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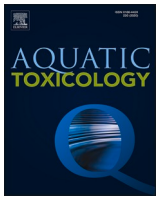

Isolated and mixed effects of pure and formulated abamectin and difenoconazole on biochemical biomarkers of the gills of *Danio rerio*

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ABSTRACT

Pesticides are released into the environment daily, and their effects on nontarget species in aquatic ecosystems have been widely reported. To evaluate the adverse effects caused in adults of *Danio rerio* species exposed to the pesticides abamectin, difenoconazole, and their commercial formulations (Kraft 36EC® and Score 250EC®), both isolated and in mixtures, biochemical biomarkers were analyzed in the gills of organisms exposed to sublethal concentrations. To this end, the activities of the enzymes 7-ethoxyresorufin-O-deethylase (EROD), glucuronosyltransferase (UDPGT), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), lipid hydroperoxide (LH), and malondialdehyde (MDA), which are indicative of oxidative stress, were measured after 48 h of exposure to the different pesticide treatments. The results showed a significant increase in EROD activity and MDA levels in the gills of fish exposed to the commercial formulation of abamectin. When the fish were exposed to difenoconazole and its commercial formulation, an increase in GST, GPx, and MDA levels and a decrease in GR activity were observed in the gills. Furthermore, the responses of the biomarkers were more pronounced in organisms exposed to mixtures of both active ingredients and commercial formulations. It is concluded that the commercial formulations Kraft 36EC® and Score 250EC® and their mixtures cause significant alterations in the detoxification metabolism of exposed organisms and induce oxidative stress in fish.

1. Introduction

The use of pesticides in agricultural areas is a recognized concern worldwide due to their potential risks to ecosystems. It was estimated that approximately 75 % of agricultural lands in the world are at risk of pesticide pollution, with 31 % of these areas at high risk and 64 % at risk from more than one active ingredient (Tang et al., 2021). Freshwater ecosystems close to agricultural fields may be contaminated due to the transport of pesticides (e.g., through spray drift and runoff), with potential negative consequences for biological communities. This is the case for the insecticide abamectin and the fungicide difenoconazole, whose simultaneous use has already been reported in agricultural areas (Burgarelli et al., 2023; Nunes and Espíndola, 2012). Abamectin belongs to the ivermectin group and is widely used in agriculture as an insecticide and as an antiparasitic agent for veterinary purposes (Dionisio and Rath, 2016). It blocks gamma-aminobutyric acid (GABA) receptors in invertebrates and vertebrates (Novelli et al., 2016). Difenoconazole is a fungicide from the triazole group that acts by interfering with the biosynthesis of ergosterol in fungi, provoking alterations in the morphology and functionality of fungal cellular membranes (Mu et al.,

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2016, 2013).

Commercial formulations of pesticides may present greater adverse effects than pure active substances to organisms, mainly due to the variable mode of action of several ingredients called inert of these products, such as surfactants, organic and inorganic solvents, emulsifiers, and adjuvants (Nagy et al., 2020; Straw et al., 2022). Although labeled "inert", these compounds are not necessarily chemically or biologically inactive, as they may increase the toxicity of the active ingredients, their mobility in the environment, skin absorption, and persistence (Cox and Surgan, 2006). Thus, comparing the negative effects of pure active ingredients with their formulations in animals may aid in understanding the implications of these inert compounds on pesticide effects to biodiversity.

Biomarker analysis enables the recognition of sublethal effects of different xenobiotics in aquatic organisms in the early days of exposure. The activities of EROD (ethoxyresorufin-*O*-deethylase, representative of isoform 1A of cytochrome P450), glutathione *S*-transferase (GST) and UDP-glucuronosyl transferase (UDPGT), are often evaluated as biomarkers of phase I and II biotransformation enzymes in fish (Schlenk et al., 2024).

During biotransformation, and as a result of increased aerobic metabolism, reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$) and hydroxyl ($^{\bullet}OH$) radicals, and other pro-oxidant molecules (e.g., hydrogen peroxide) are generated as by-products. ROS are mostly free radicals that can quickly react with biomolecules, causing their disintegration, which often results in the loss of their function (Costantini, 2008). ROS production is balanced by an antioxidant defense system that converts ROS into nonreactive molecules. This system includes, among other enzymes catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). When an imbalance between ROS production and the action of antioxidant defenses occurs, oxidative stress can be observed through indicators such as increased lipid hydroperoxide (LH) and malondialdehyde (MDA) levels.

The analysis of biomarkers for the evaluation of pesticide effects has been extensively used to provide a connection between external levels of exposure to contaminants, internal contamination levels in tissues, and early adverse effects in organisms (Kroon et al., 2017). Therefore, studies involving this approach are highly recommended. Previous studies have reported the lethal effects of abamectin and difenoconazole in *Danio rerio*, including their synergistic interactions when mixed (Sanches et al., 2017), demonstrating the importance of understanding the effects of these compounds at sublethal concentrations on nontarget species. Thus, the aim of this study was to evaluate the sublethal effects of abamectin and difenoconazole administered as pure active ingredients and their commercial formulations Kraft 36EC® and Score 250EC®, respectively, both isolated and in mixtures, on biotransformation and oxidative stress biomarkers of gills of adult *Danio rerio*. Therefore, the study hypotheses were that (i) the effects of pesticide formulations on biochemical biomarkers are greater than that of the pure ingredients they contain due to the presence of inert products and that (ii) the mixture of abamectin and difenoconazole, both of which administered as pure active ingredients and formulations, provokes increased biomarker responses, and enhances oxidative stress in organisms.

2. Materials and methods

2.1. Test organisms and acclimation

Adult zebrafish (*D. rerio*) were acquired from a local commercial hatchery in Sao Carlos (São Paulo State, Brazil). The organisms were acclimatized and maintained in the laboratory of the Nucleus of Ecotoxicology and Applied Ecology (NEEA) of the Centre for Water Resources and Environmental Studies (CRHEA/EESC) (NEEA/CRHEA) under a controlled temperature (25 ± 2 °C) and light regime (12 h light:12 h dark; light intensity ± 1000 lux). The fish were kept in 90 L glass aquaria containing reconstituted water prepared according to ABNT (2022) with a pH ranging from 7.0 to 7.6 and a hardness ranging from 40 to 48 mg $CaCO_3$ L^{-1}. Water was constantly aerated (dissolved oxygen > 6.0 mg L^{-1}), and food (Tetramin® flakes) was provided *ad libitum* daily until one day before the start of the test. The initial fish size (2.8 ± 0.4 cm) and weight (0.4 ± 0.1 g) were determined based on 10 % of the fish in each lot used. The use of the fish was approved by the Ethics Committee on the Use of Animals (CEUA/EESC-USP).

2.2. Laboratory exposures for biomarker analysis

The analytical standards of the active ingredients (a.i.) of abamectin PESTANAL® (purity: ≥ 95 %; CAS: 71751–41–2) and difenoconazole PESTANAL® (purity: ≥ 95 %; CAS: 119446–68–3) were acquired from Sigma–Aldrich. The standards were diluted in acetone, and from stock solutions of 1 mg abamectin mL^{-1} and 10 mg difenoconazole mL^{-1}, dilutions were made for the desired concentrations by using culture water. The commercial products used were Kraft 36EC® (a.i. abamectin; purchased from Cheminova) and Score 250EC® (a.i. difenoconazole; purchased from Syngenta). Stock solutions of Kraft 36EC® and Score 250EC® were prepared in distilled water according to the concentrations recommended by the manufacturers for strawberry crops, i.e., 10.8 mg abamectin L^{-1} for the Kraft 36EC® solution and 100 mg difenoconazole L^{-1} for the Score 250EC® solution. From these stock solutions, dilutions were made to obtain the desired test concentrations.

The concentrations of the insecticide and fungicide, from the pure active ingredient and commercial formulation, were set at the no-observed effect concentrations – NOEC (Table 1) as determined in a previous study in our laboratory (Sanches et al., 2017). Eight treatments were performed: (i) Control (ctrl) – culturing water only, (ii) Solvent control (ctrl solv.) – 0.01 % acetone in culture water, (iii) abamectin (aba) – pure a.i., (iv) Kraft 36EC® (kraft) – formulation with a.i. abamectin, (v) difenoconazole (difeno) – pure a.i., (vi) Score 250EC® (score) – formulation with a.i. difenoconazole, (vii) mixture of abamectin and difenoconazole (aba + difeno), and (viii) mixture of Kraft 36EC® and Score 250EC® (kraft+score).

The exposures were performed in five replicates per treatment (n = 5) in 1 L nontoxic plastic containers containing three organisms each (pools), with a proportion of 1 g of fish per liter, at a constant temperature (25 °C ± 2 °C) and a light regime (12 h:12 h light: dark) (NBR 15088, 2016). After 48 h, immobility/mortality was recorded, and the living individuals were collected and anesthetized in phenoxyethanol (2 mL L^{-1}). The organisms were decapitated, and their gills were removed, immediately frozen in liquid nitrogen, and stored (−80 °C) until the day of analysis.

2.3. Biomarker analysis

All biomarker analyses were performed at the Laboratory of Aquatic

Table 1
Sublethal concentrations (48 h NOEC) used in exposure experiments for biomarker analysis in the gills of *Danio rerio* fish (Sanches et al., 2017). The median lethal concentrations (LC_{50}) determined by Sanches et al. (2017) are also provided.

Treatments	LC_{50}	NOEC
Abamectin	58.6 μg L^{-1} (53.9 - 63.3)	20 μg L^{-1}
Kraft 36EC®	49.3 μg a.i. L^{-1} (46.6 - 52.1)	5.4 μg a.i.L^{-1}
Difenoconazole	1.41 mg L^{-1} (1.40 – 1.43)	1.0 mg L^{-1}
Score 250EC®	1.66 mg a.i.L^{-1} (1.64 – 1.68)	0.4 mg a.i.L^{-1}
Abamectin + Difenoconazole	30 μg L^{-11} + 1.05 mg L^{-1}	20 μg L^{-11} + 0.2 mg L^{-1}
Kraft 36EC® + Score 250EC®	7.57 μg a.i. L^{-11} + 1.74 mg a.i. L^{-1}	5.4 μg a.i. L^{-11} + 0.8 mg a.i. L^{-1}

Contamination Biomarkers (LABCA) of the Institute of Biosciences, Letters, and Exact Sciences of UNESP (IBILCE), São José do Rio Preto Campus (Brazil). The activity of the biotransformation and antioxidant defense enzymes and parameters of oxidative stress were analyzed in the gills of *D. rerio*. The gills were transported from São Carlos to São José do Rio Preto in liquid nitrogen and immediately stored in an LABCA freezer at −80 °C. The tissues were homogenized in 50 mM Tris buffer (pH 7.4) at a 1:4 v:v ratio containing the protease inhibitor PMSF. The samples were then centrifuged at 10,000 × *g* for 20 min. The collected supernatant was centrifuged again at 50,000 × *g* for one hour, after which the cytosolic and microsomal fractions were obtained. The activities of EROD and glucuronosyltransferase (UDPGT) were measured in the microsomal fraction, and the other enzymatic activities were measured in the cytosolic fraction.

2.3.1. Biotransformation enzyme analysis

The activity of EROD was measured in a microplate reader using a fluorometric method that involves the formation of resorufin, which is fluorescent (excitation = 537 nm, emission = 583 nm) and catalyzed by EROD. Potassium phosphate buffer (80 mM; pH 7.4) and a solution of 7-ethoxyresorufin were added to the wells of the microplates, along with the microsomal extract. The reaction was observed for 5 min at 30 °C.

The activity of UDPGT was measured using the method described by Clarke et al. (1992) with modifications. This method is based on the conjugation of the hydroxyl group of the p-nitrophenol substrate with α-D-glucuronic acid to form β-D-glucuronosyl, which is catalyzed by UDPGT. The solution contained 15.5 mM UDPGA, 50 mM Tris–HCl buffer, 10 mM MgCl (pH 7.4), microsomes, 0.2 M TCA, and 7 mM PNP. The solution was incubated at 30 °C in a water bath for 30 min. Then, 0.2 M TCA was added, and the mixture was centrifuged at 3600 × *g* for 15 min. The supernatant was collected, and 10 N KOH was added. The mixture was centrifuged again at 3600 × *g* for 15 min, and the resulting supernatant was used for measurement. A decrease in absorbance was observed using a spectrophotometer at 405 nm.

The activity of GST was evaluated using the method of Keen et al. (1976) adapted for a microplate reader. The sample was added to a reaction medium containing 0.1 M potassium phosphate buffer (pH 6.5), 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 100 mM GSH. The increase in absorbance was monitored at 340 nm at room temperature.

2.3.2. Analysis of antioxidant defense enzymes

The activity of CAT was measured using the method of Beutler (1975) by quantifying the rate of hydrogen peroxide decomposition by the enzyme through the decrease in absorbance at 240 nm at 30 °C. Quartz cuvettes were filled with 10 mM H_2O_2, 1 M Tris, 5 mM EDTA (pH 8.0), and a sample in the cytosolic fraction. The analysis of GR was performed at 340 nm by adding a sample in the cytosolic fraction and 0.1 M potassium phosphate buffer, 5 mM EDTA (pH 7.0), 2 mM GSSG, 0.15 mM GSH, and 0.1 mM NADPH solution to the microplate wells.

The analysis of GPx was performed using the technique of Sies et al. (1979), adapted for a microplate reader, which is based on the measurement of the decrease in absorbance at 340 nm during the reduction of oxidized glutathione (GSSG). This reduction is catalyzed by glutathione reductase (GR) in the presence of NADPH at 30 °C. To this end, a sample in the cytosolic fraction and 0.1 M potassium phosphate buffer, 5 mM EDTA (pH 7.0), 0.2 mM NADPH, 0.1 U mL^{-1} GR, and 1 mM tBOOH solution were added to the microplate wells.

2.3.3. Analysis of lipid peroxidation

Lipid hydroperoxide levels were analyzed using the xylene orange (FOX) method. This method is based on the principle that hydroperoxides oxidize ferrous iron to ferric iron, which reacts with xylene orange, producing a chromophore that has maximum absorption at 560 nm (Jiang et al., 1991).

The product formed between MDA (a product of lipid peroxidation) and 2-thiobarbituric acid was also detected via HPLC-UV (Nogueira et al., 2011). For HPLC, MDA-TBA samples were directly injected (20 μL) and monitored at 532 nm at 40 °C. The mobile phase consisted of a 50 mM potassium phosphate monobasic solution at pH 7.0 with 40 % methanol and was pumped isocratically (1 mL min^{-1}). An LC-18 column (150 × 4.6 mm, 5 μm pore diameter) was used. MDA levels were quantified based on a standard curve obtained by previously injecting known concentrations of MDA-TBA into the HPLC. The data are expressed as pmol of TBARS/*g* tissue (TBARS: thiobarbituric acid reactive substances).

2.4. Total protein quantification

Protein quantification is necessary for the final calculation of enzymatic activities and was performed using the Bradford (1976) method. This method is based on the binding of the Coomassie Brilliant Blue/G-250 dye (Bradford Reagent/Sigma–Aldrich) to the protein molecules in the sample, resulting in the formation of a blue complex that is measured at 595 nm using a spectrophotometer. The protein concentration was determined based on a calibration curve using known concentrations of bovine serum albumin (BSA).

2.5. Chemical analysis and physicochemical variables

To confirm the nominal test concentrations, the stock solutions were analyzed via high-performance liquid chromatography (HPLC/MS/MS Agilent® 6490 series). The chromatographic analysis conditions were as follows: Agilent Zorbax ODS C18 column (250 mm × 4.6 mm × 5 mm) and a temperature of 25 °C. The isocratic mobile phase utilized was acetonitrile and water (0.1 % formic acid; 90:10 v/v) for 6 min at an injection volume of 20 μL and a flow rate of 1.0 mL min^{-1}. Analyses were carried out in three replicates. Based on the absorbance signals observed in the diode array detection (DAD) spectrum of the standard solutions, abamectin, and difenoconazole were detected and quantified at 246 nm and 230 nm, respectively, with retention times of 2.9 and 2.2 min, respectively. The precision in terms of repeatability, expressed as the relative standard deviation (RSD), was 2.05 % for abamectin and 1.88 % for difenoconazole. The detection limits were 22.2 μg L^{-1} abamectin and 27.2 μg L^{-1} difenoconazole. The analytical recovery of abamectin was 87.2 % ± 11.03 %, and that of difenoconazole was 142.7 % ± 3.49 %.

Some physicochemical variables were analyzed in the water at the beginning and end of the experiments, such as temperature (°C), pH (Micronal B374 potentiometer), dissolved oxygen (YSI meter), and ammonia concentration (Hansen and Koroleff, 2007).

2.6. Statistical analysis of data

The enzymatic activity and oxidative stress parameter data were statistically compared between the groups using the Statistica 7.0 program. Tests were performed to check whether the data were normally distributed (Shapiro–Wilk) or had homogenous variances (Levene), and the biochemical parameters between different treatments and concentrations were compared using analysis of variance (ANOVA), followed by post hoc Fisher's least significant difference (LSD) test for parametric data. For nonparametric data, the Kruskal–Wallis's test was used, followed by the post hoc Student–Newman–Keuls test. Significant differences between groups were accepted at $p < 0.05$. The integrated effect analysis of all biomarkers was performed using the Integrated Biomarker Response (IBR) developed by Beliaeff and Burgeot (2002) and adapted by Sanchez et al. (2013) as IBR v2 (version 2). The biomarkers GPx, GR, and HL were excluded from the IBR analysis to improve statistical strength and allow for better visualization of the results in the star plots that were constructed with the average value of each treatment.

3. Results and discussion

3.1. Water parameters and test validation

The water parameters measured throughout the experiments are presented in the supplementary material (Table S1). At the end of the 48-h exposure, the pH ranged from 7.1 to 7.2, the ammonia concentration ranged from 165 to 466 μg L^{-1} and the dissolved oxygen concentration remained above 6 mg L^{-1}. In addition, the mortality of organisms in the controls was less than 10 %; thus, the experiments were valid (NBR 15088, 2016). Furthermore, no mortality was registered in any of the treatments; thus, the results presented are relative to sublethal doses of pure and mixed pesticides.

3.2. Biomarkers

The responses of the detoxification-associated enzymatic activity measured in *D. rerio* gills after exposure to pesticides are presented in Fig. 1, and the values are detailed in the supplementary material (Table S2). The activity of the enzyme EROD in the gills of organisms exposed for 48 h to a.i. abamectin, the commercial formulation Kraft 36EC® alone, or its mixture with Score® was greater than that in the gills of both controls ($p < 0.05$). No significant differences were detected in the UDPGT response ($p > 0.05$) except for the increase observed in the a.i. difenoconazole-treated group compared with the control group ($p < 0.05$). On the other hand, increases in the activity of the enzyme GST occurred after exposure to i.a. difenoconazole and this based formulation Score 250EC®, to the commercial formulation Kraft 36EC®, and both mixture scenarios ($p < 0.05$). Furthermore, GST activity increased in the gills of the fish after exposure to the a.i. mixture compared to the a.i. abamectin alone, and in the formulation mixture regarding the responses caused by both formulations alone. The increases in the activities of the biotransformation enzymes EROD and GST show that the detoxification system of *D. rerio* exposed to contaminants has been activated to transform the absorbed compounds into more soluble compounds and facilitate their excretion. Abamectin and its commercial formulation Kraft 36EC® seem to activate the phase I metabolism pathway (EROD), while difenoconazole and its commercial formulation Score 250EC® activate the phase II pathway (GST).

Effects of abamectin on fish biotransformation enzymes are insipient in the scientific literature. On the other hand, up-regulation of both phase I and phase II biotransformation enzymes due to abamectin exposure have been often observed in insects (Chen et al., 2024; Ghazawy et al., 2023; Hu et al., 2019; Liu et al., 2024; Xuan et al., 2015), strongly suggesting their protective effects against abamectin toxic effects. Moreover, a previous study using mammals such as rats and ruminants (Skálová et al., 2001) have shown that the cytochrome P450 family are the main enzymes involved in the metabolism of ivermectin, and they are known to increase the activity of CYP1A (EROD) (Albérich et al., 2014). However, contrary to our study, other studies reported a decrease in GST activity in *D. rerio* embryos exposed to ivermectin (Domingues et al., 2016; Oliveira et al., 2016), which indicates that different life stages of the same species exposed to the same test compounds may trigger different metabolic pathways. These differences in life stages in several biomarkers and mortality were also previously reported after exposure to difenoconazole (Mu et al., 2015).

Although it did not significantly alter the antioxidant defense system (Fig. 2), exposure to Kraft 36EC® produced ROS during the biotransformation of compounds, as evidenced by the increase in MDA. These unbalanced ROS, not counteracted by antioxidant enzymes, possibly attacked the cell membranes, causing oxidative stress, as evidenced by the increase in MDA levels in the gills of organisms exposed to Kraft 36EC® compared to the controls ($p < 0.05$, Fig. 3). Reports on oxidative stress in fish caused by exposure to abamectin are scarce; however, studies with *Columba livia* birds have shown that oxidative stress is caused by exposure to ivermectin (Liu et al., 2014; Zhu et al., 2013). An increase in MDA levels was also observed in the snail *Physella acuta* exposed to ivermectin (Ma et al., 2014).

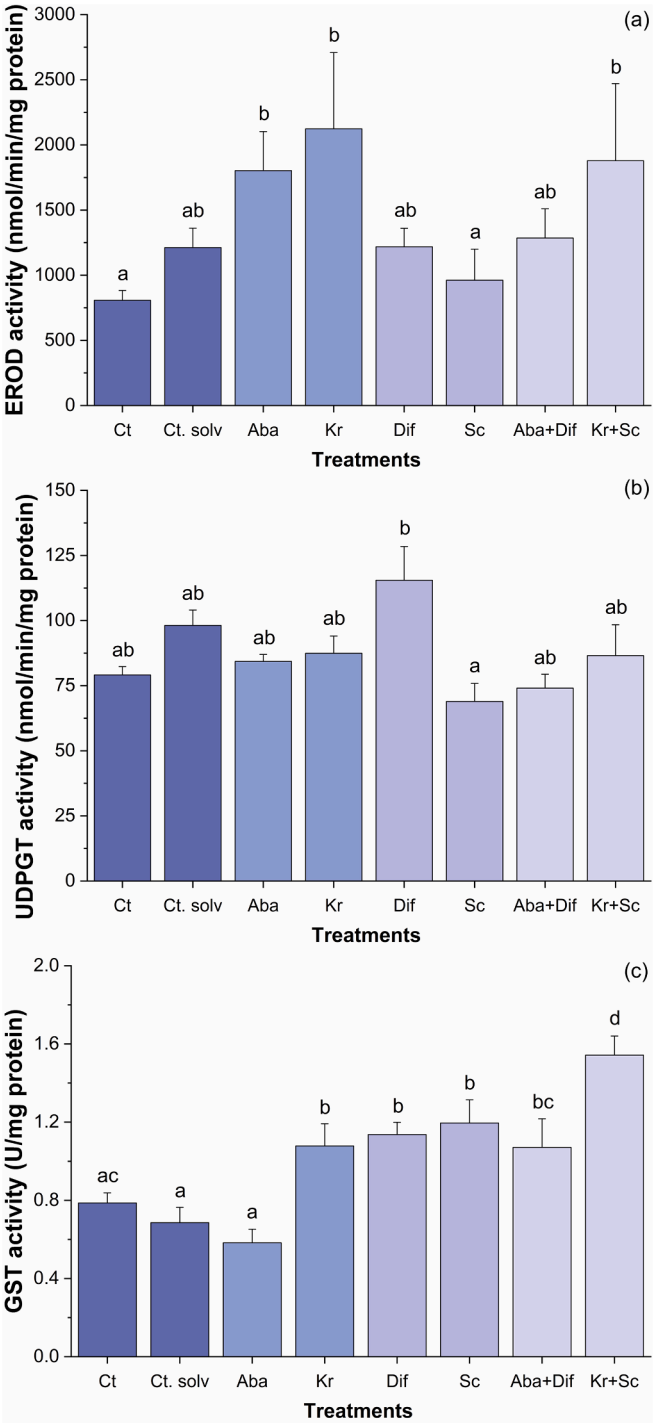

Fig. 1. Activities of the biotransformation enzymes ethoxyresorufin-O-deethylase (EROD), glucuronosyltransferase (UDPGT), and glutathione-S-transferase (GST) analyzed in the gills of *Danio rerio* exposed for 48 h to the NOEC of each compound. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Studies involving fish exposed to difenoconazole have shown that this compound usually activates both the EROD and GST detoxification pathways (Zhang et al., 2017). In the present study, only phase II enzymes exhibited increased activity, and the biotransformation of difenoconazole possibly generated ROS as GPx activity increased ($p < 0.05$, Fig. 2). On the other hand, the GR activity in all treatments that included

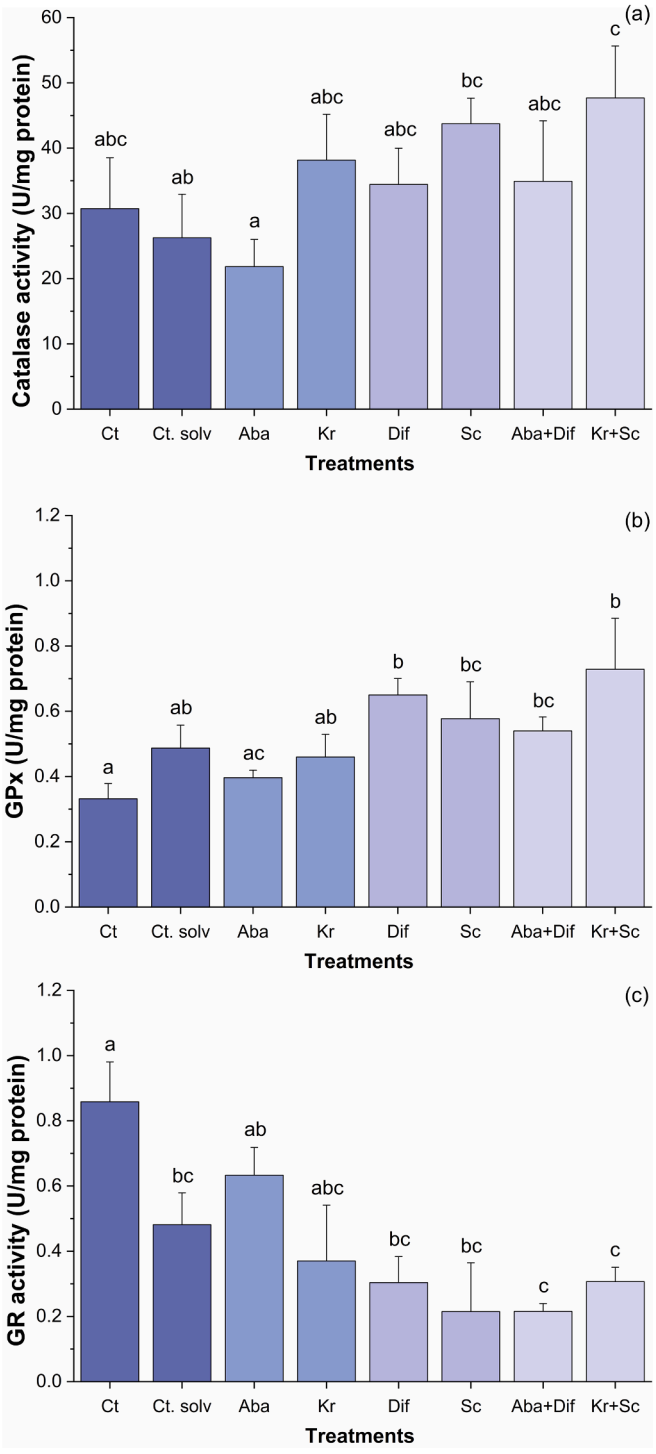

Fig. 2. Activity of the antioxidant defense enzymes catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) analyzed in the gills of *Danio rerio* exposed for 48 h to the NOEC of each compound. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

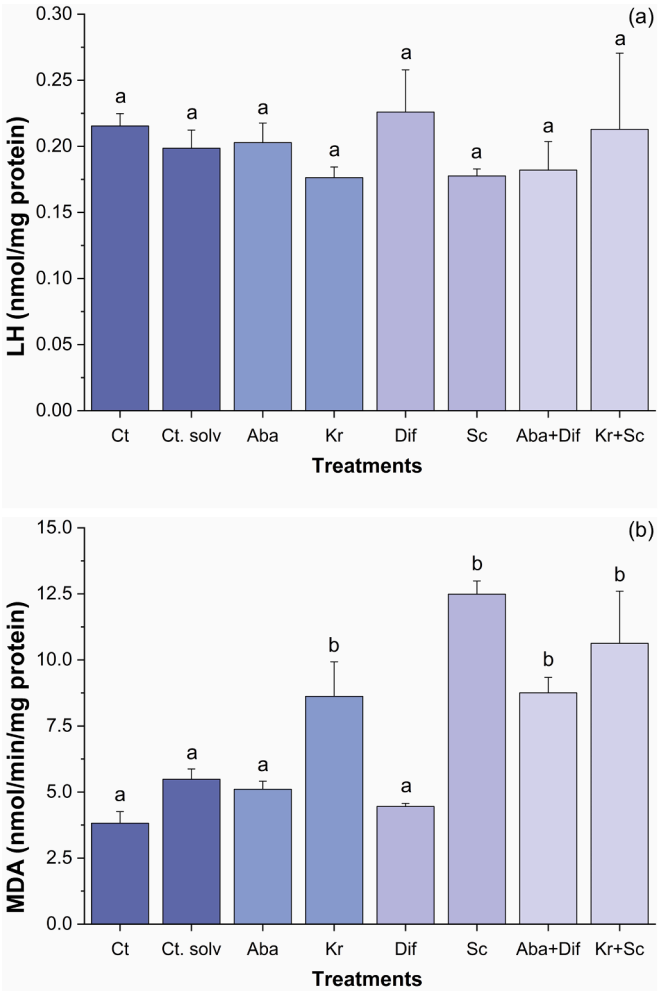

Fig. 3. Lipid peroxidation measured by malondialdehyde (MDA) and lipid hydroperoxide (HL) levels in the gills of *Danio rerio* exposed for 48 h to the NOEC of each compound. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

the fungicide (either alone or mixed with abamectin or for both the active ingredient and formulation) decreased relative to that in the control ($p < 0.05$, Fig. 2). We highlight that in treatments containing commercial formulations, no solvent was used; thus, the comparison is relevant only to the control, which showed reductions in this parameter. No alterations in the activity of the antioxidant defense enzyme catalase occurred in any of the exposure scenarios ($p > 0.05$). An increase in GPx and a decrease in GR indicate that excess ERO was generated and that the antioxidant capacity of the organisms exposed to difenoconazole, its commercial formulation, and the mixtures were affected.

Oxidative stress also occurred in organisms exposed to Score 250EC® and the mixture treatments containing difenoconazole or Score 250EC®, as these treatments significantly increased the MDA levels in the gills ($p < 0.05$, Fig. 3). The excessive generation of ERO during phase II biotransformation was possibly not efficiently counteracted by antioxidant defenses, leading to lipid peroxidation and consequent oxidative stress (Fig. 3). Previous studies have also revealed that oxidative stress in adult *D. rerio* results from increased MDA levels. Mu et al. (2015), for example, reported oxidative stress in adult *D. rerio* exposed to abamectin encountered by increased MDA levels. MDA is an aldehyde commonly used as a biomarker of pollution because it is a byproduct of lipid peroxidation caused by the attack of ROS. Excess ROS can be generated when organisms are exposed to toxic compounds and absorb and metabolize these products. When their antioxidant defenses are not sufficient to counteract the attack of ROS, lipid peroxidation occurs, leading to oxidative stress. Therefore, a significant increase in MDA levels in certain organisms indicates a state of pollution alert in the form of a metabolic response that precedes irreversible damage such as lethality (Nogueira et al., 2015). By analyzing biomarkers such as MDA, it is possible to detect whether concentrations are considered 'safe' or without observed effect (NOEC) in standard toxicity tests.

In addition to the individual biological responses of each biomarker,

the integrated biomarker response (IBRv2) was also evaluated. These calculations are based on the concept of reference deviation. The IBRv2 test uses control results to determine the values of other groups, and the IBR number is the sum of the deviation of each parameter. Exposure to commercial formulations and both mixture treatments significantly influenced the tested biomarkers, as evidenced by the significant increases in the IBR index ($p < 0.05$; Fig. 4). To further analyze these IBR results, star plots were constructed to evaluate how much the treated group differed from the control group (Fig. 5). Thus, the star plots show the extent of the effects of the treatments on the different biomarkers compared to the control (Felício et al., 2018; Sanchez et al., 2013). The significant IBR values for the isolated commercial formulations and all mixture treatments can also be observed through the star plots. These treatments caused a differential increase in the analyzed biomarker parameters compared to those of the control, except for a decrease in UDPGT in the case of the Score 250EC® group (Fig. 5).

Based on the IBR results, it can be concluded that the commercial formulations of abamectin and difenoconazole were produced more adverse effects to *D. rerio* than their active ingredients, as they caused greater deleterious effects on the metabolism of exposed organisms (Fig. 4). In addition, the concentrations of abamectin and difenoconazole after treatment with the commercial formulations were approximately 4 and 2.5 times lower, respectively, than those after treatment with the pure active ingredient (see Table 1), reinforcing the greater deleterious effects of the formulated products. These compounds may have more toxic effects on nontarget organisms than isolated active ingredients due mainly to the different modes of action (MoA) of the numerous additive compounds present in commercial formulations, called "inert compounds" by manufacturers (Coors and Frische, 2011; Cox and Surgan, 2006). Although they are called inert, it is known that these compounds often increase the toxicity of formulated products compared to that of active ingredients by increasing their cutaneous absorption, compound mobility in the environment, and persistence (Cox and Surgan, 2006). In addition, little is known about these compounds and the effects of mixtures of different "inert" ingredients present in commercial pesticide formulations on nontarget organisms. In Sanches et al. (2017), the 48 h lethal toxicity of Kraft 36EC® and Score 250EC® were compared to that of their respective active ingredients. No significant difference in lethal toxicity was observed between the commercial formulations and the active ingredients. However, when the effects of these compounds on the metabolism of *D. rerio* were evaluated at sublethal concentrations, the toxicity of Kraft 36EC® and Score 250EC® was greater than that of their respective active ingredients. This finding further reinforces the importance of evaluating the biochemical toxicity of pesticides, especially when commercial products are evaluated.

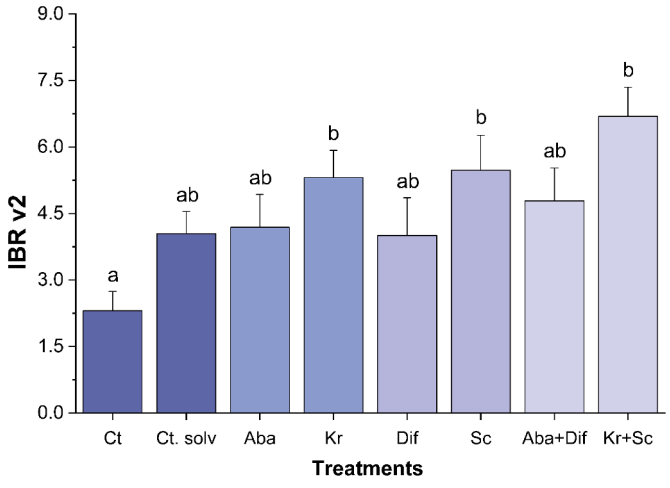

Fig. 4. Integrated biomarker response (IBR) index calculated by integrating biomarkers. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Additionally, due to the greater deleterious effects on the metabolism of *D. rerio*, the greater biochemical alterations seen in fish exposed to the mixtures was verified by the significant increase in IBR and the greater response of biomarkers when compared to the control. We highlight that the concentrations used in the present study were based on the NOEC values obtained in a previous study using the same products. In line with this, in the experiment with pure active ingredients, the concentration of difenoconazole in the mixture was reduced (from 1 mg L^{-1} to 0.2 mg L^{-1}, Table 1), compared with the fungicide alone, to avoid mortality. Thus, the increased response observed in the mixture relative to the single exposure occurred at lower doses of the fungicide, demonstrating the greater deleterious effects when both compounds occur together. The synergistic effects of mixtures of these pesticides have been reported previously in aquatic organisms (Moreira et al., 2017; Sanches et al., 2017). On the other hand, based on the NOEC values from, the concentration of difenoconazole in the mixture of the commercial formulation was greater than that in the single-exposure group (from 0.4 to 0.8 mg L^{-1}). This indicates that the greater toxicity in this treatment may be associated with the increased concentration of the fungicide, in addition to the stress related to the pesticide mixture.

Considering all the results presented, it is highlighted that the use of these compounds in the environment must be administered with caution, as they are extremely toxic and dangerous compounds that cause deleterious effects on the detoxification metabolism of nontarget organisms (Moreira et al., 2017; Oliveira et al., 2018). In addition, the biomarker results found in this study with *D. rerio* show that the adverse effects of abamectin and difenoconazole are potentiated when in mixtures, even at metabolic levels, which corroborates all the concerns regarding the safety and environmental risk involved in the indiscriminate use of these pesticides together or in a short period in crop areas. The application of both pesticides in the same crops is allowed (Burgarelli et al., 2023), increasing the probability of their co-occurrence in aquatic ecosystems on the edge of fields with implications for the indigenous organisms..

4. Conclusions

Based on the biomarker analyses using adult *D. rerio*, it can be affirmed that the commercial formulations Kraft 36EC® and Score 250EC® caused more pronounced acute biochemical effects than the pure active ingredients, as they promote significantly greater alterations in the detoxification metabolism of exposed organisms and cause oxidative stress in fish at lower concentrations, confirming our first hypothesis. In the same way, deleterious responses were more evident in the mixtures in both scenarios (pure active ingredient and commercial formulation), confirming the second hypothesis and revealing a concerning scenario in which these pesticides are licensed for use on the same crops, increasing the probability of co-occurrence in aquatic environments at the edge of agricultural areas.

CRediT authorship contribution statement

Ana Letícia Madeira Sanches: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Thandy Junio da Silva Pinto:** Writing – original draft, Formal analysis. **Michiel Adriaan Daam:** Writing – review & editing, Supervision, Formal analysis. **Fabrício Barreto Teresa:** Software, Formal analysis, Data curation. **Bruna Horvath Vieira:** Formal analysis, Data curation. **Marina Vanderlei Reghini:** Formal analysis, Data curation. **Eduardo Alves de Almeida:** Resources, Methodology. **Evaldo Luiz Gaeta Espíndola:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

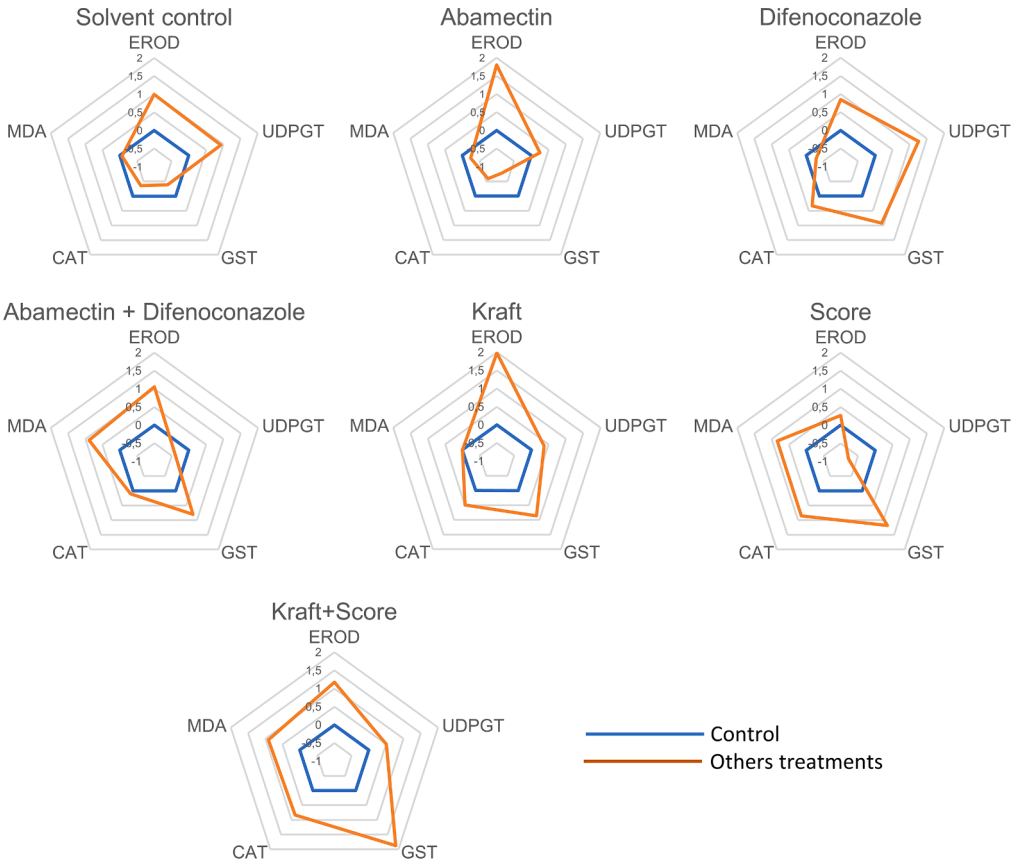

Fig. 5. Starplots constructed from the IBR analysis showing the influence of each biomarker on the response to different exposures to the control.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ana Leticia Madeira Sanches reports financial support was provided by State of Sao Paulo Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.106978.

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