Ambiente & Água - An Interdisciplinary Journal of Applied Science
ISSN 1980-993X – doi:10.4136/1980-993X
www.ambi-agua.net
E-mail: ambi.agua@gmail.com

Use of a fast toxicity test to determine whether carbendazim, fipronil, and sulfentrazone and their mixtures affect early zebrafish embryonic development

ARTICLES doi:10.4136/ambi-agua.2990

Received: 30 Jan. 2024; Accepted: 29 May 2024

Samara da Silva Gomes[1]; Bruno Andre Fernandes de Jesus da Silva Nunes[2]; Pabyton Gonçalves Cadena[1*]

[1]Departamento de Morfologia e Fisiologia Animal. Universidade Federal Rural de Pernambuco (UFRPE), Avenida Dom Manoel de Medeiros, s/n, CEP: 52171-900, Recife, PE, Brazil. E-mail: gomesamara27@gmail.com
[2]Departamento de Biologia. Centro de Estudos do Ambiente e do Mar. Universidade de Aveiro (UA), Campus de Santiago, 3810-193, Aveiro, Portugal. E-mail: nunes.b@ua.pt
[*]Corresponding author. E-mail: pabyton.cadena@ufrpe.br

ABSTRACT

Because of the exacerbated use of pesticides and their mixtures, and the difficulty of evaluating their toxicity, it is necessary to identify sensitive tools for the early detection of potential environmental risks related to the use of these chemicals. Our objective was therefore to evaluate the effects of these chemicals using a rapid 24 h toxicity test in zebrafish embryos. After exposure of individual and mixtures of the pesticides carbendazim (carb), fipronil (fipr), and sulfentrazone (sulf), the lethality and sublethally at 24 hpf, the effects of the mixtures on epiboly and on coagulation of the embryos at 8 hpf were evaluated. This model can be extrapolated to several vertebrate groups, including humans. All three pesticides were toxic in both exposure periods, at 24 hpf, all concentrations used affected the embryos, with lethal and sublethal effects occurring. At 8 hpf, epiboly analysis indicated that the mixtures did not potentiate the effects compared to the pesticides alone, being toxic in the order: carb > sulf > fipr > fipr + sulf > carb + sulf > carb + fipr + sulf > carb + fipr + sulf > carb + fipr. We conclude that the most toxic pesticide was sulfentrazone, since it was highly toxic to embryonic development at 24 hpf and caused both delayed epiboly and high percent clotting at 8 hpf. Thus, we see that the rapid toxicity test was effective in assessing the toxicity of pesticides and was sensitive in assessing the effects of pesticides at low concentrations.

Keywords: epiboly, pesticides, teratogenicity.

Uso de um teste rápido de toxicidade para determinar carbendazim, fipronil e sulfentrazona e suas misturas afetaram o desenvolvimento embrionário inicial do peixe-zebra

RESUMO

Devido ao uso exacerbado dos agrotóxicos e suas misturas e à dificuldade de avaliação de toxicidade, faz-se necessária a busca de ferramentas sensíveis para detectar precocemente potenciais riscos ambientais relacionados ao uso desses produtos químicos. Portanto, nosso

objetivo foi avaliar os efeitos desses produtos usando um teste rápido de toxicidade de 24 h em embriões de *zebrafish*. Após exposição dos pesticidas carbendazim (carb), fipronil (fipr) e sulfentrazona (sulf) isolados e misturados, foi avaliado a letalidade e subletalidade em 24 hpf, e os efeitos na epibolia e coagulação das misturas nos embriões em 8 hpf, com este, essa metodologia pode ser extrapolada para diversos grupos de vertebrados, inclusive humanos. Os três pesticidas foram tóxicos em ambos os períodos de exposição, a 24 hpf, todas as concentrações utilizadas afetaram os embriões, ocorrendo efeitos letais e subletais. Aos 8 hpf, a análise de epibolia indicou que as misturas não potencializaram os efeitos em relação aos agrotóxicos isoladamente, sendo tóxicas na ordem: carb > sulf > fipr > fipr + sulf > carb + sulf > carb + fipr + sulf > carb + fipr + sulf > carb + fipr. Concluímos que o pesticida mais tóxico foi o sulfentrazona, uma vez que foi altamente tóxico para o desenvolvimento embrionário em 24 hpf e causou retardo na epibolia e alto percentual de coagulação em 8 hpf. Assim, vemos que o teste rápido de toxicidade foi eficaz na avaliação da toxicidade dos pesticidas e foi sensível na avaliação dos efeitos destes pesticidas em baixas concentrações.

Palavras-chave: epibolia, pesticidas, teratogenicidade.

1. INTRODUCTION

Pesticides are a topic of great interest in environmental ecotoxicology, as in recent years there has been a significant increase in the number of new products marketed and registered by competent international agencies (Disner *et al.*, 2021). Among the environmental problems caused by pesticides, water pollution is attracting considerable concern (Wan *et al.*, 2021), since these chemicals have been frequently detected in natural or artificial water bodies, where they can enter ecosystems and cause various health problems to animals and humans (Pereira *et al.*, 2015). These chemicals reach water bodies through several processes or diffuse sources, such as runoff, leaching, spraying, or drainage (Demirci *et al.*, 2018).

Pesticides are rarely detected in aquatic environments as single chemicals, as they are often applied together in a mixture, or because a single water body supplies the aquifer demand of more than one agricultural crop (Gungordu *et al.*, 2016) or due to the common agricultural practice of annual crop rotation, during which the overlapping occurrence of these chemicals in soils, surface, and groundwater can occur (Thorngren *et al.*, 2017). The current assessment of the toxicity of these chemicals is predominantly based on testing the isolated chemicals, rather than in combination, so the ecological relevance may be underestimated (Brodeur *et al.*, 2014).

Therefore, these chemicals when in combination with others make it difficult to understand how ecological communities respond to them. This also challenges toxicity assessment, as the effects of these interactions can produce additive (synergistic or antagonistic) effects (Jackson *et al.*, 2016). Studies on pesticide mixtures are indispensable for understanding the effects of these mixtures in realistic conditions. Ecotoxicological tests are, thus, of great importance as they provide information for ecological risk analysis and management. This makes them widely useful in the formulation and endorsement of regulatory policies and administrative measures that define the tolerable emission and concentration limits of chemicals in the environment (Chapman *et al.*, 2002).

Historically, Brazil is a country with a strong agricultural economy, based on monoculture, and is the largest consumer of pesticides (Disner *et al.*, 2021), among which the class of herbicides is the most used, followed by insecticides and fungicides (ANVISA, 2016). For this study we selected two of the pesticides reevaluated and re-released in Brazil in 2019, sulfentrazone and fipronil (Gilson *et al.*, 2020); in addition, we also chose to study the one identified as the pesticide with the highest concentration in Brazilian food, carbendazim (Anvisa, 2016).

Sulfentrazone ($C_{11}H_{10}Cl_2F_2N_4O_3S$) is a herbicide, widely used in several crops in Brazil (Freitas, 2017). It has been identified and measured in surface runoff (10.3 mg L^{-1}), and groundwater (7.5 mg L^{-1}) in Illinois, USA (Thorngren *et al.*, 2017). In addition, sulfentrazone can have adverse effects on exposed biota; studies indicate that phytoplankton, aquatic invertebrates, and fish may be more susceptible to sulfentrazone (Thorngren *et al.*, 2017).

Fipronil ($C_{12}H_4Cl_2F_6N_4OS$) is an emerging insecticide (Wang *et al.*, 2016) banned in several European countries since 2004 for causing bee mortality (EFSA, 2013), but it is still widely used in several countries. This chemical has been detected in the water of Red River, Vietnam, at levels up to 0.02 ng/L (Wan *et al.*, 2021), and in the Rivers Vacacaí (0.5 µg L^{-1}) and Vacacaí-Mirim (0.6 µg L^{-1}), Brazil (Marchesan *et al.*, 2010). Exposure to fipronil concentrations (15 mg L^{-1}) was responsible for impairing angiogenesis, neurogenesis, and developmental processes in *Danio rerio* (Park *et al.*, 2020).

Carbendazim ($C_9H_9N_3O_2$) is a systemic fungicide, banned in the United States (Singh *et al.*, 2016), but is still used in many other countries. Its presence has been reported in the waters of the Taquari River in Brazil (0.4 µg L^{-1}) (Kronbauer *et al.*, 2021) and in Lake Hanoi in Vietnam (86.7 µg L^{-1}) (Wan *et al.*, 2021). This pesticide has been found responsible for causing adverse developmental effects (0.16 µg L^{-1}) in zebrafish (Andrade *et al.*, 2016).

The zebrafish (*Danio rerio*) has been widely used in toxicological studies because of the several advantages that make it a model species, among them genetic similarity and embryonic development with humans (Fukushima *et al.*, 2020). The fish embryotoxicity test (FET) is a toxicological assessment based on the use of embryos or newly hatched eggs of zebrafish, which has been replacing the acute test with adult fish and has become mandatory for effluent monitoring in Europe (Braunbeck *et al.*, 2015).

Among the parameters that can be easily evaluated in zebrafish embryos when exposed to pesticides, epiboly allows for rapid analysis of many experimental groups including mixtures of chemicals (Cadena *et al.*, 2020). During embryonic development, gastrulation is the stage in which epiboly occurs, a critical moment in development because it corresponds to the establishment of the main embryonic axes (dorsal-ventral, anterior-posterior, and left-right) (Solnica-Krezel and Sepich, 2012). Recently, some authors have shown that pesticides alone and their mixtures can cause teratogenic effects at several stages of zebrafish development (Wan *et al.*, 2021; Wang *et al.*, 2017; 2018).

Therefore, the purpose of this research was to evaluate the effects caused by mixtures of the pesticides carbendazim (fungicide), fipronil (insecticide), and sulfentrazone (herbicide), on zebrafish embryonic development at 8 and 24 hpf. This objective ultimately intends to establish the early development of zebrafish as a rapid method for toxicity and interaction evaluation between different chemicals, and as a responsive methodology to test the mixture of diverse pesticides.

2. MATERIAL AND METHODS

2.1. Reagents and solutions

The pesticides used for preparing the test solutions were Carbendazim (Carb.) (lot # 002-18-54392, CAS: 10605-21-7, 50% (w/v), 95% purity) was purchased from Adama Brasil (BRA), Fipronil (Fipr.) (lot # 001/19, CAS: 120068-37-3, 2.5 (w/w), 99% purity) was purchased from Rogama Indústria e Comércio LTDA (BRA) and Sulfentrazone (Sulf.) (lot # 1041-19-13767, CAS: 122836-35-5, 50% (w/v), >99% purity) was purchased from FMC Corporation (USA) and from commercial suppliers. The three pesticides were initially diluted in 1 mL of acetone (CAS: 67-64-1, ≥ 99.9%, Quimex, Brazil) and 99 mL of distilled water, and then two serial dilutions were performed only with water to reach the desired concentrations described in Table 1. The concentration of acetone in the final solutions is less than 0.01% (v/v) showing no toxicity for the animals. Pesticide concentrations were based on values equal to or

lower than the maximum residue limits (MRL) of these pesticides in foods allowed by the Brazilian Health Regulatory Agency - ANVISA. The pesticide residues were treated by advanced oxidation process (AOP) in a reactor using hydrogen peroxide/ultraviolet irradiation (16w) before final discard.

Table 1. (**A**) Concentrations of the individual compounds used in the toxicity test to assess teratogenic effects in zebrafish embryos at 24 hpf. (**B**) Concentrations of the single compounds, binary and ternary mixtures used to measure the toxic effects of the pesticides carbendazim (Carb), fipronil (Fipr), and sulfentrazone (Sulf) in the zebrafish model during epiboly. The layout of a simplex centroid design method for evaluation of interactions between chemical compounds.

A – Toxicity test for the evaluation of teratogenic effects at 24 hpf			
Components	Carbendazim (mg L^{-1})	Fipronil (mg L^{-1})	Sulfentrazone (mg L^{-1})
Control	0.00	0.00	0.00
Carb 0.20	0.20	-	-
Carb 0.50	0.50	-	-
Carb 1.50	1.50	-	-
Fipr 0.15	-	0.15	-
Fipr 0.20	-	0.20	-
Fipr 0.25	-	0.25	-
Sulf 0.01	-	-	0.01
Sulf 0.10	-	-	0.10
Sulf 1.00	-	-	1.00
B - Simplex centroid design for epiboly evaluation at 8 hpf			
Components	Carbendazim (mg L^{-1})	Fipronil (mg L^{-1})	Sulfentrazone (mg L^{-1})
Carb	0.20	0.00	0.00
Fipr	0.00	0.20	0.00
Sulf	0.00	0.00	0.20
Carb + Fipr	0.10	0.10	0.00
Carb + Sulf	0.10	0.00	0.10
Fipr + Sulf	0.00	0.10	0.10
Carb + Fipr + Sulf	0.06	0.06	0.06

2.2. Zebrafish husbandry

All protocols involving the animals were approved by the Ethics Committee for the Use of Animals (License 061/2019). Adult wild-type progeny (≈ 1 year) fish were reared and housed at the *Laboratório de Ecofisiologia e Comportamento Animal* – LECA, *Universidade Federal Rural de Pernambuco* – UFRPE. These animals were maintained in 80 L aquaria where they were quarantined to detect and confirm the absence of pathogens or diseases before the experiments. They were housed under the following physicochemical conditions: Artificial aeration of 11 mg/L DO; Ammonia (0 –5 mg L^{-1}); Nitrite (0.025 – 1 mg L^{-1}); Nitrate (0 – 140 mg L^{-1}) (Lammer *et al.*, 2009); pH 7.5 ± 0.5 (OECD, 2013); Temperature of 25 ± 1°C; and, 14/10 h light/dark cycle (OECD, 2013). The water was partially renewed once a week. Physicochemical conditions were maintained within ideal ranges during all experiments. The animals were fed Fort Color® fish feed (30% crude protein) twice daily and live brine shrimp nauplii (*Artemia* ssp) once daily.

2.3. Egg production of zebrafish

To obtain eggs, male and female zebrafish were placed in the crossing chambers (Alesco® Zebclean) at a ratio of 2:1 (Westerfield, 2000). 30 minutes after spawning the eggs were

collected, the unfertilized eggs were removed, and the fertilized eggs (normal blastula development) (Oecd, 2013) were washed and proceeded to the exposure chambers.

2.4. Exposure to chemicals

We propose a new 24 h toxicity test for fast evaluation of different chemicals' toxicity. Embryos at 2 hpf were randomly added into sterile polystyrene chambers and exposed to 50 mL of the test solutions (Table 1). The tests were divided into two experiments with different exposure times and evaluated endpoints. The first experiment evaluated lethal (coagulation) and sublethal (teratogenic effects) effects at 24 hpf (embryos exposed for 22 h from 2 hpf) and the second experiment evaluated the epiboly process at 8 hpf (embryos exposed for 6 h from 2 hpf). Pesticide-free distilled water was used as a control in both tests.

2.5. 24 h acute toxicity test

Preliminary tests were conducted to determine the concentrations of the pesticides (data not shown) used in the definitive experiment. The 24 h acute toxicity tests were carried out following OECD 236 (2013) guidelines with modifications. Animals with 2 hpf were exposed to pesticides for 22 h. The study was performed with 10 animals per experimental group in triplicate (n = 30 per group with 8 experimental groups ≈ 240 embryos). Abiotic conditions were controlled using an incubator with the physicochemical conditions described above. We considered that animals were affected by pesticides when they exhibited at least one lethal or sublethal effect (Cadena *et al*., 2020). These effects were observed using a BIO2B SSI microscope with an LED lamp. Mortality was recorded for lack of embryonic development or coagulation (OECD 236, 2013), while teratogenic effects analyzed were general growth retardation (delay), lack of pigmentation, pericardial edema, yolk sac edema, spinal deformation, tail deformation, lack of spontaneous movement, and rickets (Cadena *et al.*, 2020; Lammer *et al.*, 2009).

2.6. 8 h epiboly test

The epiboly test was based on the protocol by Cadena *et al*. (2020) and was assessed at 8 hpf, after 6 h the onset of pesticide exposure. The individual concentrations and mixtures of pesticides are described in Table 1. Embryos were washed 3 times in a phosphate buffer (100 mM, pH 7.4) and fixed in 4% paraformaldehyde (PFA) diluted in the same buffer for 24 h (Cadena *et al*., 2020). Thirty-five embryos were used in each experimental group (n = 35 per group with 8 experimental groups ≈ of 280 embryos). Photographs were recorded with a Hayear HY 2307 14 MP digital camera attached to a BIO2B SSI microscope with an LED lamp. The percentage of epiboly was determined by the distance between the animal pole and the blastodermal margin, divided by the distance between the animal pole and the plant pole. Measurements were made with Image J software (Version 1.52A, 2019, National Institutes of Health, MD).

2.7. Statistical analysis

Statistical planning for the epiboly test was performed by the simplex centroid design method (Cadena *et al.*, 2020) as shown in Table 1, as a basis for determining the number of experimental groups and evaluating the interaction between pesticides. A special cubic model was adjusted to analyze the data. All data were presented by mean ± SD. The acute toxicity test was performed in triplicate. The results were analyzed by one-way ANOVA. When the difference was significant, the means were compared by Tukey's test with $p < 0.05$. Statistical analyses were performed using Origin Pro Academic 2015 (Origin Lab. Northampton, MA, USA) and Statistica 14 Version (TIBCO, USA) (Cadena *et al.*, 2020).

3. RESULTS

3.1. 24 h acute toxicity test

We analyzed the toxic effects exhibited by the animals, in terms of embryonic development after 24 hpf of exposure. The pesticides caused a wide range of teratogenic effects, with the most common being developmental delay and lack of pigmentation, as seen in Figure 1.

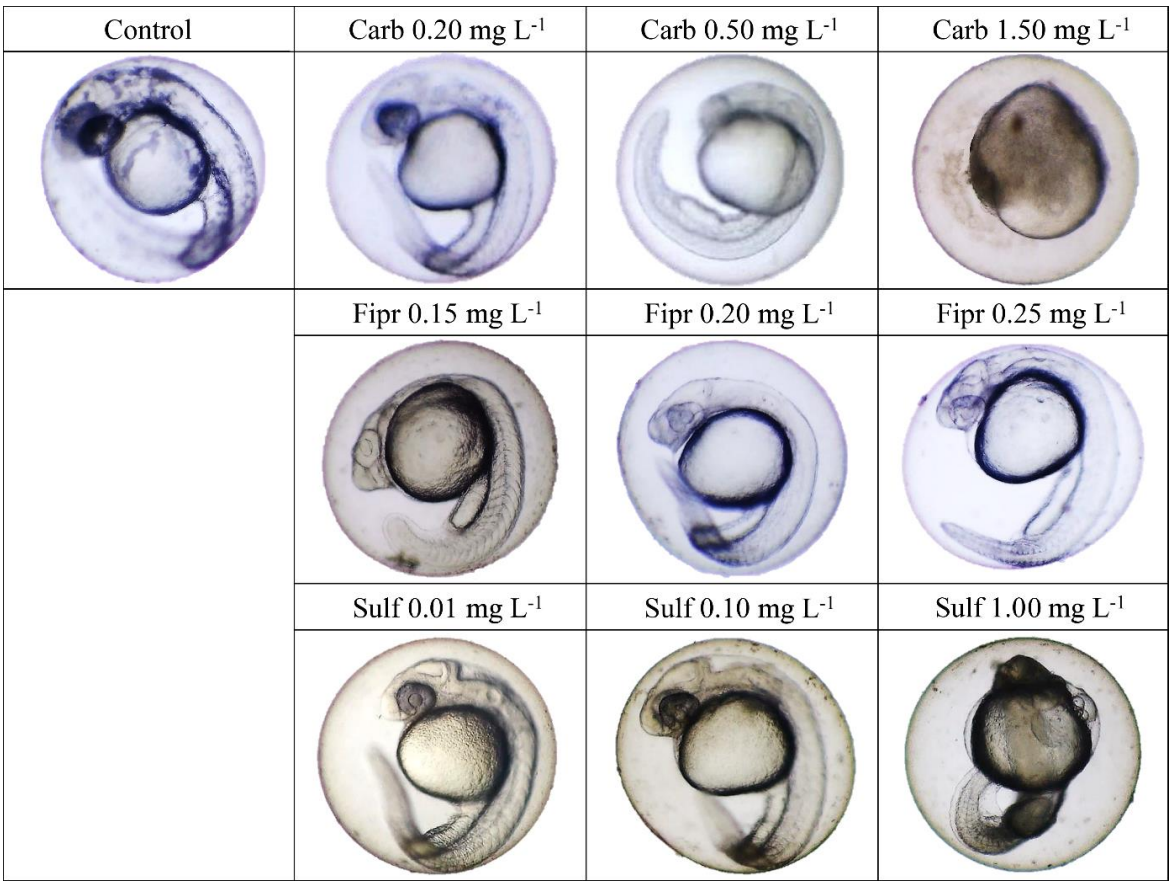

Figure 1. Teratogenic effects were observed in zebrafish embryos exposed to different concentrations of the pesticides carbendazim, fipronil, and sulfentrazone after 24 hpf compared to a normal embryo (control group). Key: Control - Control; Carb - Carbendazim; Fipr - Fipronil; Sulf - Sulfentrazone.

Furthermore, we observed that the pesticides affected the embryos almost in their entirety at the three concentrations used (Figure 2A). When analyzing the sublethal and lethal effects after exposure to a single pesticide, a dose-dependent lethality effect could be observed, in which the increase in lethal effects occurred with increasing concentrations of the tested pesticides (Figure 2B and Figure 2C).

The most frequent sub-lethal effect was a developmental delay. Sulfentrazone showed dose-dependent lethality and it was the most toxic compound among those studied here. Carbendazim showed low lethality and high sub-lethality in lower concentrations, while fipronil was the least toxic among the pesticides studied, presenting only sub-lethal effects. Regardless that the epiboly and acute toxicity tests were performed at different post-fertilization periods, the ranking of toxicity among pesticides was maintained.

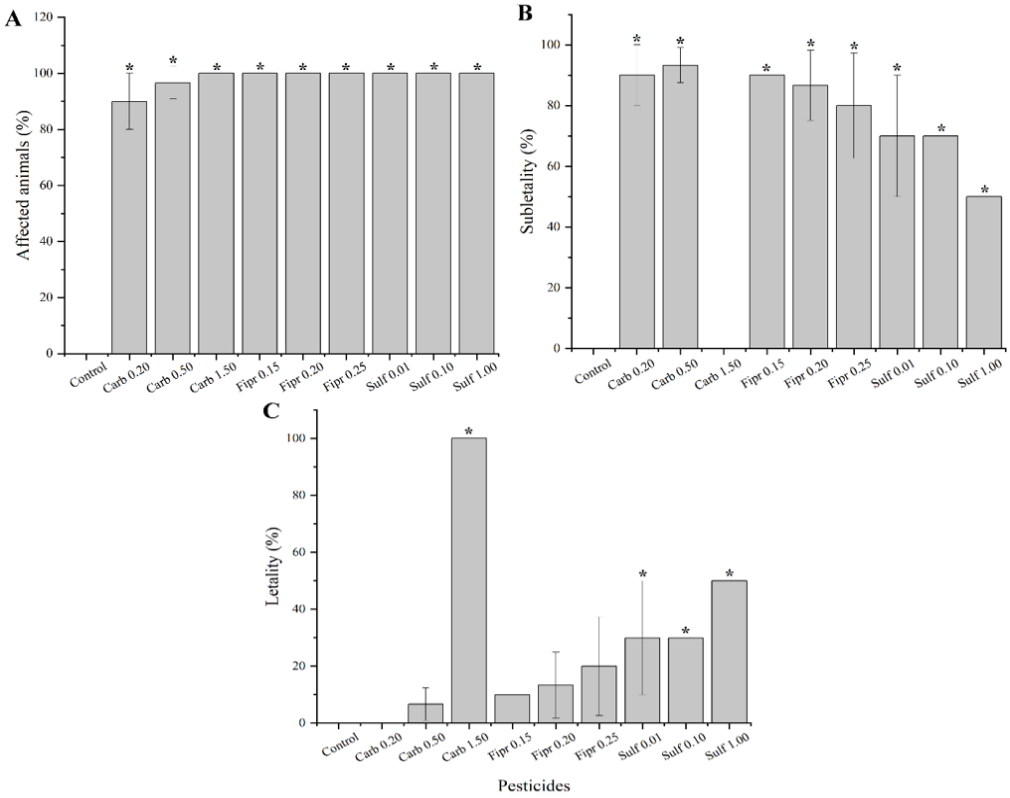

Figure 2. Results of the toxicity test of the pesticides carbendazim, fipronil, and sulfentrazone in zebrafish embryos at 24 hpf where (**A**) represents the percentage of affected animals, including lethal and sub-lethal effects, compared to the control group by one-way ANOVA ($F(9,29) = 220.67$ $p < 0.001$); (**B**) percentage of animals that showed sub-lethal effects compared to the control group by one-way ANOVA ($F(9,29) = 39.42$ $p < 0.001$); and (**C**) percentage of animals that showed lethal effects compared to the control group by one-way ANOVA ($F(9,29) = 31.78$ $p < 0.01$). * Denotes a significant difference by Tukey's test ($p < 0.05$). Key: Control - Control; Carb - Carbendazim; Fipr - Fipronil; Sulf – Sulfentrazone.

3.2. 8 h epiboly test

The three pesticides affected epiboly in different ways, based on qualitative analysis of animal images (Figure 3).

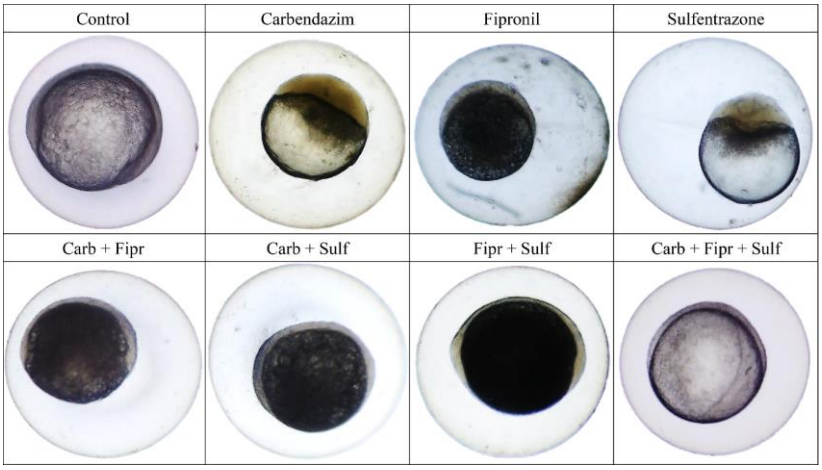

Figure 3. Toxic effects were observed in zebrafish embryos exposed to the single compounds and binary and ternary mixtures after 8 hpf compared to a normal embryo (control group). Key: Control - Control; Carb - Carbendazim; Fipr - Fipronil; Sulf – Sulfentrazone.

The pesticides carbendazim and sulfentrazone were the ones that significantly affected the epiboly process (Figure 4A). Kimmel *et al*. (1995) reported that zebrafish embryos with normal development after 8 hpf are at 75% epiboly. Considering this assumption, it can be inferred that the embryos exposed to carbendazim and sulfentrazone evidenced delayed development. When evaluating epiboly, in parallel we also observed the coagulation of the embryos (Figure 4B) and verified that the experimental group most affected concerning coagulation was fipronil. This was the pesticide that caused the least delay in terms of epiboly, despite causing the highest coagulation, indicating high lethality. Additionally, carbendazim caused the greatest delay in epiboly and the lowest percentage of coagulated embryos. Our data also allowed us to conclude that the most toxic pesticide for embryos after 8 hpf was sulfentrazone since it significantly reduced epiboly, but simultaneously was responsible for a large percentage of coagulated animals. The Ternary contour plot (Figure 4C) summarizes the interaction between the three pesticides, and their effects in terms of the reduction of epiboly, individually and in combination. It is possible to observe the change caused by carbendazim and sulfentrazone on epiboly individually. The mixture between them, however, caused less reduction in the percentage of epiboly.

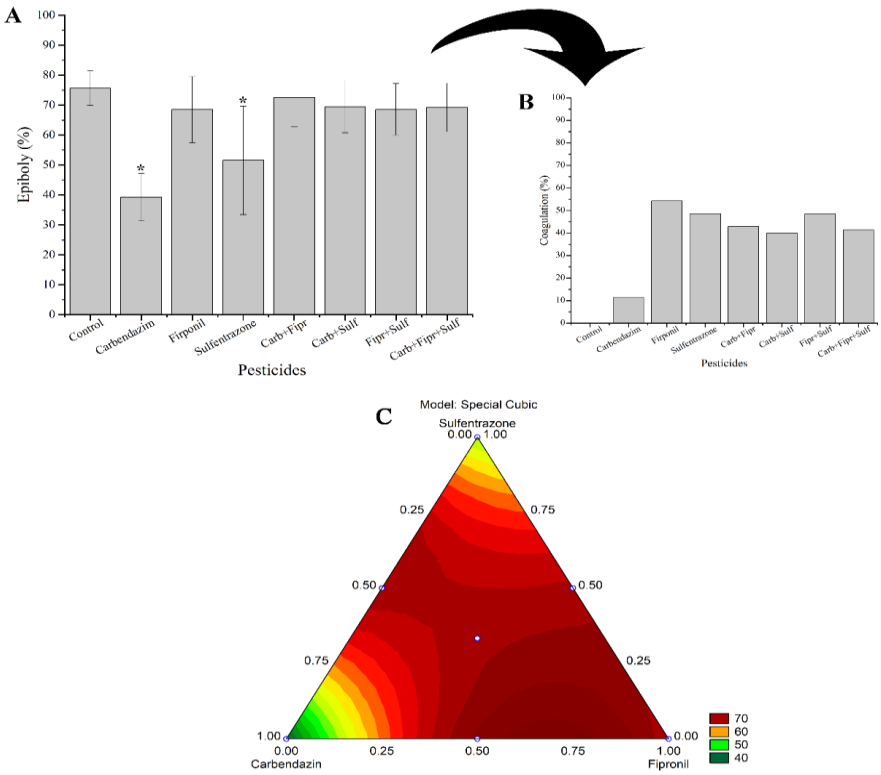

Figure 4. Exposure of zebrafish to the pesticides carbendazim, fipronil, and sulfentrazone during embryogenesis after 8 hpf. (**A**) the epiboly process was considered affected when Tukey's test = $p < 0.05$ (*). Each experimental group was compared with the control group by one-way ANOVA ($F(7,190) = 47.29$ $p < 0.001$) followed by Tukey's test (Carbendazim $p = 0.00$; Fipronil $p = 0.19$; Sulfentrazone $p < 0.001$; Carb + Fipr $p = 0.93$; Carb + Sulf $p = 0.30$; Fipr + Sulf $p = 0.16$; Carb + Fipr + Sulf $p = 0.12$). (**B**) Percentage of coagulated animals visualized during the epiboly test in the single and mixed pesticides. (**C**) Ternary contour plot of the variables Carbendazim, Fipronil, and Sulfentrazone for a special cubic model ($F(1,150) = 6.02$, $p = 0.01$) evaluating the individual effects and interactions of these pesticides in their mixtures. Carbendazim and Sulfentrazone were more toxic by reducing the percentage of epiboly (green areas). Key: Control - Control; Carb - Carbendazim; Fipr - Fipronil; Sulf – Sulfentrazone.

4. DISCUSSION

In our research, we evaluated the toxic effects of pesticides (a fungicide, an insecticide, and an herbicide), when isolated but also of their mixtures, through a rapid toxicological analysis to identify the early effects of these chemicals on zebrafish embryos.

A 24-hour acute toxicity test is the first step to identifying the toxic effects of environmental contaminants. However, acute tests are sometimes not entirely adequate to study the environmental release of chemicals and their adverse effects on exposed biota. Responses drawn from acute exposure usually overestimate toxicity data but are often ecologically relevant, such as in the case of our study. Accidental spills or releases or runoff of pesticides will result in conditions that are comparable to the results of acute testing, and toxicity tests that utilize the early life stages of the organism, tend to be sensitive, and reduce the cost and duration of such tests (Wang *et al.*, 2017). Following this assumption, our acute test aimed at assessing adverse effects after a short period of exposure – acute exposure. Our data showed that all concentrations of the tested pesticides affected embryos after 22 hours of exposure, similar also to the test performed by Wang *et al*. (2018). Therefore, our toxicity data using the embryonic stage provides valuable information on toxicity over a short period so that these methods can serve as a model for emergency screening and as an assessment of the interaction between different chemicals.

When we separately evaluated the effects as sublethal and lethal, we saw some differences in the toxicological response, especially concerning pesticide concentrations. The most frequent sub-lethal effect was a developmental delay, which was more pronounced in the groups exposed to lower concentrations of pesticides. Regarding the lethality of pesticides used on embryos, it was possible to observe high lethality in the animals exposed to carbendazim. Andrade *et al*. (2016), when investigating the effect of carbendazim on zebrafish embryos, found no mortality at concentrations < 1.0 mg L^{-1} and found 100% mortality at > 2.0 mg L^{-1}, determining a 96 h LC50 of 1.75 mg L^{-1}. These authors also observed teratogenic effects when fish were exposed to concentrations between 0.85 and 1.6 mg L^{-1}. The authors found no significant mortality at 24 hpf but reported developmental abnormalities, tail and spinal cord deformities, and edema at 24 hpf that persisted to 96 hpf. The embryos used in our study were shown to be more sensitive, as when exposed to concentrations above 0.75 mg L^{-1} of carbendazim, they showed 100% mortality and teratogenic effects occurred at concentrations below 0.50 mg L^{-1} at 24 hpf. Given this, they proved more resistant than zebrafish embryos used by Palanikumar *et al*. (2013), who reported a 96 h LC50 value for carbendazim of 0.013 mg/L, who reported a 100% mortality effect after 24 h exposure to 0.216 mg L^{-1} carbendazim. The data corroborate the results obtained here for the epiboly test, suggesting that the effects of carbendazim tend to be more relevant in the early stages of embryonic life due to its mode of action.

Park *et al*. (2020), estimated the 72 h LC50 value for fipronil to be 13.47 mg L^{-1}, with significant lethality observed at concentrations above 15 mg L^{-1}, and teratogenic effects starting at 7.5 mg L^{-1}, with developmental delay, yolk sac, and pericardial edema, spinal deformity, and decreased body size, and a decreased hatching rate in embryos exposed to fipronil. Kim and Lee (2023) in their research they used concentrations of up to 5.0 mg L^{-1}, embryos exposed to fipronil exhibited significant mortality at 96 hpf, as well as body length and cardiac activity were affected. Yan *et al*. (2016) observed significant lethality at fipronil concentrations above 400 g L^{-1}, not observed at concentrations below 200 g L^{-1}, and teratogenic effects were observed only at concentrations above 100 g L^{-1} at 120 hpf, such as non-inflated swim bladder, reduced body length, and spinal deformities. The concentrations used in our research were lower than those used in the three studies mentioned above, possibly for this reason no significant lethality was observed since the lethality of fipronil was shown to be dose-dependent. However, we observed that the group exposed to isolated fipronil and those that contained it as a component

in the mixture showed a higher percentage of coagulation (Figure 4B) at 24 hpf and similar teratogenic effects at lower concentrations than those used in previous research.

The results obtained in the 8 h epiboly test showed that the pesticide that caused the longest delay in this process was carbendazim. The toxic effects of carbendazim tend to be more relevant in the early stages of embryonic life, where major cell division is underway, since this chemical acts by inhibiting tubulin/microtubule formation in fungus and mammals (Lim and Miller, 1997). The adverse effects of carbendazim on similar processes in zebrafish have not been previously described, but it is known that the progression of epiboly in zebrafish is driven by microtubule and actin filament forces, and the effects on the development of these structures may delay the epiboly process (Cheng *et al*., 2023; 2004), which would explain the results obtained in our research. The sensitivity of the early phases of embryonic development of zebrafish has already been demonstrated. Sarmah *et al*. (2013) showed the adverse effects of ethanol on the epiboly process in zebrafish, reporting that abnormal epiboly development is associated with microtubule formation and radial intercalation cell movement. Considering the already described mechanisms of toxic action caused by carbendazim (interference with tubulin polymerization, and with the formation of microtubules), it is possible to suggest that the here-observed delay in epiboly may have been caused by carbendazim.

From our results, we may consider the herbicide sulfentrazone as the most toxic to zebrafish embryos among all tested compounds, since it significantly reduced epiboly, while also causing the coagulation of more than 50% of the exposed embryos. Jiang *et al*. (2022) estimated the 96-h LC50 value for sulfentrazone at 2.02 mg L^{-1} for zebrafish embryos, in addition to observing a delay in absorption from the yolk sac, it affected hatching and heart rate during the zebrafish embryonic stage, while concentrations between 0.01–0.10 mg L^{-1}did not cause teratogenicity in embryonic development. In our study, it was possible to observe significant lethality at concentrations of 0.01 and 0.10 mg L^{-1}sulfentrazone, as well as showing teratogenicity in exposed embryos.

According to Bianchi *et al.* (2016), sulfentrazone exposure can lead to both chromosomal breaks and chromosomal losses, and this genotoxicity may be related to the formation of reactive oxygen species (ROS). Li *et al.* (2020) also observed that sulfentrazone induced ROS production, as well as altered the activity of antioxidant enzymes (superoxide dismutase, catalase, guaiacol peroxidase, and glutathione-S-transferase) indicating that sulfentrazone exposure, may cause oxidative stress and damage to biomolecules (such as proteins, lipids, and DNA) in earthworm (*Eisenia fetida*) cells. The study by Freitas (2017) evaluated ROS production in two tadpole species (*Eupemphix nattereri* and *Rhinella schneideri*) after exposure to sulfentrazone and also showed that exposure to this pesticide caused ROS overproduction. We speculate, therefore, that the overproduction of ROS caused by exposure to sulfentrazone may be responsible for the lethality and delay in epiboly observed in the present study. Since ROS accumulation has been found to induce DNA damage, trigger abnormalities in cell proliferation, and cause malformations and increased cell apoptosis in zebrafish embryonic development (Wei *et al.*, 2021).

Fipronil was the pesticide that caused the most coagulation in the embryos; however, the embryos that survived showed less delay in epiboly when compared to the other pesticides tested here. Fipronil is a γ-amino-butyric acid (GABA) receptor antagonist, it acts by blocking GABA receptors in the CNS, which prevents GABAergic synaptic transmission, leading to excessive neuronal stimulation resulting in death (Wu *et al.*, 2021). Blockade of these receptors by fipronil induces cell apoptosis and alters gene transcription by modifying the cell cycle (Park *et al.*, 2020); when this cell cycle dysregulation occurs, various regulators, such as cyclins, induce cell cycle arrest and senescence, causing apoptosis. This cell cycle arrest blocks differentiation in developing zebrafish, which require rapid DNA replication and mitosis for morphogenesis and partial CNS development (Verduzco and Amatruda, 2011), this arrest may

account for the high coagulation found in embryos that were exposed to fipronil in our test. The embryos that developed and showed less delay in epiboly. Overmyer *et al*. (2007), when evaluating the toxicity of fipronil, observed that *Palaemonetes pugio* larvae were generally less sensitive than adults, suggesting that the sensitivity is related to the differentiation of GABA receptors at this stage of development of the organisms, indicating that the development of the receptors progresses over time, so that fipronil binds later with the advancement of the development of these receptors, causing greater toxicity at later stages than those evaluated in this study.

We also noted that epiboly was a practical and fast parameter for the evaluation of pesticide toxicity in zebrafish embryos, but we did not observe the interactions between the pesticides used in this study. Silva *et al.* (2015) used different endpoints measured in the microcrustacean *Daphnia magna* to analyze the effects of mixtures of the pesticides triclosan and carbendazim and observed different responses regarding the toxicity of the mixture, including synergy and antagonism. The toxicity of mixtures can be expressed in different ways depending on the parameter analyzed, which is interconnected with the mode of action of each chemical but is also related to the potential mechanism and the interaction pathways among the chemicals within test organisms. Given this, we can suggest that the absence of potentiation of effects of mixtures in the endpoints used in our research does not mean that the studied mixtures do not cause synergistic effects that could be putatively observed if other endpoints were selected.

From the results obtained in the present study, we observed that all pesticides individually and in mixtures caused sublethal effects in zebrafish. This is particularly important if we assume that such effects can lead to their lethality in the short or long term. These results also demonstrate that the rapid 24 h toxicity test was sensitive to the chemicals used even at low concentrations and can be used as a warning sign or as a guide for further toxicity studies with zebrafish embryos.

5. CONCLUSION

Our proposal for a rapid toxicity test was effective in addressing the toxicity of pesticides, different chemical groups and their mixtures, proving to be as sensitive as the traditional lethality analysis. The toxicity of the pesticides found in this research registered the same in the two-time intervals, where the isolated compounds carbendazim and sulfentrazone presented greater lethality at 24 hpf, and a greater delay in epiboly at 8 hpf, respectively. They caused effects both at the limited concentrations permitted for consumption and at concentrations three times lower than this limit. In addition to lethality, these substances also cause teratogenic effects that compromise these organisms in several ways. In this way, our research provides valuable toxicological information about the effects caused in the first hours of development of the zebrafish, being able to detect the toxicity of these chemical products early to determine the potential risks of exposure to these substances, since such alterations manifest themselves before lethality.

6. ACKNOWLEDGEMENTS

The Universidade Federal Rural de Pernambuco for allowing the development of this research. We thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the productivity grant to Pabyton Cadena (302399/2023-2), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the grant to Samara Gomes (88887.528986/2020-00). Thanks also to "ECO-R-pharmplast - Ecotoxicity of realistic combinations of pharmaceutical drugs and microplastics in marine ecosystems", FCT (Fundação para a Ciência e a Tecnologia) reference POCI-01-0145-FEDER-029203, and to CESAM (Centro de Estudos do Ambiente e do Mar) for financial support

(UIDB/50017/2020+UIDP/50017/2020), by FCT/MCTES through national funds (PIDDAC), and for co-financing by FEDER, within the PT2020 Partnership Agreement and Compete for 2020.

7. REFERENCES

ANDRADE, T. S.; HENRIQUES, J. F.; ALMEIDA, A. R.; MACHADO, A. L. *et al.* Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos. **Aquatic Toxicology**, v. 170, p. 390-399, 2016

ANVISA (Brasil). **Programa de análise de resíduos de agrotóxicos em alimentos**. Brasília, 2016

BIANCHI, J.; FERNANDES, T. C.; MARIN-MORALES, M. A. Induction of mitotic and chromosomal abnormalities on Allium cepa cells by pesticides imidacloprid and sulfentrazone and the mixture of them. **Chemosphere**, v. 144, p. 475-483, 2016

BRAUNBECK, T.; KAIS, B.; LAMMER, E.; OTTE, J. *et al.* The fish embryo test (FET): origin, applications, and future. **Environmental Science and Pollution Research**, v. 22, n. 21, p. 16247-16261, 2015

BRODEUR, J. C.; POLISERPI, M. B.; D'ANDREA, M. F.; SANCHEZ, M. Synergy between glyphosate- and cypermethrin-based pesticides during acute exposures in tadpoles of the common South American toad Rhinella arenarum. **Chemosphere**, v. 112, p. 70-76, 2014

CADENA, P. G.; SALES CADENA, M. R.; SARMAH, S.; MARRS, J. A. Protective effects of quercetin, polydatin, and folic acid and their mixtures in a zebrafish (Danio rerio) fetal alcohol spectrum disorder model. **Neurotoxicology and Teratology**, v. 82, p. 106928, 2020

CHAPMAN, P. M.; WANG, F.; GERMANO, J. D.; BATLEY, G. Pore water testing and analysis: the good, the bad, and the ugly. **Marine Pollution Bulletin**, v. 44, n. 5, p. 359-366, 2002

CHENG, J. C.; MILLER, A. L.; WEBB, S. E. Organization and function of microfilaments during late epiboly in zebrafish embryos. **Developmental Dynamics**, v. 231, n. 2, p. 313-323, 2004

CHENG, J. C.; MILLER, A. L.; WEBB, S. E. Actin-mediated endocytosis in the E-YSL helps drive epiboly in zebrafish. **Zygote**, v. 31, n. 6, p. 517-526, 2023

DEMIRCI, O.; GUVEN, K.; ASMA, D.; OGUT, S. *et al.* Effects of endosulfan, thiamethoxam, and indoxacarb in combination with atrazine on multi-biomarkers in Gammarus kischineffensis. **Ecotoxicology and Environmental Safety**, v. 147, p. 749-758, 2018

DISNER, G. R.; FALCAO, M. A. P.; ANDRADE-BARROS, A. I.; LEITE DOS SANTOS, N. V. *et al.* The Toxic Effects of Glyphosate, Chlorpyrifos, Abamectin, and 2,4-D on Animal Models: A Systematic Review of Brazilian Studies. **Integrated Environmental Assessment and Management**, v. 17, n. 3, p. 507-520, 2021

EFSA. Evaluation of the FERA study on bumble bees and consideration of its potential impact on the EFSA conclusions on neonicotinoids. **EFSA Journal**, v. 11, n. 6, p. 3242, 2013

FREITAS, J. S. **Influência da temperatura na toxicidade dos herbicidas sulfentrazone (Boral 500SC®) e clomazone (Gamit®) em larvas de anuros Eupemphix nattereri (Leiuperidae) e Rhinella schneideri (Bufonidae)**. 2017. 195 f. (Ph.D.) - Programa de Pós Graduação em Biologia Animal, Universidade Estadual Paulista, São José do Rio Preto, 2017

FUKUSHIMA, H.; BAILONE, R. L.; BAUMGARTNER, I.; BORRA, R. C. *et al.* Potenciais usos do modelo animal Zebrafish Danio rerio em pesquisas na Medicina Veterinária. **Revista de Educação Continuada em Medicina Veterinária e Zootecnia do CRMV-SP**, v. 18, n. 1, 2020

GILSON, I. K.; ROCHA, L. G.; SILVA, M. R. V. d.; WAMMES, S. W. *et al.* Agrotóxicos liberados nos anos de 2019-2020: Uma discussão sobre a uso e a classificação toxicológica. **Brazilian Journal of Development**, v. 6, n. 7, p. 49468-49479, 2020

GUNGORDU, A.; UCKUN, M.; YOLOGLU, E. Integrated assessment of biochemical markers in premetamorphic tadpoles of three amphibian species exposed to glyphosate- and methidathion-based pesticides in single and combination forms. **Chemosphere**, v. 144, p. 2024-2035, 2016

JACKSON, M. C.; LOEWEN, C. J.; VINEBROOKE, R. D.; CHIMIMBA, C. T. Net effects of multiple stressors in freshwater ecosystems: a meta-analysis. **Global Change Biology**, 22, n. 1, p. 180-189, 2016

JIANG, J. et al. Health risks of sulfentrazone exposure during zebrafish embryo-larvae development at environmental concentration. **Chemosphere**, v. 288, p. 132632, 2022

KIM, C.; LEE, S. Developmental toxicity of fipronil and its two metabolites towards zebrafish (*Danio rerio*) embryos. **Environmental Pollution**, v. 333, p. 122119, 2023

KIMMEL, C. B.; BALLARD, W. W.; KIMMEL, S. R.; ULLMANN, B. *et al.* Stages of embryonic development of the zebrafish. **Developmental Dynamics**, v. 203, n. 3, p. 253-310, 1995

KRONBAUER, E. A.; BIONDO, E.; ZANETTI, C.; KOLCHINSKI, E. M. Agrotóxicos em água do rio e água tratada no Município de Encantado/RS. **Ambiente: Gestão e Desenvolvimento**, v. 14, n. 2, 2021

LAMMER, E.; CARR, G. J.; WENDLER, K.; RAWLINGS, J. M. *et al.* Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, v. 149, n. 2, p. 196-209, 2009

LI, M.; MA, X.; SALEEM, M.; WANG, X. *et al.* Biochemical response, histopathological change and DNA damage in earthworm (Eisenia fetida) exposed to sulfentrazone herbicide. **Ecological Indicators**, v. 115, p. 106465, 2020

LIM, J.; MILLER, M. G. The Role of the Benomyl Metabolite Carbendazim in Benomyl-Induced Testicular Toxicity. **Toxicology and Applied Pharmacology**, v. 142, p. 401–410, 1997

MARCHESAN, E.; SARTORI, G. M. S.; AVILA, L. A. d.; MACHADO, S. L. d. O. *et al.* Resíduos de agrotóxicos na água de rios da Depressão Central do Estado do Rio Grande do Sul, Brasil. **Ciência Rural**, v. 40, p. 1053-1059, 2010

OECD. **Education at a Glance 2013: OECD Indicators**. OECD Publishing. 2013

OVERMYER, J. P.; ROUSE, D. R.; AVANTS, J. K.; GARRISON, A. W. *et al.* Toxicity of fipronil and its enantiomers to marine and freshwater non-targets. **Journal of Environmental Science and Health, Part B**, v. 42, n. 5, p. 471-480, 2007

PALANIKUMAR, L.; KUMARAGURU, A. K.; RAMAKRITINAN, C. M.; ANAND, M. Toxicity, biochemical and clastogenic response of chlorpyrifos and carbendazim in milkfish Chanos. **International Journal of Environmental Science and Technology**, v. 11, n. 3, p. 765-774, 2013

PARK, H.; LEE, J. Y.; PARK, S.; SONG, G. *et al.* Developmental toxicity of fipronil in early development of zebrafish (*Danio rerio*) larvae: Disrupted vascular formation with angiogenic failure and inhibited neurogenesis. **Journal of Hazardous Materials**, v. 385, p. 121531, 2020

PEREIRA, L. C.; DE SOUZA, A. O.; FRANCO BERNARDES, M. F.; PAZIN, M. *et al.* A perspective on the potential risks of emerging contaminants to human and environmental health. **Environmental Science and Pollution Research**, v. 22, n. 18, p. 13800-13823, 2015

SARMAH, S.; MURALIDHARAN, P.; CURTIS, C. L.; MCCLINTICK, J. N. *et al.* Ethanol exposure disrupts extraembryonic microtubule cytoskeleton and embryonic blastomere cell adhesion, producing epiboly and gastrulation defects. **Biology Open**, v. 2, n. 10, p. 1013-1021, 2013

SILVA, A. R.; CARDOSO, D. N.; CRUZ, A.; LOURENCO, J. *et al.* Ecotoxicity and genotoxicity of a binary combination of triclosan and carbendazim to Daphnia magna. **Ecotoxicology and Environmental Safety**, v. 115, p. 279-290, 2015

SINGH, S. *et al.* Toxicity, monitoring and biodegradation of the fungicide carbendazim. **Environmental Chemistry Letters,** v. 14, n. 3, p. 317-329, 2016

SOLNICA-KREZEL, L.; SEPICH, D. S. Gastrulation: making and shaping germ layers. **Annual Review of Cell and Developmental Biology**, v. 28, p. 687-717, 2012

THORNGREN, J. L.; HARWOOD, A. D.; MURPHY, T. M.; HUFF HARTZ, K. E. *et al.* Fate and risk of atrazine and sulfentrazone to nontarget species at an agriculture site. **Environmental Toxicology and Chemistry**, v. 36, n. 5, p. 1301-1310, 2017

VERDUZCO, D.; AMATRUDA, J. F. Analysis of cell proliferation, senescence, and cell death in zebrafish embryos. **Methods in Cell Biology**, v. 101, p. 19-38, 2011

WAN, Y.; TRAN, T. M.; NGUYEN, V. T.; WANG, A. *et al.* Neonicotinoids, fipronil, chlorpyrifos, carbendazim, chlorotriazines, chlorophenoxy herbicides, bentazon, and selected pesticide transformation products in surface water and drinking water from northern Vietnam. **Science of The Total Environment**, v. 750, p. 141507, 2021

WANG, Y.; LV, L.; YU, Y.; YANG, G. *et al.* Single and joint toxic effects of five selected pesticides on the early life stages of zebrafish (Denio rerio). **Chemosphere**, v. 170, p. 61-67, 2017

WANG, Y.; WU, S.; CHEN, J.; ZHANG, C. *et al.* Single and joint toxicity assessment of four currently used pesticides to zebrafish (*Danio rerio*) using traditional and molecular endpoints. **Chemosphere**, v. 192, p. 14-23, 2018

WANG, Y.; YANG, G.; DAI, D.; XU, Z. *et al.* Individual and mixture effects of five agricultural pesticides on zebrafish (*Danio rerio*) larvae. **Environmental Science and Pollution Research**, v. 24, n. 5, p. 4528-4536, 2016

WEI, Y.; MENG, Y.; HUANG, Y.; LIU, Z. *et al.* Development toxicity and cardiotoxicity in zebrafish from exposure to iprodione. **Chemosphere**, v. 263, p. 127860, Jan 2021

WESTERFIELD, M. **The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*)**. 4th ed. Eugene, OR: University of Oregon Press, 2000

WU, C. H.; LU, C. W.; HSU, T. H.; WU, W. J. *et al.* Neurotoxicity of fipronil affects sensory and motor systems in zebrafish. **Pesticide Biochemistry and Physiology**, v. 177, p. 104896, 2021

YAN, L.; GONG, C.; ZHANG, X.; ZHANG, Q. *et al.* Perturbation of metabonome of embryo/larvae zebrafish after exposure to fipronil. **Environmental Toxicology and Pharmacology**, v. 48, p. 39-45, 2016