in general, may increase the generation of reactive oxygen species (ROS) and unbalance between ROS production and antioxidant activity results in oxidative stress. The increasing activity of antioxidant defense system may avoid oxidative stress (Barreiros et al., 2016). Pesticides also may alter the activity of acetylcholinesterase (AChE), the enzyme involved in cholinergic synapses, and affect fish behavior as already observed in *D. rerio* (Lopes et al., 2017).

Pesticides into water directly or indirectly interfere in the organ morphology and function (Fernandes et al., 2007). In addition to gas exchange, gills are the sites of ionic and acid-base regulation, via chloride (CC) and pavement (PVC) cells. Disturbances in any homeostatic mechanisms generate biochemical and morphological responses in fish, which can affect

animal at individual and population levels making them excellent bioindicators (Van der Oost et al., 2003).

Among fish, *D. rerio* is a model organism to toxicological studies due to the extensive knowledge of its biology and physiology and that 70% of human genes have, at least, one ortholog from this species (Howe et al., 2013). Thus, the aim of this study was to evaluate the biochemical and morphological alterations induced by the pesticides fipronil and 2,4-D isolated and mixture, in their commercial formulation (Regent® 800 WG and DMA® 806 BR, respectively) in the tropical fish *D. rerio*. Whole body pesticide bioconcentration, whole body and gill antioxidant defense system, acetylcholinesterase response in muscle and, the morphological changes in the gills with emphasis on CC and PVC were analyzed. The occurrence of synergistic or antagonistic interactions between the effects of these compounds when exposed in association was also evaluated in order to predict the effects in non-target organisms at a scenario of high agricultural production.

2. MATERIALS AND METHODS

2.1. Fish acquisition and maintenance

Adult specimens of *D. rerio* (body mass: 0.4 ± 0.1 g, total length: 3.5 ± 0.3 cm) were acquired in the Pisciculture Studio Submerse Aquarismo (Ibama Record No. 7044057) and acclimated for 10 days in the laboratory, according to the recommendations of ABNT (2016). The fish were randomly distributed in aquariums with 200 L dechlorinated water with continuous aeration and controlled temperature. Every two days, 50% of aquarim water was renewed. During the acclimation period, temperature (23 ± 0.1 ºC), dissolved oxygen (7.4 ± 0.2 mg L^{-1}), pH (6.8 ± 0.04) and conductivity (33.5 ± 14 μS cm^{-1}) were measured daily using a YSI 556 MPS multiparameter probe (Yellowspring). The photoperiod was maintained with a cycle

of 12 h: 12 h (light:dark). The fish were fed *ad libitum* once a day with commercially artificial diet (TetraMin Tropical Flakes®, 48% protein).

2.2. Pesticides

The pesticides fipronil and 2,4-D were used in the commercial formulation. The source of fipronil was the insecticide Regent® 800 WG (800 g/kg, BASF S.A., Brazil), as dispersive granules, containing 80% active ingredient (fipronil) and 20% inert ingredients. The exposure dosage was calculated following the indication recommended by the manufacturer (500 g/hectare) for sugarcane monocultures. A fipronil stock solution was prepared by dissolving 4 mg of the commercial chemical in 1 L of Milli-Q water® and the exposure solution was prepared by diluting the stock solution in the aquarium water. The source of 2,4-D was the commercial formulation DMA® 806 BR (80.6 g L^{-1} of 2,4-D, Dow Agrosciences Ltda, Brazil), as soluble concentrate, containing in its formulation 67% of active ingredient (acid 2,4-D, dimethylamine salt) and 43% of inert ingredients. The exposure dosage was calculated considering the recommendation of manufacturer for sugarcane crops (3.5 L/hectare). The 2,4-D stock solution was prepared by diluting 1 mL of the commercial chemical in 100 mL of Milli-Q water® and the exposure solution was obtained by diluting the stock solution in the aquarium water. The quantification of pesticides was performed with Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) using chromatography Agilent 1200 coupled to a Triple Quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 6410) in negative mode. The Limit of Quantification (LOQ) of the method to fipronil was 0.1 μg/L and 2,4-D was 1 μg/L, respectively.

2.3. Experimental design

The experimental design was randomized with four treatments: control (water without pesticides), fipronil = 63.5 μg a.i. L^{-1} (proportional to 500 g/hectare), 2,4-D = 447 μg i.a L^{-1} (proportional to 3.5 L/hectare) and Mixture: 63.5 μg a.i. L^{-1} fipronil + 447 μg a.i. L-1 2,4-D. The experiments were carried out with three replicates (10 fish per repetition/experimental aquarium) in static systems, photoperiod 12 h:12 h (light:dark). Fish were no fed 24 hours before and during the experiment.

After the experimental period (96 h), the fish were irreversibly anesthetized with benzocaine (2 mg L^{-1}). Four whole fish from each replication were collected to determine the bioconcentration of agrochemicals and immediately frozen (-20 ºC). Subsequently, the gills and the rest of the body of two fishes from each replication and the muscle of other two fishes per replication were collected and were immediately frozen in liquid nitrogen for biochemical analyses and stored in an ultra-freezer (-80 ºC). The gills of two fishes, by repetition, were removed and fixed in 2.5 % glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 7.4, for 24 hours and kept in glutaraldehyde 0.5 % in the same buffer for morphological analyses.

All experiments and procedures were carried out according to ethical principles in animal experimentation and approved by the protocol of the Ethics Committee on The Use of Animals (CEUA) No. 2358080918 of the Federal University of São Carlos (UFSCar), São Carlos, Brazil.

2.4. Determination of pesticides in fish and bioconcentration factor

The bioconcentration of each pesticide in *D. rerio*, was done after the whole fish be lyophilized in a micromodulyo-115 (Thermo Electron Corporation) connected to an oil filter (VPOF 110) of the vacuum pump (VLP 200) at 60 Hz, 20 A, for 48 hours. Subsequently, the

fish were macerated and pooled (n = 3 fishes) to reach the approximate weight of 0.5 g necessary for analysis.

For extraction of the compounds from macerated and lyophilized fish were used the ultrasonic extraction method with water:acetonitrile (W:ACN) by solid phase extraction (SPE) pH 2.5 with Oasis HLB cartridges (200 mg, 6 cc). The Oasis HLB cartridges were conditioned with 5 mL of methanol (MeOH) and 5 mL of HPLC grade water, pH 2.5 as described Serra-Compte et al. (2017) modified by Portruneli (2020).

The extracted samples were dissolved in 6 mL of MeOH, completely evaporated under a nitrogen stream, reconstituted in 1 mL of MeOH: H_2O (1:1) and analyzed in a UHPLC-MS/MS system (Acquity H-Class, Waters®) coupled to a triple quadruple mass spectrometer (Xevo TQ-S micro, Waters®) according to Álvarez-Muñoz et al. (2014). The detection limit (LOD) for fipronil was 0.0109 μg L^{-1} and 2.4-D of 0.191 μg L^{-1} and the limit of quantification (LOQ) for fipronil was 0.0363μg L^{-1} and 2.4-D of 0.638 μg L^{-1}

The bioconcentration factor (BCF) was determined by the equation according to Opperhuizen (1991): **BCF = C_O/C_S,** where C_O represents the quantified concentration in the organism and C_S is the concentration determined in the water.

2.5. Biochemical biomarkers analyses

After defrost, gill and body samples were weighed and homogenized in 0.2 M potassium phosphate buffer (pH 7.8) in a 1:10 ratio (mass:volume). Samples were centrifuged at 10000 x*g* for 10 minutes at 4 ºC, and the supernatant was used for biochemical analyses. All biochemical analyses were performed in microplate using a Spectra Max-M5 microplate reader (Molecular Devices®, San Jose, CA, USA).

Protein concentration (pt) was quantified using the Bradford reagent and the bovine serum albumin as standard and the absorbance was measured at 595 nm (Bradford, 1976). The

activity of the enzyme glutathione S-transferase (GST) in the gills was determined according to Habig and Jakoby (1981) using 1-chloro-2,4-dinitrobenzene (CNDB) as substrate. The enzymatic activity was determined by increasing absorbance at 340 nm against the blank, for 4 minutes with reading intervals every 30 seconds. Catalase (CAT) activity in the gills was determined according to Beutler (1975), following the H_2O_2 consumption for 2 minutes, at intervals of 15 seconds, via decreasing absorbance at 240 nm. The activity of acetylcholinesterase (AChE) in the muscle was measured by the thiol produced by the enzyme in the sample reacting with NBD (5.5'-dithio-bis-(2-nitrobenzoic) for 5 min, at intervals of 30 seconds, at 412 nm (Ellman et al., 1961).

2.6. Gill morphological analyses

For morphological analyses of gills, the gill arches on one side of the animal were used for histopathological analysis under light microscopy and those on the other side were used for the chloride cell analysis using scanning electron microscopy.

2.6.1. *Gill Histopathology and histopathological index*

The fixed gills were washed in 0.1 M phosphate buffer for the removal of the excess fixator, dehydrated in increasing ethanol series (70 to 100 %) for 4 hours, diaphanized in xylol (1 h) and embedded in paraplast® (Sigma Aldrich). Thereafter, sections (5 μm thickness) were made in semi-serial sequence (1 cut: 10 μm disposal) in automatic microtome (Thermo Scientific Microm, HM-360) using disposable knives. The histological sections were stained with toluidine blue according to Behmer et al. (1976). Randomly digital images of 10 distinct filament fields in each cut were obtained under a light microscope (Olympus BX61, Olympus, USA) with a video camera attached to a computer.

The frequency of each histopathological damage and the degree of severity of this damage were recorded and then the histopathological alteration index (HAI) was calculated. The type and severity of the lesions were classified according to Poleksic and Mitrovic-Tutundzic (1994) with minor modifications. The damage was classified into three degrees of severity: I, not very severe injuries, which do not affect the functioning of the organ, reversible and punctual; II, moderately severe lesions that may affect the functioning of the organ may be irreversible, but in general they are punctual; III, very severe and irreversible lesions that affect the functioning of the organ. Subsequently, the HI was calculated as the sum of the lesion types in each of the three severity stages (S) and multiplied by the index of each stage (1, 10 and 100, respectively) using the equation proposed by Poleksic and Mitrovic-Tutundzic (1994).

2.6.2 *Chloride cells analyses*

Chloride cells (CC) are also denominated as mitochondria-rich cells and/or ionocytes. In this study, we will use CC. Samples of fixed gills were dehydrated in a gradual sequence of ethanol up to 100 %. Subsequently, the samples underwent two baths (30 seconds each) of 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and dried at room temperature. Pairs of filaments were glued to metal stubs and coated with gold (Degussa, 99%, Brazil) in a vacuum sputter (Sputter Coater SCD 040, Balzers, Germany). Digital images of the samples were obtained in a scanning electron microscope (Philips XL-30 FEG, Field Emission Gun, USA), at 15 kV and 4000 to 8000x magnification.

Density (CC number mm^{-2}) and fractional area of CC (CCFA, %) in the epithelium surface of the filaments were estimated in five fields of each sample from each fish using Adobe Photoshop software (version 14.0, 2019). The apical surface perimeter of each CC, whole or partial, was determined as well as the total area of the filament image of the gill. The CC density and CC fractional area were calculated according to Moron et al. (2003):

CC Density = CCFA/mean apical CC surface area of image

CCFA = Σ area of total CC (whole or partial)/Total epithelium surface area in the image

2.7. Determination of synergism and antagonism

Interactions between the compounds may occur when exposure are performed in mixture. It was calculated by the ratio between the expected (EE) and observed (OE) effects. Values lower than those predicted were considered as antagonism and those higher than predict were synergism. To perform this classification, calculations of the independent action model were performed in all parameters that presented significant difference between treatments according to Gottardi et al. (2017). The fish exposure EE to pesticides fipronil (F) and 2.4-D (D) isolated was calculated in relation to the non-exposed fish (controls, C): **EE = F/C * D/C**. The OE was calculated from the mixture (M) of contaminants fipronil and 2,4-D in relation to the control (C): **OE = M/C**. The synergy/antagonism ratio was the ratio between the OE and EE of each analysis: Ratio = EE/OE.

2.8. Statistical analysis

All data were expressed as mean ± SD. The normality test (Shapiro-Wilk) and homogeneity of variances (Bartlett) were applied to data. One-way variance analysis (ANOVA) followed by Tukey's test were applied at significance level of $P < 0.05$. Statistical analyses were performed using the R program (version 3.6.1.) (R development core team, 2019).

3. RESULTS

There was no mortality during the experiments and the fish showed no clinical signs or behavioral differences when exposed to pesticides isolated and in mixture.

3.1. Abiotic variables of the toxicity tests and chemical analyses

The water quality parameters evaluated were in accordance with the criteria established by ABNT (2016): pH (6.8 ± 0.1) and dissolved oxygen (7.4 ± 0.2 mg L^{-1}). The concentration of active ingredients in the stock solutions for fipronil and 2,4-D were 1.3 ± 10.4 mg a.i.L^{-1} and 5.3 ± 0.16 g a.i.L^{-1}, respectively. The real values for the two pesticides calculated from the quantification of the stock solution are 354.0 µg L^{-1} for 2,4-D and 25.8 µg L^{-1} for fipronil

3.2. Bioconcentration

Fish exposed for 96 hours to fipronil, 2,4-D isolated and mixture of them accumulated these compounds into the body. Fipronil and 2,4-D residues were not detected in fish from the control group. The fipronil residues in whole body fish exposed to fipronil isolated and mixture of fipronil+2,4-D were 69.31 and 80.04 µg kg^{-1}, respectively. The 2,4-D residues determined in whole-body fish exposed to 2,4-D isolated and mixture of fipronil+2,4-D were 613.70 and 449.09 µg kg^{-1}, respectively. The calculated BCF in whole body fish were 2.69 and 1.73, respectively for fish exposed to fipronil and 2,4-D isolated and 3.10 and 1.27, respectively for fish exposed to mixture of these pesticides. Although there was no significant difference in the BCF values for fipronil and 2,4-D isolated and in mixture, there was a tendency to increase fipronil and reduce 2,4-D concentrations in animals exposed to mixture of the two pesticides.

3.3. Biochemical biomarkers

Fish exposed to fipronil and 2,4-D isolated and mixed increased total protein concentration in the gills (ANOVA, $F = 30.01$ $p < 0.05$; Tukey $p < 0.05$) and muscle (ANOVA, $F = 11.4$ $p < 0.05$; Tukey $p < 0.05$) compared to those from the control group (Fig. 1A, B, 2A). The whole-body protein concentration was higher in the group exposed to 2,4-D and group

exposed to the mixture of contaminants in relation to the control group, but did not differ from the group exposed to fipronil (One Way ANOVA, $F = 6.11$ $p < 0.05$; Tukey $p < 0.05$) (Fig. 1B).

CAT activity decreased in the gills of fish from the group exposed to fipronil and increased in fish exposed to 2,4-D and mixture of contaminants compared to the control group (One Way ANOVA, $F = 26.73$ $p < 0.05$; Tukey $p < 0.05$) (Fig. 1C). Whole-body Cat activity was unchanged (One Way ANOVA, $F = 0.093$ $p > 0.05$) (Fig. 1D). The GST activity increased in the gills of fish from group exposed to fipronil and 2,4-D isolated compared to the control group and group exposed to mixture of pesticides (One Way ANOVA, $F = 26.88$ $p < 0.05$; Tukey $p < 0.05$) (Fig. 1E). Considering the whole-body fish, the activity of GST increased in all groups exposed to fipronil, 2,4-D and mixture of pesticides (One Way ANOVA, $F = 8.43$ $p < 0.05$; Tukey $p < 0.05$) (Fig. 1F).

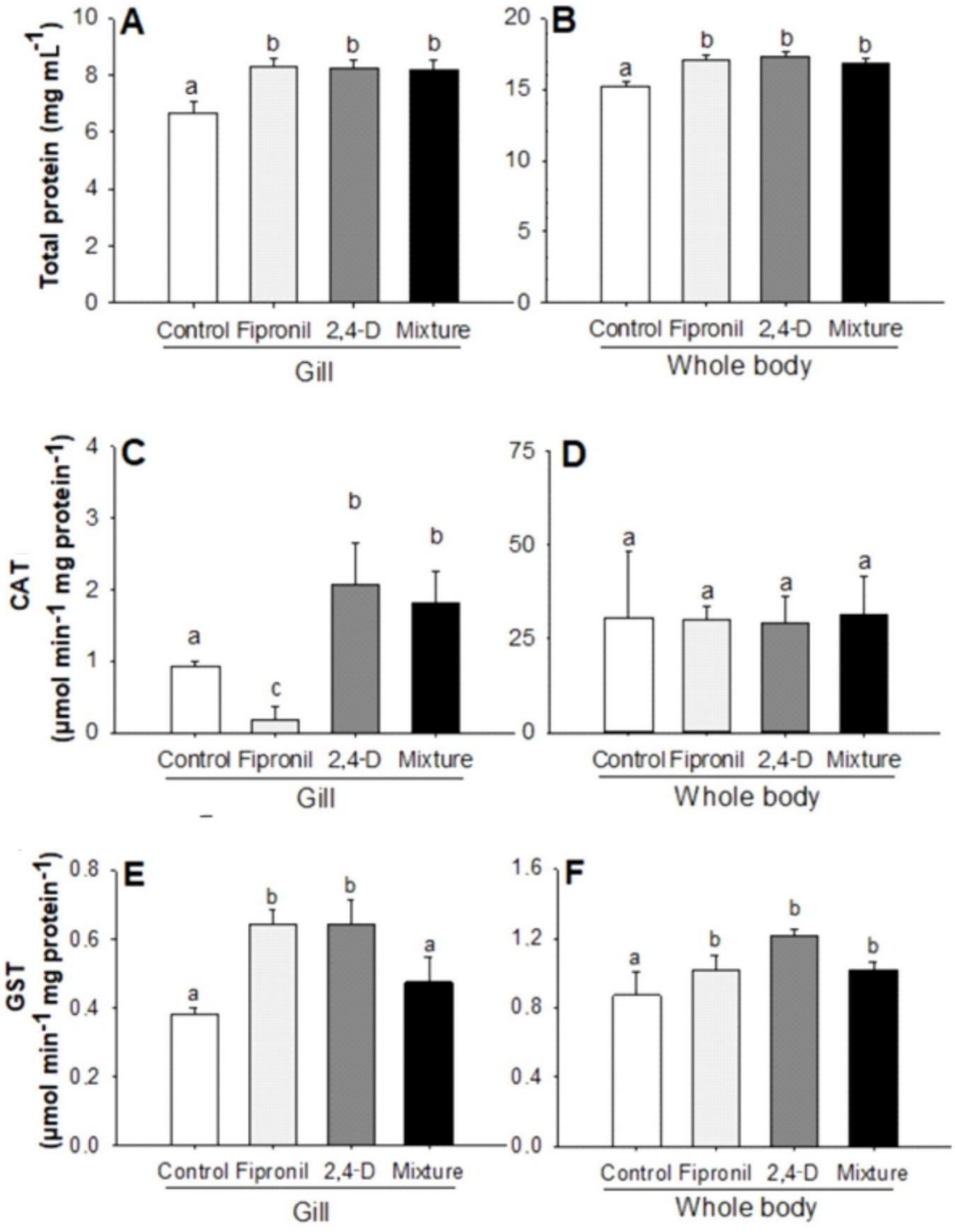

Figure 1. *Danio rerio.* Total protein concentration in the gills (A) and whole-body (B), catalase (CAT) activity in the gill (C) and whole-body (D) and, glutathione S-transferase (GST) in the gill (E) and whole-body (F) of fish from control group and groups exposed to fipronil and 2,4-D, isolated and in

their mixture, for 96 hours. Bars represent mean ± standard deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

In muscle, AChE activity increased in the groups exposed to fipronil, 2,4-D and mixture of contaminants in relation to the control group (One Way ANOVA, $F = 21.35$ $p < 0.05$; Tukey $p < 0.05$) (Fig. 2B).

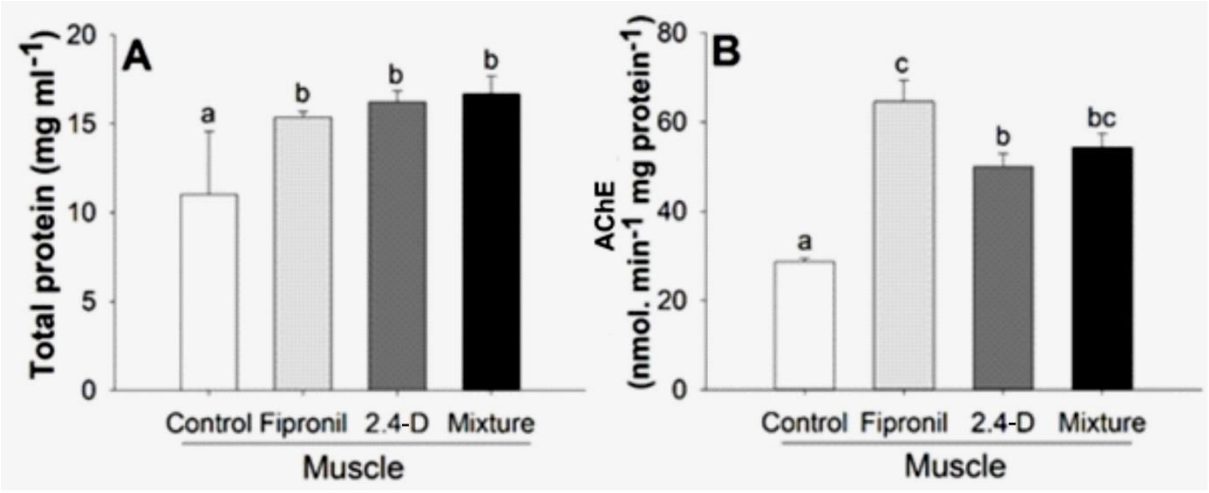

Figure 2. *Danio rerio.* Total protein (**A)** and activity of acetylcholinesterase (AChE) (**B)** in the muscle in fish from control group and groups exposed to fipronil and 2,4-D, isolated and in their mixture, for 96 hours. Bars represent mean ± standard deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

3.4. Gill morphology

3.4.1 *Histopathology*

The gill structure of *D. rerio* is similar to other teleost, there are 4 gill arches on the right and left sides of head, each one constituted by two rows of gill filaments and each filament has lamellae, regularly spaced from each other, above and below the filament horizontal axis. The filament epithelium is stratified, and the lamellar epithelium consists of two cell layers. The gills of fish from control group presented very few histological changes (Fig. 3A).

The histopathology classified as severity grade I and II were identified in the lamellar epithelium and blood vessels. Lamellar epithelial lifting (Fig. 3B) was observed in fish exposed to 2,4-D (0.2 %) and mixture (1 %). Cell hypertrophy (0.7%) and hyperplasia (67,8 %) were more frequent in fish exposed to fipronil (Fig. 3C). Hyperplasia resulted, sometimes, in partial or total lamellar fusion (Fig. 3D) which were classified as severity grade II. Changes in lamellar blood pathway structures such as congestion (Fig. 3E) and aneurysm (Fig. 3F), severity grade I and II respectively, were more frequent in fish exposed to 2,4-D alone (1 %). CC localized in the interlamellar epithelium presented hypertrophy (Fig. 3G) and proliferation (Fig. 3H).

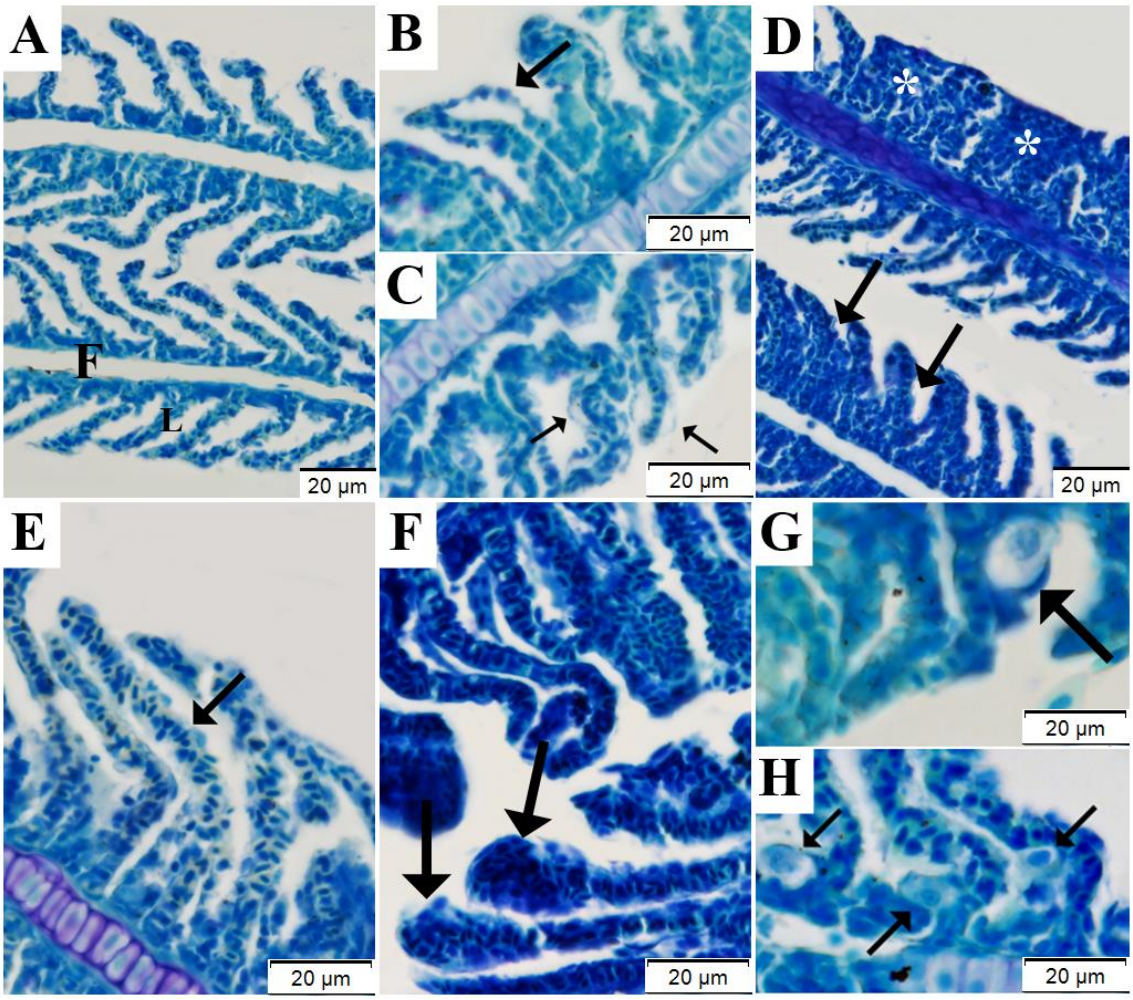

Figure 3. *Danio rerio*. Gill filament and lamella of *Danio rerio* from control group and groups exposed to fipronil, 2,4-D and their mixture. **A**: Control group. Note the filament (F) and lamella (L) organization. **B**: Lamellar epithelium lifting (arrow) of fish from no group mixture. **C**: Cellular hypertrophy of lamellar epithelium (arrow) in fish exposed to fipronil. **D**: Hyperplasia resulting in partial fusion (arrows) and total fusion of lamella (*) in fish exposed to fipronil. **E**: Lamellar congestion (arrow) in fish exposed to 2,4-D. **F**: Apical aneurysm (arrows) in fish exposed to 2,4-D. **G**: Chloride cell hypertrophy (arrow) in fish exposed to 2,4 -D. **H**: Chloride cell hyperplasia in the lamellar epithelium in fish exposed to fipronil.

Fish exposed to fipronil, 2,4-D and mixture increased gill histopathological indices compared to the controls (One Way ANOVA; $F = 10.16$ $p < 0.05$; Tukey, $p < 0.05$) after the 96 hours exposure (Fig. 4).

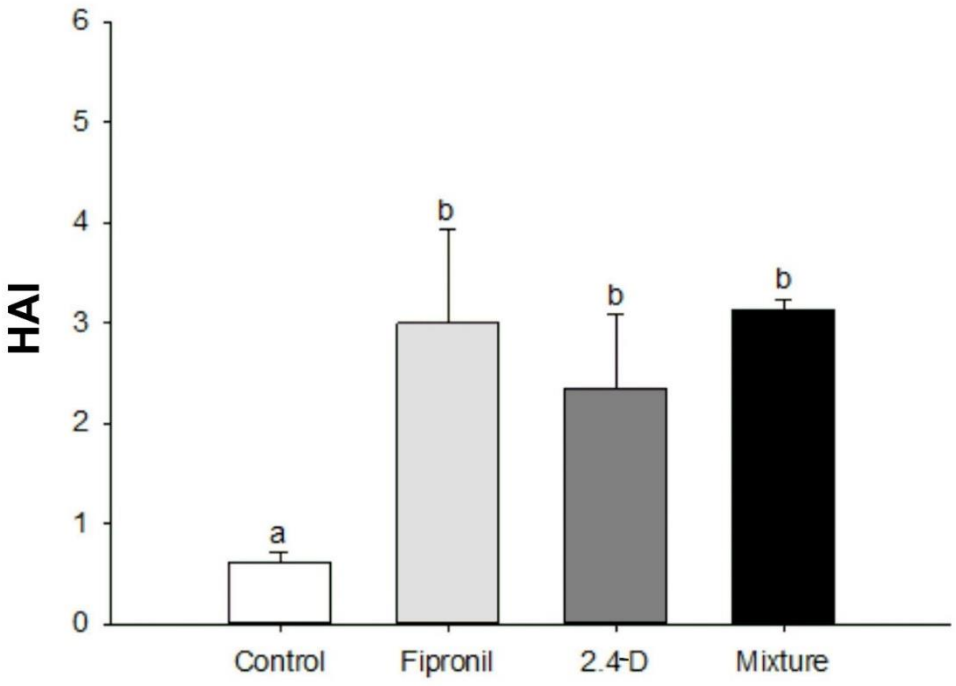

Figure 4. *Danio rerio*. Histopathological alteration index (HAI) of gills of fish from control group and groups exposed to fipronil, 2,4-D and their mixture. Bars are the mean ± standard deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

3.4.2. *Pavement and chloride cell apical surface*

The pavement cells (PVC) of *D. rerio* constitute the outmost cell layer of filament and lamellar epithelia; among them were identified the CC. The PVC are polygonal in shape and have numerous microridges irregularly distributed throughout cell surface and long microridge limiting cell boundary; in the interlamellar and lamellar epithelia, microridges are sparced on cell surface and the cell limits are easily identified by continuous microridge (Fig. 5A). The CC have different apical morphological surface contacting water. In control fish, they have well defined microvilli and are limited by the PVC (Fig. 5B), while after exposure to fipronil, the microvilli of CC and the PVC were not well defined (Fig. 5C and D). Fish exposed to 2,4-D presented microvilli and the cells were compressed among PVC. In fish exposed to the mixture

of pesticides, the CC surface area contacting water have microvilli were compressed along PVC as in fish exposed to 2,4-D or no apical microvilli (Fig. 5E, F).

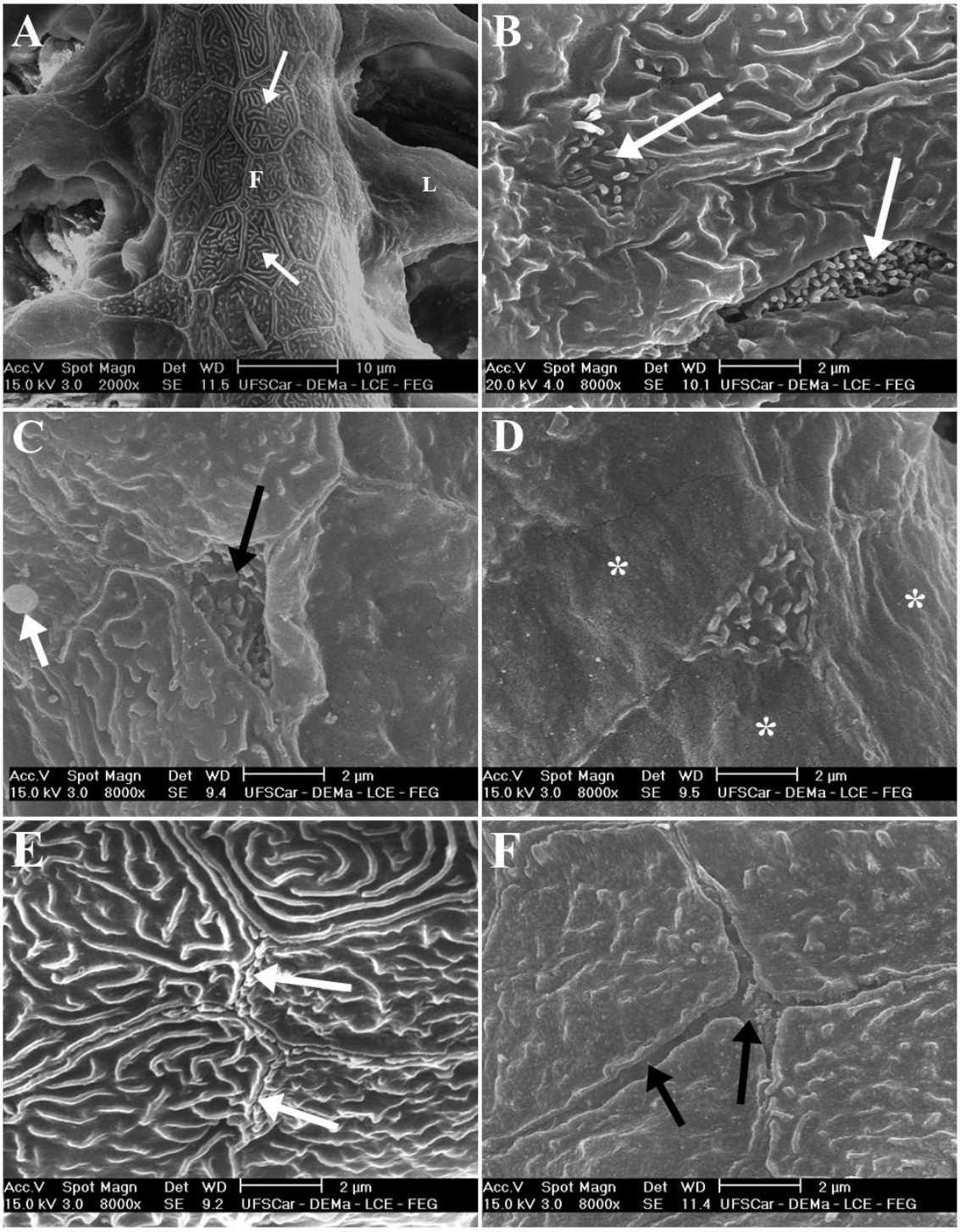

Figure 5. Scanning electron microscopy (MEV) of the gills of *Danio rerio* from control group and groups exposed to fipronil, 2,4-D and their mixture. **A and B**: Control group: filament (F) and lamella (L). Note the pavement cell (PVC) shape and surface microridges (arrows). B: Chloride cells (CC) with

microvilli (arrows) in the filament epithelium. **C**: CC having low microvillus (black arrow) and mucus dropped (white arrow). **D**: PVC (*) and CC having low microvillus in fish exposed to fipronil, **E**: CC among PVCs cells in the interlamellar epithelium in fish exposed to 2,4-D. Note short and few microvilli among PVC (arrow). **F**: CC without microvillus and undefined shape at epithelial surface in fish exposed to the mixture fipronil + 2,4-D (arrows).

The CCFA increased significantly in fish exposed to the mixture of contaminants compared to fish from the control group, but did not differ from fish of group exposed to fipronil and 2,4-D (One Way ANOVA; $F = 5.23$ $p < 0.05$; Tukey, $p < 0.05$) (Fig. 6A). There was no significant difference in the density of CC (One Way ANOVA; $F = 0.32$ $p > 0.05$) compared to the control group and those exposed to fipronil and 2,4-D isolated and in mixture (Fig. 6B).

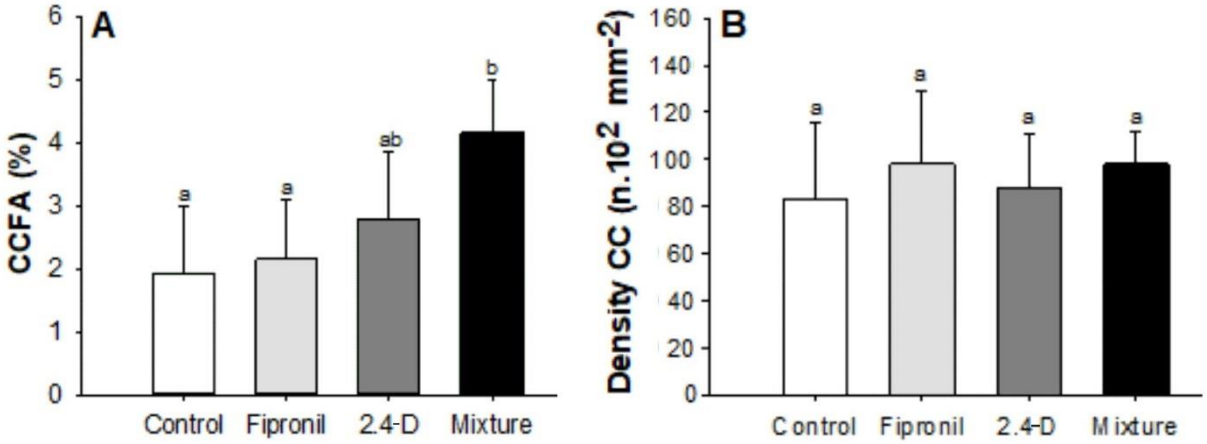

Figure 6. Chloride cell fractional area (CCFA, **A**) and density (**B**) in the gill filaments of *Danio rerio* from control group and groups exposed to fipronil, 2,4-D and their mixture. Bars are the mean ± standard deviation. Different letters indicate significant difference among treatments (ANOVA, Tukey, $p < 0.05$).

3.5. Synergism and antagonism

The synergism or antagonism interactions between contaminants calculated according to the independent action model were summarized in Table 1. In the gills, synergistic effect was

identified in the activity of CAT which was 451.42% higher and in CCFA which the synergistic ratio was 8.64 % when fish were exposed to mixture. All other analyses occurred antagonism.

Table 1. Synergistic(S) and antagonist (A) interactions between fipronil and 2,4-D and their mixture in *Danio rerio* after 96 hours exposure.

Treatment	HAI	CCFA (%)	Total Protein (mg mL^{-1})			GST (µmol min^{-1} mg $protein^{-1}$)		CAT (µmol min^{-1} .mg $protein^{-1}$)	AChE (nmol.mi^{-1} .mg $protein^{-1}$)
			Gills	Whole-body	Muscle	Gills	Whole-body	Gills	Muscle
Control	0.61±0.22	1.91±1.06	6.66±0.38	15.21±0.39	11.01±3.56	0.38±0.01	0.93±0.13	0.93±0.05	28.7±1.79
Fipronil	3.0±2.09	2.14±0.95	8.32±0.27	17.05±0.38	15.36±0.31	0.64±0.04	1.13±0.03	0.18±0.18	64.6±10.73
2,4-D	2.34±1.66	2.77±1.07	8.26±0.25	17.32±0.3	16.24±0.6	0.64±0.06	1.06±0.08	2.07±0.58	49.98±6.59
Mixture	3.12±0.25	4.15±0.82	8.17±0.36	16.85±0.35	16.69±0.98	0.47±0.07	0.98±0.04	1.81±0.43	53.3±7.18
Expected Effect	18.44	1.62	1.54	1.27	2.05	2.83	1.38	0.43	3.92
Observado effect	5.05	2.16	1.22	1.10	1.51	1.24	1.05	1.93	1.89
Interaction	A	S	A	A	A	A	A	S	A
Ratio	3.64	0.74	1.26	1.15	1.35	2.28	1.31	0.22	2.07

4. DISCUSSION

The exposure to fipronil and 2.4-D, isolated or mixture, at the doses indicated by the manufacturers for application in sugarcane crops were not lethal for *D. rerio*, however the biochemical and morphological changes suggested that these concentrations has certain degree of toxicity.

Fipronil and 2,4-D and/or they metabolites have lipophilic characteristic, facilitating the diffusion through the cell membrane (Simon-Delso et al., 2015). The fipronil and 2,4-D

concentrations in the animals' bodies evidenced that both pesticides were absorbed by fish through the gills and/or body surface area, as they were not fed during the experiments. Inside organism, the pesticides can be bioconcentrated or metabolized and, the metabolites excreted. The BCF that indicates the degree of affinity of a contaminant to a living organism (Rand et al., 1995) was validated, *in vitro*, by exposing eggs of the *D. rerio* to lipophilic substances (Schreiber et al., 2009). The low BCF values of fipronil and 2,4-D isolated or in mixture, after 96 hours exposure, in the present study, corroborate the hypothesis proposed by Call et al. (1983) that organic pesticides can be metabolized and quickly elimination of the organism. For example, the BCF values were also low for methrin (1.73), tebutiurom (0.98), hexazonin (0.32) and diuron (4.78) in *Oreochromis niloticus* exposed to the mixture of these herbicides in their commercial formulation, for 7 and 14 days (Jonsson et al., 2019). In frog *Phelophylax kl. esculentus* exposed to 10 µg fipronil, the BCF was 517 ± 218 in the gallbladder, but it was lower than 100 in other organs as liver, kidneys and intestine (Reynauld et al., 2012). Bioconcentration may be higher in some organs than other, it may explain the low BCF values found in the present study using the whole body of the fish to determine bioconcentration.

The large surface area of gills in contact with water favors the entry of contaminants dissolved into water which can bioconcentrate or be metabolized in these organs or reach the bloodstream and be distributed throughout the body. Therefore, the presence of stressors such as the pesticides both, in their primary form or their metabolites, can induce various disorders in the body. According to Van der Oost et al. (2003), organs such as gills, liver, kidneys, and intestine play the important role of biotransformation and excretion foreign substances. The enzymatic and morphological alterations observed in the gills of *D. rerio*, in the present study, indicated the action of the defense mechanism to avoid that these pesticides reach the blood stream.

The increased protein levels in the gills, muscle and whole body after 96 hours exposure to fipronil, 2,4-D and mixture corroborate with Oruç and Üner (1999) which suggested that pesticide exposure induces an increasing of protein synthesis in fish. The antioxidant enzymes play important role in the tissue defense by neutralizing the reactive oxygen species (ROS) formed during the xenobiotic metabolism (Barreiros et al., 2006) as the pesticides fipronil and 2,4-D and, other substances and metals. CAT enzyme is part of the first line of antioxidant defense performing ox-reduction reaction in which H_2O_2 is decomposed into O_2 and H_2O (Coelho et al., 2011). The inhibition of CAT in the gills after exposure to fipronil alone indicated sharp reduction in the capacity of these organs to degrade H_2O_2, which may lead in oxidative stress in this organ if other mechanism was not activate as the glutathione peroxidase that was not analyzed in this study. CAT inhibition also occurred in the liver of *Cyprinus carpio* exposed to 650 µg L^{-1} fipronil (Clasen et al., 2012). Conversely, the increased CAT activity in the gills after exposure to 2,4-D isolated and to the mixture of fipronil + 2,4-D suggested possible cellular metabolism of 2,4-D and H_2O_2 production in the gills, at a level that stimulates CAT activity but not high enough to inhibit the enzyme. Atamaniuk et al. (2013) reported an increased CAT activity in the gills of *C. auratus* exposed to 100 mg L^{-1} of 2,4-D, for 96 hours, but not after exposure to 1 and 10 mg L^{-1} of 2,4-D. Cat was not activated at very low level of H_2O_2 and can be inhibited at very high levels of H_2O_2 (Zhang et al. 2004). The unchanged CAT activity in whole-body of *D. rerio* exposed to both pesticides isolated and mixture of them suggested compensatory mechanism in other organs to maintain the detoxification capacity of whole organism.

GST is a family of enzymes involved in the detoxification of phase II; GST catalyze the GSH conjugation with xenobiotic becoming the product less toxic and more water soluble, facilitating it excretion (Wheatley et al., 1994; Rao, 2006; Huber et al., 2008). The increased activity of GST in the gills and whole-body fish exposed to fipronil and 2,4-D isolated in *D.*

rerio evidenced a process to increase the elimination of them, although when fish were exposed to a mixture of both pesticides, an antagonism effect was observed and GST activity did not differ from the controls. Increasing in GST activity was reported in the intestine of *Dicentrarchus labrax* feed for 14 days with a diet content 10 mg kg $^{-1}$ food of fipronil in the same commercial formulation (Regent® 800WG, 80% fipronil) used in this study (Dallarés et al., 2020). Nevertheless, the GST activity unchanged in the gills of *Carassius auratus* exposed to 1, 10 e 100 mg L^{-1} 2,4-D (Atamaniuk et al., 2013).

The AChE is an important enzyme in the transmission of nervous action potential in the neuromuscular junctions and cholinergic synapses acting in the acetylcholine hydrolysis. In the present study, the AChE activity in the muscle of *D. rerio* exposed, for 96 hours, to fipronil and 2,4-D isolated and in mixture increased, although antagonistic response was detected in the AChE exposed to the mixture. AChE was inhibited in *D. rerio* embryos exposed to low 2,4-D (20 µg L^{-1}) (Gaaied et al., 2019) but, no change was observed in whole-body of adult *D. rerio* and the neotropical serpae tetra fish *Hyphessobrycon eques* exposed to 200, 400, 800, 1500, 2000 and 2500 µg a.i. L^{-1} 2,4-D for 10 hours (Moreira et al., 2021). AChE inhibition was reported in whole adult *D. rerio* exposed to 75 and 100 µg a.i. L^{-1} fipronil and, unaffected AChE activity was observed in *H. eques* exposed to the same fipronil concentrations (Moreira et al., 2021). The pesticide interference in this enzyme compromise the swimming activity for searching food, avoiding predation and affect reproduction behavior.

The different responses of antioxidant enzymes and AChE in fish exposed to pesticides are related to differences in the species sensibility to them, time and concentration exposures, form of exposure and to pure active pesticide or commercial formulation in which the inert ingredients, in general, contain surfactants and other chemical favoring pesticide absorption by the animal.

In relation to gill morphology, the hypertrophy, lamellar epithelial lifting, hyperplasia leading to total or partial fusion of lamellae reported in this study, are general defense mechanisms which increase the water-blood diffusion distance reducing the transference of stressor agent as the pesticides from the lamellar surface to the blood (Lins et al., 2010; Fernandes and Moron, 2020). Previous studies by exposing carp, *Cyprinus carpio,* to 0.04 e 0.06 mg L^{-1} for 8 to 12 days reported similar tissue responses in the gills (Ghaffar et al., 2018). Furthermore, Qureshi et al. (2016) reported gill changes classified as severity grade I, II e III in *C. carpio* L. exposed to 400 µg L^{-1} fipronil, almost the maximum value (465 µg L^{-1} fipronil) reported in aquatic systems close to sugarcane crops in the in the São Paulo state, Brazil (CETESB, 2018). Blood congestion and aneurysm observed only in fish exposed to 2,4-D isolated and mixture are related to circulatory flow disturbance. Blood congestion is the dilatation of blood spaces formed by the flanges of adjacent pillar cells in the lamella due to numerous factors related to blood flow and aneurysm results in a rupture of such spaces and may lead to local hemorrhages. Both pathologies alter the gas exchange, O_2 uptake and CO_2 excretion, if occur in large areas of gills. Most of observed alterations in the gill epithelium were considered progressive and reversible; progressive if the environment contamination unchanged or increased, the pathologies may progress to next stage and affect the organ function depend on extension area damaged of gills; reversible if the stressor agent was removed from water, the damage is repairable (Poleksic and Mitrovic-Tutundzic, 1994; Bernett, 1999). The HI in fish exposed to fipronil and 2,4-D isolated or in mixture, always lower than 10, indicated normal gill structure although the gill alterations increased after exposure. Then, it is possible to infer that these pesticides, isolated or in mixture, at concentrations recommended for application in sugarcane crops, did not cause severe lesions in the gills during 96 hours exposure. Such changes help to reduce the gill absorption of such by *D. rerio* but,

under continuous exposure may progress and affect the gas exchange efficiency leading to intern hypoxia (Fernandes et al., 2007).

In freshwater fish, the CC have important role in the active ion uptake to maintain ionic homeostasis as the fish are continuously loss ions to hypoosmotic environment by passive diffusion process (Hwang et al., 2011; Fernandes, 2019). These cells varying in size, apical morphology, and distribution throughout the gill epithelium depending on species and environmental ion concentration (Moron et al., 2003). The presence of stressor agent into water, as the pesticides, may change the CC density and apical surface area contacting water (Paulino et al., 2012). In *D. rerio* embryos, there are, at least, three functional types of ionocytes immunocytochemical identified throughout the skin (Hwang et al., 2011). In the *D. rerio* surface gills only one morphological CC type was identified; all the CC presented high microvilli density at the apical surface. In *P. lineatus* and other Neotropical fish, two morphologically CC type has been identified, one having microvilli and other having a sponge-like apical surface (Paulino et al., 2012, Portruneli, 2020) The efficiency of ion transport depends on apical surface area in contact with surround water being the CCFA directly related to increasing ion uptake (Perry et al, 1992; Paulino et al., 2012). The changes induced in the apical surface of CC due to exposure to fipronil and 2,4-D isolated did not alter significantly the CCFA but, the exposure to the mixture increased 8.6 % the CCFA in the gills compared to the control and those exposed to fipronil and 2,4-D isolated, which suggested that the exposure of both pesticides in mixture affected ion regulation. Smejteck (1979) reported that the neutral form of 2,4-D increased cations and inhibit anion transport through the cellular membrane which may result in ion unbalance; the ionized form of 2,4-D did not modify the ion transport. The increasing CCFA in the gills of fish exposed to the mixture of both pesticides may be a compensatory mechanism to maintain ionic homeostasis.

The changes in PVC cells having not well defined microridges in fish exposed to fipronil may interfere in the cellular function. The microridges at apical surface of PVC increased it surface area and favor mucus retention which protect the cell against physical injuries and avoiding chemicals to reach cell surface. Microridges increasing on PVC surface was reported in *Prochilodus lineatus* exposed to 10 e 25 µg L^{-1} atrazine (Paulino et al., 2012) while reduction of microridge density in the PVC apical membrane was described in *Lepomis macrochirus* exposed to 60 µg L^{-1} to diazinon (Dutta et al., 1997) and in *Rhamdia quelen* exposed to 10 and 100 µg L^{-1} atrazine (Mela et al., 2013). PVC are involved in Na+ uptake during the process of H+ excretion via apical vacuolar H+-ATPase (Evans et al., 2005) and changes in cell surface may affect such function.

5. CONCLUSION

The 96 hours exposure of *D. rerio* to fipronil (Regent® 800 WG) and 2,4-D (DMA® 806 BR) isolated and in mixture are not lethal however, bioconcentration occurred in whole body and biochemical and morphological changes were induced in the fish gills. Fipronil inhibited CAT activity while 2,4-D increase it activity and, both pesticides, isolated and in mixture, increased the GST activity suggesting responses to avoid oxidative stress and favor the pesticide elimination. AChE activity increasing in the muscle probably affects neurological function. Both pesticides and they mixture caused histopathology in the gills and the lamellar hyperplasia was the most frequent change. Nevertheless, the HI indicated normal gill structure. Changes in PVC and CC apical surface suggested some effect on ion regulation. In general, the action of mixture of both pesticides showed antagonistic interactions, except CAT activity and the CCFA exhibited synergistic interaction. The recommended doses of fipronil and 2,4 D, isolated and mixture for sugarcane crops induced biochemical changes in the gills and muscle of *D. rerio* and minor histopathology in the gills. However, such effects can trigger other long-term

damage, especially in the natural environment where there are a series of natural and anthropogenic variables that can interfere with fish homeostasis. Further studies are needed to evaluate the action of both pesticides, isolated and in mixture, in other organs which may be more sensible to them.

Compliance with Ethical Standards: The study was approved by the protocol of the Ethics Committee on The Use of Animals (CEUA) No. 2358080918 of the Federal University of São Carlos (UFSCar), São Carlos, Brazil.

Conflict of interest: The authors declare that they have no conflict of interest.

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REFERENCES

ABNT. **Associação Brasileira de Normas Técnicas**. Toxicidade aguda – Método de ensaio com peixes (Cyprinidae), 2016.

ÁLVAREZ-MUÑOZ, D.; AL-SALHI, R.; ABDUL-SADA, A.; GONZÁLEZ-MAZO, E.; HILL, E. M. Global metabolite profiling reveals transformation pathways and novel metabolomic responses in *Solea senegalensis* after exposure to a non-ionic surfactant. **Environ. Sci. Technol**. v. 48, p. 5203–5210, 2014. https://doi.org/10.1021/es501276g

ANVISA. Consulta pública nº 164, de 03 de fevereiro de 2016, publicada no D.O.U de 12/04/2016. Disponível em: <http://portal.anvisa.gov.br/documents/10181/2719308/Relat%C3%B3rio+de+An%C3%A1lise+de+Contribui%C3%A7%C3%B5es+-+CP+164.pdf/41a725b5-37af-4a39-b809-f9f74807062c?version=1.0>

ANVISA. Consulta pública nº 692, de 23 de agosto de 2019, publicada no D.O.U de 28/08/2019. Disponível em: <http://portal.anvisa.gov.br/documents/10181/5612748/CONSULTA+P%C3%9ABLICA+N+692+GGTOX.pdf/332022d9-cb9b-4ddf-bcd7-197e629636ae>

ARCAUTE, C. R. de.; SOLONESKI, S.; LARRAMENDY, M. L. Toxic and genotoxic effects of the 2,4-dichlorophenoxyacetic acid (2,4-D)-based herbicide on the Neotropical fish *Cnesterodon decemmaculatus.* **Ecotoxicol. Environ. Safe** v. 128, p. 222-229, 2016. https://doi.org/10.1016/j.ecoenv.2016.02.027

ATAMANIUK, T. M.; KUBRAK, O. I.; STOREY, K. B.; LUSHCHAK, V. I. Oxidative stress as a mechanism for toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D): studies with goldfish gills. **Ecotoxicology.** v. 22, p. 1498-1508, 2013. https://doi.org/10.1007/s10646-013-1136-z

BARREIROS, A. L. B. S.; DAVID, J. M.; DAVID, J. P. Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. **Quím. Nova.** v. 29, p. 113-123, 2006. https://doi.org/10.1590/S0100-40422006000100021

BEHMER, A.O., TOLOSA, E.M.C., FERITAS-NETO, A.G. **Manual de técnicas para histologia normal e patológica.** 1ª edição. São Paulo: EDART/USP, 1976.

BERNET, D. et al. Histopathology in fish: Proposal for a protocol to assess aquatic pollution. **J. Fish Dis**, v. 22, n. 1, p. 25–34, 1999. https://doi.org/10.1046/j.1365-2761.1999.00134.x

BEUTLER, E. **Red Cell Metabolism: Manual of Biochemical Methods.** 2nd Edition. New York: Grune & Stratton, 1975.

BRADFORD, M. M. A rapid and sensitive method for the quantification of microgram of protein utilizing the principal of protein-dye binding. **Anal Biochem.** v., 72, p. 248-254, 1976. https://doi.org/10.1016/0003-2697(76)90527-3

CALL, D. J.; BROOKE, L. T.; KENT, R. J.; KNUTH, M.L.; ANDERSON, C.; MORIARITY, C. Toxicity, bioconcentration, and metabolism of the herbicide propanil (3',4'-Dichloropropionanilide) in freshwater fish. **Arch Environ Contam Toxicol**. v. 12, p. 352-358, 1983. https://doi.org/10.1007/bf01059578

CETESB (2018). Qualidade das águas interiores no estado de São Paulo, 2017.

CLASEN, B.; LORO, V. L.; CATTANEO, R.; MORAES, B.; LÓPES, T.; AVILA, L. A.; ZANELLA, R.; REIMCHE, G. B.; BALDISSEROTTO, B. Effects of the commercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: Implications for rice-fish cultivation. **Ecotoxicol Environ Safe** v. 77, p. 45-51, 2012. https://doi.org/10.1016/j.ecoenv.2011.10.001

COELHO, S.; OLIVEIRA, R.; PEREIRA, S.; MUSSO, C.; DOMINGUES, I.; BHUJEL, R. C.; SOARES, A. M. V. M.; NOGUEIRA, A. J. A. Assessing lethal and sub-lethal effects of

trichlorfon on different trophic levels. **Aquat. Toxicol.** v. 103, p. 191-198, 2011. https://doi.org/10.1016/j.aquatox.2011.03.003

DALLARÉS, S.; DOURADO, P.; SANAHUJA, I.; SOLOVYEV, M.; GISBERT, E.; MONTEMURRO, N.; TORREBLANCA, A.; BLÁZQUEZ, M.; SOLÉ, M. Multibiomarker approach to fipronil exposure in the fish *Dicentrarchus labrax* under two temperature regimes. **Aquat Toxicol**. v. 219, 105378, 2019. https://doi.org/10.1016/j.aquatox.2019.105378

DUTTA, H. M.; MUNSHI, J. S. D.; ROY, P. K.; SINGH, N. K.; MOTZ, L.; ADHIKARI, S. Effects of Diazinon on bluegill sunfish, *Lepomis macrochirus*, gills: scanning electron microscope observations. **Exp. Biol. Online.** v. 2, p. 1-11, 1997. https://doi.org/10.1007/s00898-997-0017-4

ELLMAN, G. L.; COURTNEY, D.; ANDRES, V. J.; FEATHERSTONE, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. **Biochem Pharmacol.** v. 7, p. 88-95, 1961. https://doi.org/10.1016/0006-2952(61)90145-9

EL-MURR, A.; IMAM, T.S.; Hakim, Y.; GHONIMI, W.A.M. Histopathological, immunological, hematological and biochemical effects of fipronil on Nile tilapia (*Oreochromis niloticus*). **J. Veterinar. Sci. Technol.** 6(5), 1000252, 2015. https://doi.org/10.4172/2157-7579.1000252

EUROPEAN FOOD SAFETY AUTHORITY (EFSA) Conclusion on the peer review of the pesticide risk assessment of the active substance 2,4-D. EFSA 2014, v. 12 (9), p. 3812, 2014.

EVANS, D. H., PIERMARINI, P. M., CHOE, K.P. The multifunctional fish gills: dominant site for gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogen wastes. **Physiol. Rev.** 85: 97-177, 2005.

FERNANDES, M.N. Gills. Respiration and ionic osmoregulation. In. **The Histology of Fish,** (Frank Kirschbaum, Krzysztof Formicki, eds). CRC Press, Boca Raton, 2019.

FERNANDES, M. N.; MORON, S.E. Breathing and respiratory adaptations. p. 217-250. In **Biology and Physiology of Freshwater Neotropical Fish**, (Bernardo Baldisserotto, Elisabeth Criscuolo Urbinati, J.E.P. Cyrino, eds.), Academic Press, London, 2020.

FERNANDES, M. N.; MORON, S. E.; SAKURAGUI, M. M. Gill morphological adjustments to environment and the gas exchange function. *In*: FERNANDES, M. N.; GLASS, M. L.; RANTIN, F. T.; KAPOOR, B. G. **Fish respiration and environment.** Enfield: Science publishers, 2007, p. 93–120.

GAAIED, S.; OLIVEIRA, M.; DOMINGUES, I.; BANNI, M. 2,4-Dichlorophenoxyacetic acid herbicide effects on zebrafish larvae: development, neurotransmission and behavior as sensitive endpoints. **Environ Sci Pollut Res**. v. 27 (4), p. 3686-3696, 2019. https://doi.org/10.1007/s11356-019-04488-5

GHAFFAR, A.; HUSSAIN, R.; ABBAS, G.; KALIM, M.; KHAN, A.; FERRANDO, S.; GALLUS, L.; AHMED, Z. Fipronil (Phenylpyrazole) induces hemato-biochemical, histological and genetic damage at low doses in common carp, *Cyprinus carpio* (Linnaeus, 1758). **Ecotoxicology**. v. 27, p. 1261-1271, 2018. https://doi.org/0.1007/s10646-018-1979-4

GOTTARDI, M.; BIRCH, M. R.; DALHOFF, K.; CEDERGREEN, N. The effects of epoxiconazole and A-cypermethrin on Daphnia magna growth, reproduction and offspring size. **Environ. Toxicol. Chem.** v. 36, p. 2155-2166, 2017. https://doi.org/10.1002/etc.3752

GUNASEKARA, A.S.; TRUONG, T.; GOH, K.S.; SPURLOCK, F.; TJEERDEMA, R.S. Environmental fate and toxicology of fipronil**. J Pest Sci.** v. 32 (3), 189-199, 2007. https://doi.org/10.1584/jpestics.R07-02

GUPTA, R. C.; ANANDÓN, A. Fipronil. *In*: GUPTA, R. **Veterinary Toxicology: Basic and Clinical Principles.** Cambridge: Academic Press, 2018. p. 533-538.

HABIG, W. H.; JAKOBY, W. B. Assays for differentiation of glutathione-S-transferases. **Method Enzymol.** v. 77, p. 398-405, 1981. https://doi.org/10.1016/S0076-6879(81)77053-8

HOWE, K.; CLARK, M.; TORROJA, C. et al. The zebrafish reference genome sequence and its relationship to the human genome. **Nature** v. 496, p. 498-503, 2013. https://doi.org/10.1038/nature12111

HUBER, P. C.; ALMEIDA, W. P.; FÁTIMA, Â. D. Glutationa e enzimas relacionadas: papel biológico e importância em processos patológicos. **Quím. Nova**. v. 31, p. 1170–1179, 2008. https://doi.org/10.1590/S0100-40422008000500046

HWANG, P-P.; LEE, T-H.; LIN, L-Y. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. **Am J Physiol Regul Integr Comp Physiol.** v. 301, p. 28–R47, 2011. https://doi.org/10.1152/ajpregu.00047.2011

ISLAM, F.; WANG, J.; FAROOQ, M.A.; KHAN, M. S. S.; XU, L.; ZHU, J.; ZHAO, M.; MUÑOS, S.; LI, Q. X.; WEIJUN, Z. Potential impact of the herbicide 2,4-dichlorophenoxyacetic acid on human and ecosystems. **Environ Int.** v. 111, p.332–351, 2018. https://doi.org/10.1016/j.envint.2017.10.020

JONSSON, C. M.; MOURA, M. A. M.; FERRACINI, V. L.; PARAÍBA, L. C.; ASSALIN, M. R.; QUEIROZ, S. C. N. Bioconcentrations of herbicides used in sugarcane crops in tilápia (*Oreochromis niloticus*) and the risk for human consumption. **Heliyon**. v. 5 (8), e02237, 2019. https://doi.org/10.1016/j.heliyon.2019.e02237

KONWICK, B. J.; GARRISON, A. W.; BLACK, M. C.; AVANTS, J. K.; FISK, A. T. Bioaccumulation, biotransformation, and metabolite formation of fipronil and chiral legacy pesticides in rainbow trout. **Environ. Sci. Technol.** v. 40, p. 2930-2936, 2006. https://doi.org/10.1021/es0600678

LI, K.; WU, J-Q.; JIANG, L-L.; SHEN, L-Z.; LI, J-Y.; HE, Z-H.; WEI, P.; LV, Z.; HE, M-F. Developmental toxicity of 2,4-dichlorophenoxyacetic acid in zebrafish embryos. **Chemosphere.** v. 171, p. 40-48, 2017. https://doi.org/10.1016/j.chemosphere.2016.12.032

LINS, J. A. P. N.; KIRSCHNIK, P. G.; QUEIROZ, V. S.; CIRIO, S. M. Use of fish as biomarkers for monitoring aquatic environment. **Rev. Acad., Ciênc. Agrár Ambient.** v. 8, p. 469-484, 2010. https://doi.org/10.7213/cienciaanimal.v8i4.11018

LOPES, F. M.; CALDAS, S. S.; PRIMEL, E. G.; da ROSA, C. E. Glyphosate Adversely Affects *Danio rerio* Males: Acetylcholinesterase Modulation and Oxidative Stress. **Zebrafish.** v. 14, p. 97-105, 2017. https://doi.org/10.1089/zeb.2016.1341

MELA, M.; GUILOSKI, I. C.; DORIA, H. B.; RANDI, M. A. F.; RIBEIRO, C. A. O.; PEREIRA, L.; MARASCHI, A. C.; PRODOCIMO, V.; FREIRE, C. A.; ASSIS, H. C. S. Effects of the herbicide atrazine in neotropical catfish (*Rhamdia quelen*). **Ecotox Environ Safe.** v. 93, p. 13-21, 2013. https://doi.org/10.1016/j.ecoenv.2013.03.026

MOREIRA, R. A.; DAAM, M. A.; VIEIRA, B. H.; SANCHES, A. L. M.; REGHINI, M. V.; MANSANO, A. S.; FREITAS, E. C.; ESPINDOLA, E. L. G.; ROCHA, O. Toxicity of abamectin and difenoconazole mixtures to a Neotropical cladoceran after simulated run-off and spray drift exposure. **Aquat Toxicol.** v. 185, p. 58-66, 2017. https://doi.org/10.1016/j.aquatox.2017.02.001

MOREIRA, R. A.; ARAÚJO, C.V.M.; PINTO, T. J. S.; SILVA, L. C. M.; GOULART, B. V.; VIANA, N. P.; MONTAGNER, C. C.; FERNANDES, M. N.; ESPINDOLA, E. L. G. Fipronil and 2,4-D effects on tropical fish: Could avoidance response be explained by changes in swimming behavior and neurotransmission impairments? **Chemosphere**. v. 263, p. 127972, 2021.

MORON, S.E.; OBA, E.T.; ANDRADE, C.A.; FERNANDES, M.N. Chloride cell responses to ion challenge in two tropical freshwater fish, the erythrinids *Hoplias malabaricus* and *Hoplerythrinus unitaeniatus*. **J Exp Zool A Ecologicol. Int. Physiol.** v. 298 (2), p. 93-104, 2003. https://doi.org/10.1002/jez.a.10259

OPPERHUIZEN A. Bioconcentration and bioaccumulation: is a distinction necessary. In: NAGET, R., R., LOSKILL, R. (Eds.), **Bioaccumulation in Aquatic Systems**. VCH Publishers, Weinheim, pp. 67 – 80, 1991.

ORUC, E. Ö; ÜNER, N. Effects of 2,4-diamin on some parameters of protein and carbohydrate metabolisms in the serum, muscle and liver of *Cyprinus carpio***. Environ Pollut,** v. 105, p. 267–272, 1999. https://doi.org/10.1016/S0269-7491(98)00206-1

PAULINO, M. G; SOUZA, N. E. S.; FERNANDES, M. N. Subchronic exposure to atrazine induces biochemical and histopathological changes in the gills of a Neotropical freshwater fish, *Prochilodus lineatus*. **Ecotoxicol Environ Safe.** v. 80, p. 6-13, 2012. https://doi.org/10.1016/j.ecoenv.2012.02.001

PERRY, S. F.; GOSS, G. G.; LAURENT, P. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. **Can. J. Zool.** v. 70, p. 1775 – 1786, 1992. https://doi.org/10.1139/z92-245

POLEKSIC, V., MITROVIC-TUTUNDZIC, V. Fish gills as a monitor of sublethal and chronic effects of pollution. In: Müller, R. &R. Lloyd (Eds.). Sublethal and Chronic effects of pollutants on freshwater fish. Oxford, Fishing News Books, p. 339-352, 1994.

PORTRUNELI, N. Efeito da exposição aguda do inseticida Regent 800 WG (Fipronil), do herbicida DMA 806 BR (2,4-D) e mistura de ambos em curimbatá, *Prochilodus lineatus* (Teleósteo, Prochilodontidae). MSc Thesis. Universudade Federal de São Carlos, São Paulo, Brazil, 40 pp., 2020.

QURESHI, I. Z; BIBI, A.; SHAHID, S.; GHAZANFAR, M. Exposure to sub-acute doses of fipronil and buprofezin in combination or alone induces biochemical, hematological, histopathological and genotoxic damage in common carp (*Cyprinus carpio L.).* **Aquat Toxicol**., v. 179, p. 103–114, 2016. https://doi.org/10.1016/j.aquatox.2016.08.012

R DEVELOPMENT CORE TEAM. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2019.

RAND, G.M.; WELLS, P.G.; MCCARTY, L.S. Introduction to aquatic toxicology. *In*: RANDY, M.G. (Ed.). **Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment**. Florida: Taylor & Francis, 1995. p.3-67.

RAO, J.V. Toxic effects of novel organophosphorus insecticide (RPR-V) on certain biochemical parameters of euryhaline fish, *Oreochromis mossambicus*. **Pestic. Biochem**. **Physiol.** v. 86, p. 78–84, 2006. https://doi.org/10.1016/j.pestbp.2006.01.008

REYNAUD, S.; WORMS, I. A. M.; VEYRENC, S.; PORTIER, J.; MAITRE, A.; MIAUD, C.; RAVETON, M. Toxicokinetic of benzo[a]pyrene and fipronil in female green frogs (*Pelophylax kl. esculentus*). **Environ Pollut**. v. 161, p. 206-214, 2012. https://doi.org/10.1016/j.envpol.2011.10.029

SANCHES, A. L. M.; VIEIRA, B. H.; REGHINI, M. V.; MOREIRA, R. A.; FREITAS, E. C.; ESPINDOLA, E. L. G.; DAAM, M. A. Single and mixture toxicity of abamectin and difenoconazole to adult zebrafish (*Danio rerio*). **Chemosphere.** v. 188, p. 582-587, 2017. https://doi.org/10.1016/j.chemosphere.2017.09.027

SCHREIBER, R.; ALTENBURGER, R.; PASCHKE, A.; SCHÜÜRMANN, G.; KÜSTER, E. A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs. **Chemosphere**. v. 77, p. 928-933, 2009. https://doi.org/10.1016/j.chemosphere.2009.08.038

SERRA-COMPTE A, ÁLVAREZ-MUÑOZ D, RODRÍGUEZ-MOZAZ S. Multi-residue method for the determination of antibiotics and some of their metabolites in seafood. Food Chem. Toxicol. v. 104, p. 3-13, 2017. https://doi.org/10.1016/j.fct.2016.11.031

SIMON-DELSO, N.; AMARAL-ROGERS, V.; BELZUNCES, L.P. et al. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. **Environ Sci Pollut Res**. v. 22, p. 5-34, 2015. https://doi.org/10.1007/s11356-014-3470-y

SMEJTEK, P.; PAULIS-ILLANGASEKARE, M. Modification of ion transport in lipid bilayer membranes in the presence of 2,4-dichlorophenoxyacetic acid. **Biophys J.**. v. 26, p. 441-466, 1979. https://doi.org/10.1016/S0006-3495(79)85265-0

VAN DER OOST,. R.; BEYER, J.; VERMEULEN, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environ Toxicol and Phar.** v. 13, p. 57-149, 2003. https://doi.org/10.1016/s1382-6689(02)00126-6

WANG, C.; QIAN, Y.; ZHANG, X.; CHEN, F.; ZHANG, Q.; LI, Z.; ZHAO, M. A metabolomic study of fipronil for the anxiety-like behavior in zebrafish larvae at environmentally relevant levels. **Environ Pollut**. v. 211, p. 252-258, 2016. https://doi.org/10.1016/j.envpol.2016.01.016

WHEATLEY, J. B.; KELLEY, M. K.; MONTALI, J. A.; BERRY, C. O.; SCHMIDT JR, D. E. Examination of glutathione S-transferase isoenzyme profiles in human liver using high-performance affinity chromatography. **J. Chromatogr. A**. v. 663, p. 53-63, 1994. https://doi.org/10.1016/0021-9673(94)80495-8

ZHANG, J.; SHEN, H.; WANG, X. WU, J.; XUE, Y. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. **Chemosphere**. v. 55, p. 167–174, 2004.

3. CONCLUSÃO GERAL

Em conclusão, os resultados obtidos sugerem que o fipronil e 2,4-D, testados isoladamente e em mistura, interferem na saúde de *Danio rerio*. A taxa de bioconcentração relatada no presente estudo indica que mesmo em baixas concentrações esses agrotóxicos podem concentrar no corpo do peixe a partir de sua biodisponibilidade no ambiente aquático. Os valores colocados nos recipientes para teste foram de concentração ambientalmente relevante e levaram a alterações nas enzimas responsáveis pela detoxificação do organismo, podendo levar ao estresse oxidativo e dificuldade do organismo em manter o funcionamento normal do corpo. Além disso, as alterações observadas em brânquias mostram que os agrotóxicos podem interferir diretamente na eficiência respiratória e de regulação iônica desses peixes. Sendo assim, fipronil e 2,4-D apresentam riscos a ecossistemas aquáticos.