Journal of Hazardous Materials Advances 11 (2023) 100334

Contents lists available at ScienceDirect

Journal of Hazardous Materials Advances

journal homepage: www.elsevier.com/locate/hazadv

# Toxicity of individual and combined effect of Mefenpyr di-ethyl safener and its co-herbicide, Fenoxaprop-P-ethyl, to zebrafish

Oluwabunmi P. Femi-Oloye[a,b,*], Femi F. Oloye[a], Sana Daneshamouz[a], Paul D. Jones[a], John P. Giesy[c,d,e]

[a] Toxicology Centre, University of Saskatchewan, Saskatoon S7N 5B4, Canada
[b] Adekunle Ajasin University, Akungba- Akoko, Ondo State, PMB 001, Nigeria
[c] Department of Biomedical Sciences, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada
[d] Department of Integrative Biology and Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA
[e] Department of Environmental Science, Baylor University, One Bear Place #97266, Waco, TX 76798-7266, USA

ARTICLE INFO

*Keywords:*
Safener
Herbicide
Mefenpyr diethyl
Mortality
Reproduction
Molecular docking

ABSTRACT

Mefenpyr diethyl (MEF), a supposed inert chemical, is a safener used in herbicide formulations with active ingredients such as Fenoxaprop-P-ethyl (FEN), has been found in surface water. Since the hazards of this safener, individually and in combination with its herbicide, toward non-target aquatic organisms, were not known,acute and chronic studies were conducted on various endpoints in embryos of zebrafish (*Danio rerio*). Endpoints during acute exposures included mortality and hatchability. During chronic exposures, the growth and survival of larvae were determined. Exposure to concentrations of MEF > 3 mg/L alone significantly decreased the rate of hatching, while exposure to FEN alone to > 3 mg/L had no significant effect on the rate of hatching. However, exposure to each of the chemicals individually caused some delay in hatching. When exposed in combination with FEN, adverse effects of MEF on the rate of hatching were mitigated in a dose-dependent manner. During the acute exposure to 3 mg/L, mortality was caused by MEF than FEN. During chronic exposures to 0.1 or 1.0 mg/L, MEF has more toxic potency than FEN. Both compounds caused some abnormalities, including pericardial edema, spinal curvature, tail malformation, and edema of the yoke sac. Based on the activities of SOD and GST, both MEF and FEN caused oxidative stress. FEN reduced the toxic potency of MEF when exposed together, but there were more deformities of greater severity in embryos exposed to MEF than those exposed to FEN. Molecular docking showed that both chemicals could potentially inhibit the activity of sex hormones and hatching enzymes. These results demonstrate that while classified as "inert", the safener, and FEN can cause various effects, including molecular responses and lethality and should be monitored and regulated.

## 1. Introduction

Safeners are compounds used in combination with herbicides to protect crops from the injurious effects of herbicides without inhibiting the efficiency of the herbicides on target plants that are competing with crop plants (Femi-Oloye et al., 2023; Jia et al., 2021; Oloye et al., 2021; Woodward et al., 2018). Safeners are classified as “inert ingredients,” and their toxicities to non-target organisms have received little or no attention (Femi-Oloye et al., 2023; Oloye et al., 2021; Woodward et al., 2018). Mefenpyr diethyl (MEF) is an emerging safener that protects cereal crop plants and other grains from effects of herbicides, such as Fenoxaprop-P-ethyl (FEN) (Jia et al., 2021; Oloye et al., 2021). FEN is a member of the aryloxyphenoxypropionate herbicide family, mainly used to control annual and perennial grass in spring barley, winter rye, and winter wheat; it is also used for control of wild oat in fallow fields (Cevik and Tutar, 2008). Relatively great concentrations of FEN have been implicated in causing injuries to plants and the death of crops (Jia et al., 2021). Due to the physical and chemical properties of safeners and the nature of the soil in which crops are grown, transportation of safeners to the aquatic environment is possible. Recently, safeners have been found in surface water and could cause potential threats to aquatic organisms (Femi-Oloye et al., 2023; McFadden and Hladik, 2021; Woodward et al., 2018). Accumulated safeners and herbicides in surface water could be toxic to humans, other non-target organisms and cause ecosystem-level responses. Pathways by which herbicides and their associated safeners could get into surface waters include

* Corresponding author at: Toxicology Centre, School of Environment and Environmental Sustainability, University of Saskatchewan, Sasakatoon, SK, S7N 5B3, Canada.
*E-mail address:* femi-oloye.oluwabunmi@usask.ca (O.P. Femi-Oloye).

https://doi.org/10.1016/j.hazadv.2023.100334
Received 16 May 2023; Received in revised form 23 June 2023; Accepted 26 June 2023
2772-4166/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

runoff and erosion, sorption, leaching, and volatilization (Oloye et al., 2021). The fact that there are potential adverse effects of safeners on non-target aquatic organisms was the justification to study potential toxic effects of these compounds on various endpoints in embryos of zebrafish (*Danio rerio*). MEF has been shown to be toxic to rainbow trout (*Oncorhynchus mykiss*) with an $LC_{50}$ of 4.2 mg MEF/L. MEF also caused lethality in water fleas (*Daphnia magna*) with and range of $EC_{50}$ values of 5.6 to 52 mg/L (National Center for Biotechnology Information, 2023). Thus, there was a need to re-evaluate its toxicity by use of alternative species. Zebrafish embryos are well-studied model organisms that have been used for testing toxicities of contaminants (Chen et al., 2011).

Effects of various chemicals on zebrafish have been well documented, but no studies on toxicity of MEF or its mixture with FEN have been reported for zebrafish. Zebrafish embryos are transparent, so identification of craniofacial, cardiac, and skeletal deformities can be assessed through a microscope. The life cycle and fecundity are other reasons Zebrafish are becoming more popular (Chen et al., 2011). Morphology of zebrafish's spinal column and vertebral structure is similar to that of humans (Boswell and Ciruna, 2017). Therefore, using this organism will provide a better understanding of the toxicity of this chemical singly and in combination with Fenoxaprop-P-ethyl (FEN).

The binding of chemicals with specific receptors (targets), such as enzymes and proteins within the body, is crucial to understanding effects of chemicals on living organisms. Molecular docking is valuable for predicting these bindings and identifying the most likely molecular initiating event. Molecular docking of MEF and some selected enzymes in plants show that MEF could compete with chlorsulfuron binding to the herbicide target enzyme, resulting in more herbicide tolerance (Fu et al., 2019). Thus, the binding affinity of MEF to the protein targets compared to FEN will determine the feasibility of various molecular events".

This study assessed toxicological effects of MEF and FEN, singly and in a mixture, on zebrafish during early development. While organisms are often exposed to various chemicals, studies consider only toxic effects during exposure to individual compounds. However, mixtures of chemicals could result in various toxic effects different from effects ofsingle chemicals. In this study, zebrafish were exposed to varying concentrations of MEF and FEN for 96 h, and the effects on survival, growth, and development were evaluated. Additionally, oxidative stress, DNA damage, and biochemical changes were assessed to determine the mechanisms of toxicity. Finally, in-silico modeling was used to assess potential effects on specific molecular targets.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

The safener, Mefenpyr diethyl and herbicide, Fenoxaprop-P-ethyl, were purchased from Sigma-Aldrich (Canada). Stock solutions were prepared with 0.01% acetone. Commercial assay kits for determining enzyme activities of superoxide dismutase (SOD) and glutathione S-transferase (GST) were purchased from Sigma-Aldrich (Canada) and Fischer Scientific. All other chemicals were of analytical grade and purchased from either of the two vendors. Chemicals concentrations were quantified using LC-MS/MS (Femi-Oloye et al., 2023).

### 2.2. Zebrafish maintenance and egg collection

Zebrafish were bred at a ratio of 2:1 (female: male), with approximately 20 females and ten males per breeding tank (Hill and Janz, 2003; Thomas and Janz, 2016). The fish were maintained at the Collaborative Science Research laboratory at the University of Saskatchewan, Canada, from which eggs less than or equal to 3 hpf were collected. Adult zebrafish were fed twice daily - alternating Skretting Gemma micro 500 fish food and thawed brine shrimp for morning feed and Gemma 500 for the afternoon feed. Fish were maintained in tanks and segregated by sex on Techniplast racks, and water quality was tested weekly. The breeding tank was a 10-gallon glass aquarium 1/3 - $\frac{1}{2}$ filled and was netted to create shallow areas with plastic plants and an air stone in the tank. Males and females were added to tanks at the end of the workday before collection the next day.

#### 2.2.1. Egg collection

Eggs were collected at approximately 9:00 AM, the day after fish were introduced to the breeding tank. The breeding fish, plants, netting, and air stones were removed, water was siphoned off until the tank could be lifted, and the feces were removed. The remaining tank water and eggs were poured through a metal sieve to catch the eggs. Eggs were put in a Techniplast 1.7 L tank with approximately 1 inch of water, several drops of methylene blue were added, and additional feces were removed with a pipet. Zebrafish cultivation and egg collection were performed according to previous procedures (Li et al., 2018). Healthy fertilized embryos were transferred to 24-well plates or clean 90 mm Petri dishes for chemical exposure.

### 2.3. Embryotoxicity test

Embryo toxicity tests were performed following the Organization for Economic Co-operation and Development (OECD, 1992) 210 guidelines with some modifications to assess the effects on some endpoints, including mortality and hatching. Briefly, embryos, at 2 hpf, were exposed to six concentrations of either MEF or FEN (0, 0.1, 0.5, 1.5, 3 and 5 mg/L; 15, 20, and 50 mg/L for MEF alone) and their mixtures of equal concentrations each of MEF and FEN (0, 0.1 and 1 mg/L) until 96 hpf. Three 24-well plates were used per chemicals and each well contained a single embryo, but four wells were intentionally left empty making the total embryo per 24-well plates twenty. Thus, a total sixty eggs were used per chemicals. Concentrations of MEF and FEN were selected based on previously determined $LC_{50}$ values of MEF and FEN to zebrafish at 96 hpf and earlier acute toxicity test data (Pubchem). Rates of hatching of eggs were calculated at 48 and 72 hpf and the heartbeat rates per 30 s at 96 hpf under an inverted Zeiss microscope.

### 2.4. Chronic toxicity - developmental toxicity test

Chronic exposures were done using static-renewal methods for 21 days, following the same method as the acute tests, but were carried out in Petri-dishes. Twenty eggs were transferred to clean petri dishes containing 60 mL of the tested chemicals, and a total of three petri dishes were used per chemicals. Feeding and water change were done twice every day. Effects of the combination of FEN and MEF on developmental toxicity were evaluated according to previous methods (Blahova et al., 2020). Lengths of larvae were measured on days 4 and 21.

### 2.5. Determination of oxidative stress biomarkers

Sixty embryos were randomly selected and placed into Petri dishes for each concentration (90 mm diameter) with 40 mL of exposure solution per 20 eggs/larvae. Two-thirds of the exposure solution was renewed every 24 h, and the dead individuals were removed daily. The fish were then homogenized with 1 ml of PBS buffer (pH 7.4) per 5–10 mg of fish in a tissue homogenizer for 15 min at 20 Hz and centrifuged at 12,000 g at 4°C for 15 min. The supernatant was used for oxidative stress-related biomarkers (SOD, 450 nm and GST, 340 nm) determined by commercial assay kits.

### 2.6. Molecular docking analyses

Sex hormone receptor (6N1H) and hatching enzyme (ZHE1 (3lqb)) in zebrafish were selected as targets for molecular initiating events. Structures of these proteins were obtained from the Protein Data Bank (PDB), and the 3D ligand structures in SDF format of MEF and FEN were obtained from PubChem Database and

were energy-minimized using the ViewerLite and Chem3D Ultra. The optimized chemical structures and receptor structures were imported into Discovery Studio 2.5 (https://accelrys-discovery-studio-visualizer.software.informer.com/2.5/), by using auto dock tools, these optimized structures were further stabilized by the addition or deletion of bonds and charges and were saved in pdbqt format. A grid (20 × 20 × 20) was created for ZHE1 and 6N1H proteins for MEF and FEN binding with optimized X, Y and Z coordinates, after which docking simulations were completed using Auto dock Vina software. The program was run in command prompt for the best fitting model of ZHE1 with the two ligands, and 6N1H and the two ligands with minimal energy. Interaction energy values were utilized to determine the strength of the interaction between protein receptors and MEF and FEN, with greater absolute binding affinities corresponding to greater binding affinity. Probable 3D models were viewed using the pyMOL molecular visualization program.

### 2.7. Binding energy calculation

Molecular mechanism with the generalized Born and surface area solvation (MM/GBSA) was used to calculate the energy of optimal free receptors, free ligands, and a complex of ligands and a receptor (Genheden and Ryde, 2010). The ligands were dissolved in a solution generated automatically by the VSGB 2.0 suit, and the ligand strain energy was calculated. The OPLS3 force field, the VSGB solvent model, and prime rotomer search methods in Maestro were employed.

The relative free binding energy was calculated according to Eq. (1) below:

$$\Delta Gbind = \Delta Gcomplex - \Delta Gprotein - \Delta Gligand \quad (1)$$

### 2.8. ADMET study

The FEN and MEF were then subjected to ADMET screening with the Pro-tox II and swissADME servers to generate data on their physiochemical properties, pharmacokinetic profile, drug-likeness, and toxicity.

### 2.9. Statistical analyses

All experiments were performed in triplicate at least, and results were expressed as means ± standard deviation (SD). There were four experimental units with 20 larvae in each unit, including the control group. The normality test was checked using the Shapiro-Wilks test in Graph Pad prism, and the assumption of homogeneity of variance was evaluated using Levenes' test. For concentrations whose data were not normally distributed, the data were either transformed using the natural log (ln) of (x + 1), and then parametric statistics applied, or appropriate non-parametric statistics, such as the Kruskal Wallis test or sample t-test or Wilcoxin's test were employed. For the parametric test, the significance of the differences between the mean values was analyzed by a one-way analysis of variance (ANOVA) following Duncan's multiple range test using Graph Pad prism. A *p*-value of 0.05 or less was considered statistically significant.

## 3. Results and discussion

### 3.1. Hatching rate

No significant differences were observed in rate of hatchingbetween the control and concentration ≤ 1.5 mg MEF/L, while significant differences wereobserved at concentrations ≥ 3 mg MEF/L (Fig. 1A). Therefore, the concentration of MEF at which no observable effect was observed on rate of hatching zebrafish embryos was 1.5 mg/L. There was no significant effect on rate of hatching of zebrafish embryos exposed to concentrations ≤ 5 mg FEN/L (Fig. 1B). Mixtures of MEF and FEN did not result in any significant changes in the hatching rate compared to the control (Fig. 1C). There was also no significant difference in zebrafish embryos hatched within the third and fourth days. However, there was delayed hatching when exposed to a mixture of 1.0 mg/L each of MEF and FEN compared to the control and exposed individually to MEF and FEN (Fig. 2). No significant difference in hatching was observed when the concentration was ≤ 1.0 mg/L in the treatment group and control at $p<0.05$. The observation was similar to the effect of benaxacor on zebrafish embryo hatching, in which lesser concentrations of 0.01 mg/L delayed hatching, while greater concentrations, such as 0.1 mg/L, resulted in inhibition of hatching(Liu et al., 2021). Chemicals such as perfluorooctanesulfonate (PFOS) have also been shown to cause delayed hatching in zebrafish (Shi et al., 2008). Another study has also shown that the hatching rates of embryos exposed to nano-ZnO and $Zn^{2+}$ decreased with increasing concentrations of >1 mg/L (Bai et al., 2010). The rate of hatching was more sensitive to MEF than FEN; nevertheless, another study has shown that larvae could be more sensitive to some chemicals than the embryos and that the early life stages of zebrafish are concentration and time-dependent (Zhao et al., 2019). The lesser rate of hatched eggs exposed to chemicals has been associated with a disturbance of the hatching enzymes and hypoxia (Bai et al., 2010). The chorion, which is the first barrier, has numerous pore canals of approximately 0.5 −0.7 μm in diameter providing a possible route for dissolved MEF and FEN to enter the egg through the pores (Bai et al., 2010). Thus, MEF (log Kow 3.9), being more soluble than FEN (log $K_{ow}$= 4.1) (PubChem), could penetrate inside the pore faster than FEN. The inability of FEN to circulate well in the system might inhibit its ability to get to the target enzyme even when it is more lipophilic. Hence, the combination of solubility and lipophilicity favors MEF over FEN. Nevertheless, according to Lipinski and Veber's rules, none of the two chemicals violated the rule and thus are bioavailable to their targets since they both have a positive bioavailability score of 0.55. This indicates that MEF and FEN are bioavailable in the living cell and thus could cause toxic stress.

**Table 1**
Lethal toxicity of Mefenpyr-diethyl and Fenoxaprop-P-ethyl.

| | MEF | | FEN | |
|---|---|---|---|---|
| | LC50 | CI | LC50 | CI |
| 24 h | 7.12 | 4.45–11.36 | 13.05 | 5.06–96.43 |
| 48 h | 3.25 | 1.94–5.37 | 11.28 | 3.45–59.73 |
| 72 h | 2.90 | 1.80–4.60 | 10.26 | 3.55- 77.91 |
| 96 h | 2.93 | 1.78–4.72 | 10.85 | 3.86- 84.09 |

### 3.2. Survival rate and morphological changes

Exposure to MEF or FEN caused lethality of zebrafish embryos at concentrations ≥ 0.1 mg/L in the 96 h study. Exposure to greater concentrations, > 3 mg MEF/L resulted in 100% mortality during the yolk sac stage (Fig. 3A), while there was 100% survival of embryos exposed to concentrations > 3 mg FEN/L (Fig. 3B). The mixture studies showed significant differences from the controls at lesser concentrations (0.1 mg/L), while no significant difference at 1.0 mg/L. This result suggests that the mixture results in lesser toxicity, which confers some form of protection up to 8.7% to zebrafish embryos (Fig. 3C). Since MEF was more potent than FEN, this result can be interpreted as FEN antagonizing the effects of MEF.

The $LC_{50}$ of MEF to Rainbow trout was reported to be 4.2 mg/L after 96 hpf (PubChem), which was greater than the value of 2.93 mg/L observed for zebrafish after 96 hpf in this study (Table 1). For FEN, the $LC_{50}$ for rainbow trout was 0.46 mg/L (PubChem), while the observed $LC_{50}$ for zebrafish was 10.85 mg/L after 96 hpf in the current study. The $LC_{50}$ for MEF falls within the range observed for R, S and Rac-benaxacor after 96 hpf (0.60–4.48 mg/L) (Liu et al., 2021); Thus, the toxic potency

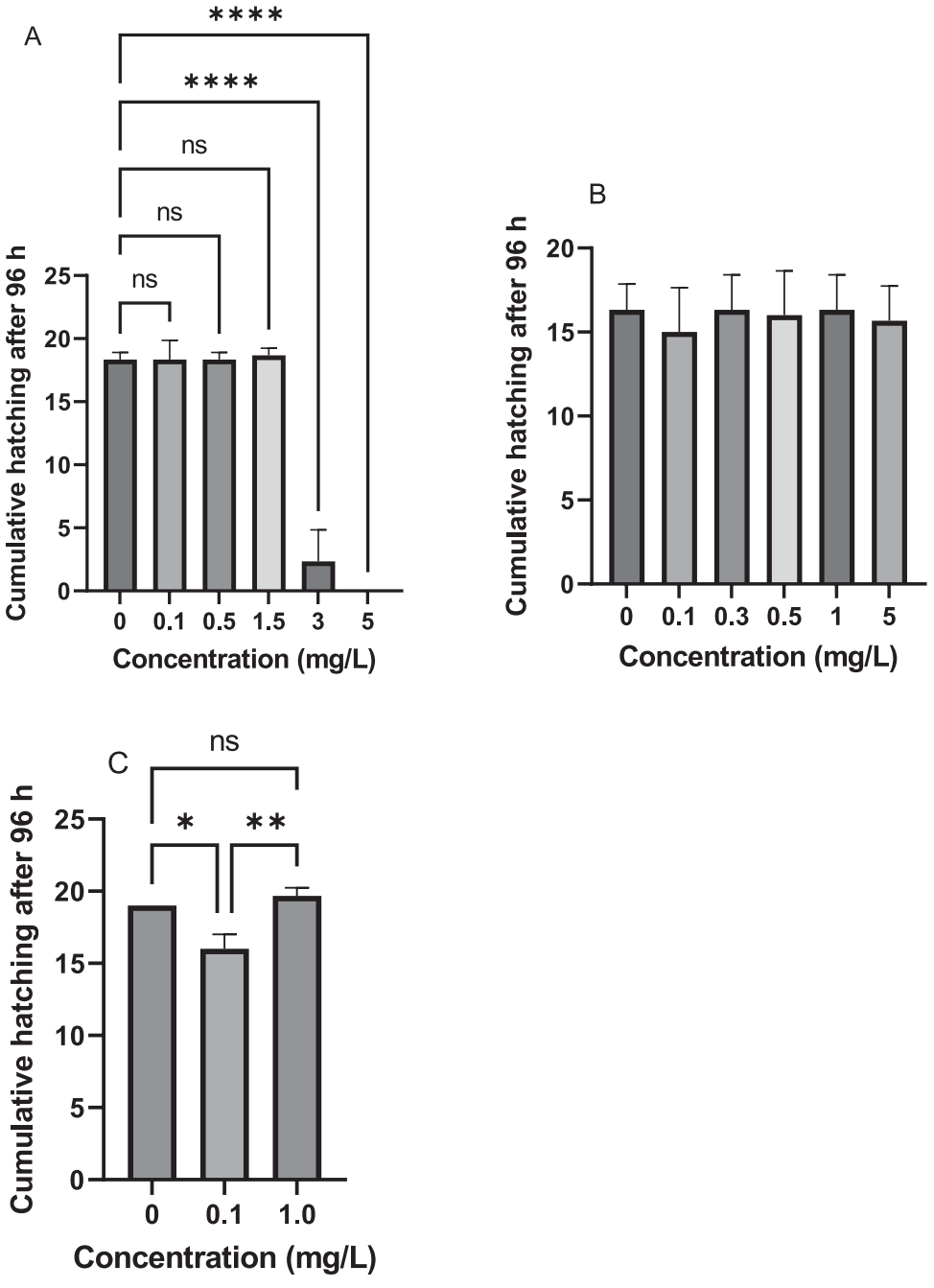



**Fig 1.** Average number of hatched Zebrafish larvae after 96 hpf exposed to a varied concentration of (A) MEF, (B) FEN, (C) MEF and FEN. *P<0.01, **P<0.005, ****P<0.0001.

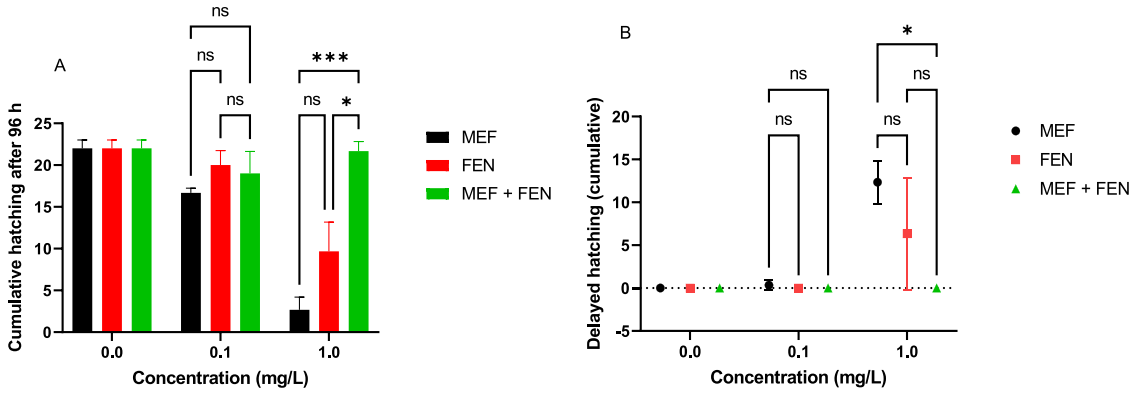



**Fig 2.** Average number of hatched Zebrafish larvae (A) during the normal hour after 96 h and (B) delayed period after 120 h for MEF, FEN and their mixture at different concentrations. *P<0.05, ***P<0.001.

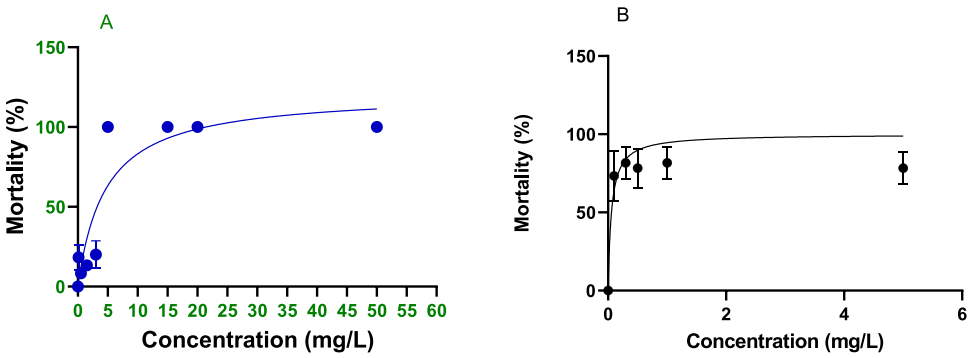
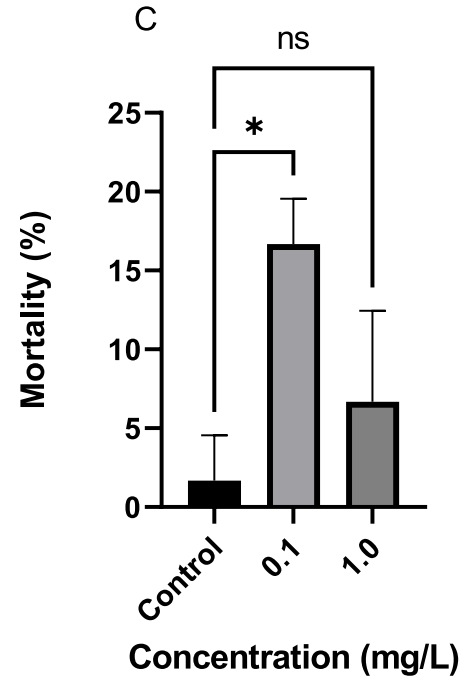



**Fig 3.** Mortality rate in zebrafish larvae exposed to (A) MEF, (B) FEN and (C) MEF+FEN after 96 hpf. *P<0.05.



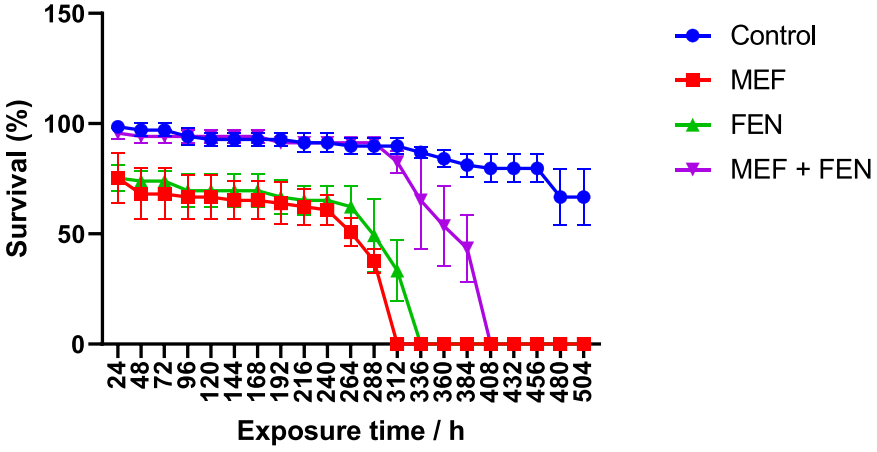




**Fig. 4.** Survival of Zebrafish exposed to MEF, FEN, mixtures of MEF and FEN for 21 days. (The plot shows how long it takes from 100% survival to 0% survival).

of MEF is similar to that of benaxacor. A report showed that < 10 mg FEN/L did not significantly alter centric diatom and chlorophyta levels after 13 days of exposure (Cevik and Tutar, 2008).

Toxicity curves for MEF and FEN (Figs. 4 and A.1) had $LC_{50}$ that were smaller at greater durations, which confirmed that toxicity to zebrafish is dependent on both duration and magnitude of exposure. The twenty-one-day chronic test results showed that no organism survived toxic stress after 312, 336 and 408 hpf when exposed to MEF, FEN individually or as a mixture, respectively. This observation implies that MEF has greater toxic potency than FEN, and since it took longer for mortality to be 100%, the toxic potency of MEF was less when embryos were exposed to a combination of FEN and MEF. This observation sug-

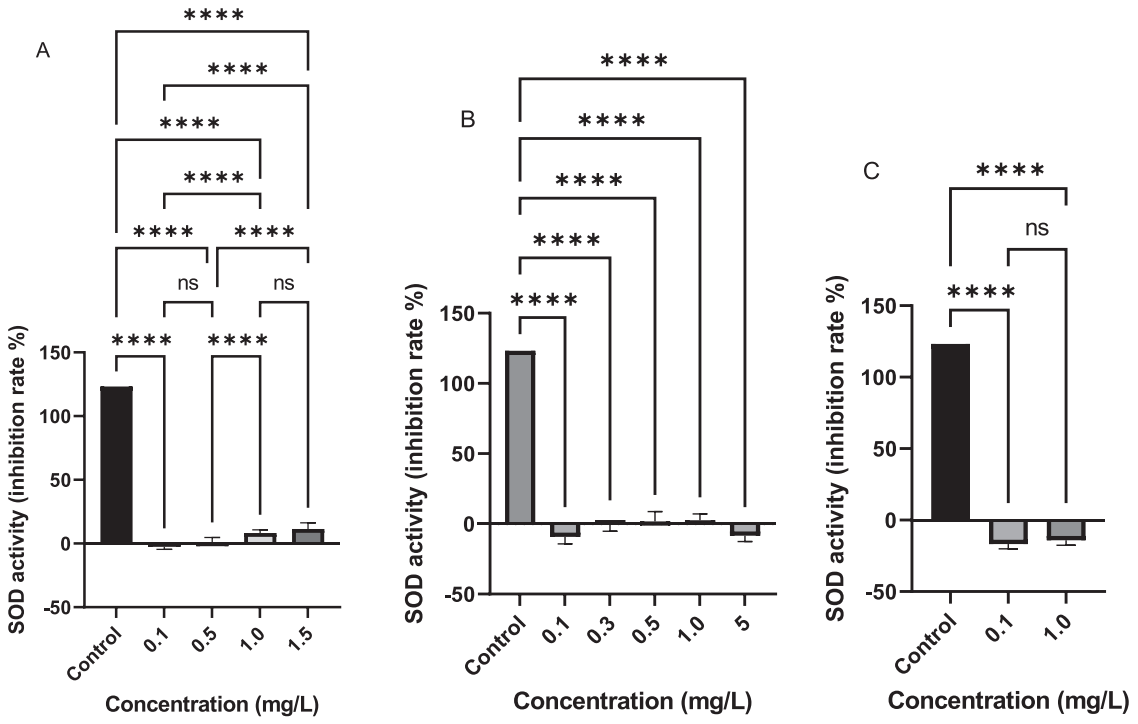



**Fig. 5.** SOD activities of Zebrafish exposed to (A) MEF (B) FEN (C) MEF + FEN. ****P<0.0001.

gests that a mixture of the two chemicals was infra-additive, and less toxicity would be expected at equivalent concentrations of MEF alone. No significant difference was observed at 0.1 mg/L for MEF, while this concentration induced toxicity in FEN.

Several deformities, including pericardial edema and spinal malformation, were observed in zebrafish larvae (Figs. A.2–A.4). The common sublethal effects observed in embryos exposed to MEF or FEN were pericardial edema, spinal curvature, tail malformation and yolk sac edema. Percentages of deformed embryos were dose-dependent at the greater concentrations of both MEF and FEN. Generally, sublethal endpoints, such as edema, malformations, and non-hatched eggs characterized the organisms exposed in the chronic study. Hence, MEF could be toxic to zebrafish larvae at environmentally relevant concentrations. These results are consistent with findings that MEF is moderately persistent in aquatic systems, and its ability to persist in the living cell could be the reason for the different degrees of morphological deformities observed (Lewis et al., 2016).

Despite the abnormalities observed, no significant differences in heart rate (Fig. A.5) and length (Fig. A.6) of organisms exposed to MEF or FEN compared to the unexposed controls. The lack of difference compared to the control group could be because the organisms tested for heart rate and length were phenotypically similar to the control group as they look healthy. Similar findings have been reported with no significant effect on the overall fitness of zebrafish in the different treatment groups, even when there were different degrees of damage in the internal organs (Yang et al., 2016).

### 3.3. Reactive oxygen species (ROS)/oxidative stress

Oxidative stress could induce cell damage and lead to cell apoptosis, immunotoxicity, neurodegeneration and liver damage. The generation of hydroxyl radicals has been implicated as a major reason why chemicals cause oxidative stress and the binding of chemicals in tissues to cytochromes and uncoupling of the electron transport chain from monooxygenase activity (Wu et al., 2011). SOD and GST are ideal indicators of oxidative stress. MEF, FEN and Mixture of MEF and FEN had a significant effect on SOD activities by reducing it in zebrafish at all concentrations compared to the control group (Fig. 5). SODs are metalloenzymes that catalyze the dismutation of superoxide radical ($O^{2-}$) into hydrogen peroxide (Pamanji et al., 2016). Inhibition of SOD activity might be due to severe oxidative stress, which leads to a decreased SOD activity in zebrafish embryos or due to the saturation of SOD during the process of converting $O^{2-}$ to hydrogen peroxide. A reduction in SOD indicates the production of excess ROS and intense SOD reutilization. MEF had a significant effect on zebrafish by altering the GST activities across concentrations when compared to the control groups (Fig. 6). Only 0.5 mg FEN/L significantly decreased GST; other FEN concentrations did not significantly affect GST activity, while the mixture of MEF and FEN significantly affect the zebrafish by causing a reduction in the activity of GST at both concentrations compared to control group (Fig. 6).GST contributes to cellular protection against oxidative damage and detoxification of many xenobiotics. It converts xenobiotics to nontoxic metabolites by conjugation with glutathione (Kavitha and Rao, 2009). Therefore, an increase or decrease in GST might indicate oxidative stress. Therefore, the difference between GST in the control and treatment groups indicates oxidative stress caused by the chemical. Under normal physiological conditions, there is a balance between the production of ROS and the activity of a family of antioxidant enzymes, so when the generation of ROS exceeds beyond the protective capacity of these enzymes, oxidative damage occurs (Pamanji et al., 2016). Oxidative stress refers to the excessive accumulation of ROS under the harmful stimulation of the internal or/and external environment, resulting in an imbalance between the oxidation and antioxidant systems (Cao et al., 2020). These observations were similar to those obtained with exposure to benaxacor in zebrafish, which induced oxidative stress, pericardial edema and decreased embryo hatching rates (Zhang et al., 2021).

### 3.4. Molecular docking

Sex hormones receptor and hatching enzymes are important factors that determine reproductive success, and any chemicals that bind strongly with proteins acting as hormone receptors or enzymes have potential to affect reproduction. Therefore, the Sex hormone recep-

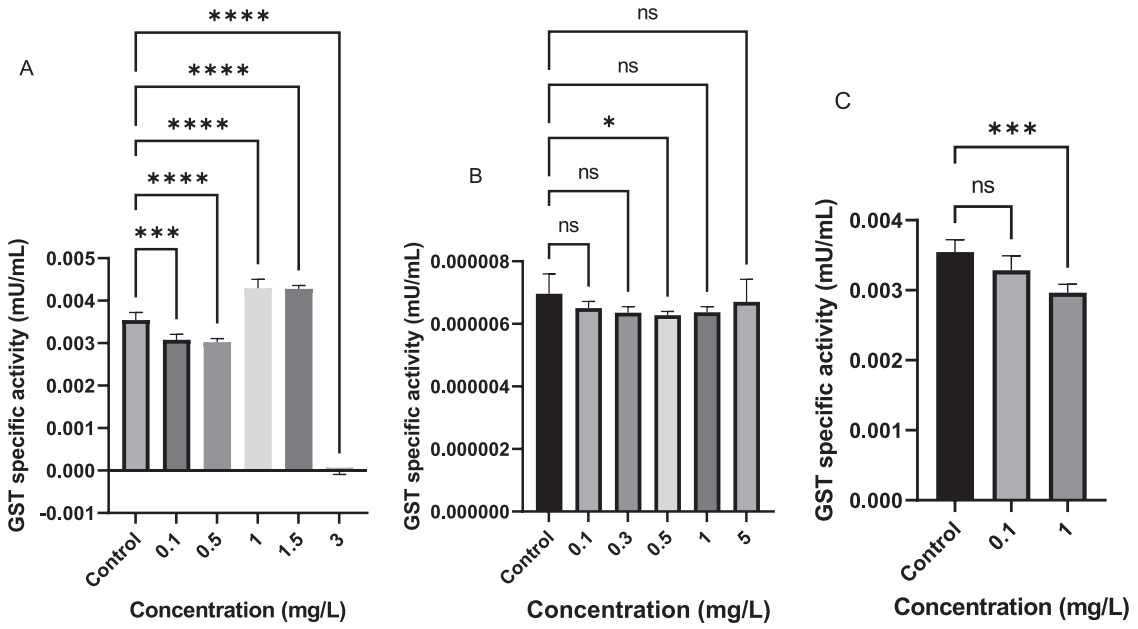



**Fig. 6.** GST activities of Zebrafish exposed to (A) MEF (B) FEN (C) MEF + FEN. ****P<0.0001, *P<0.05, ***P=0.0001.

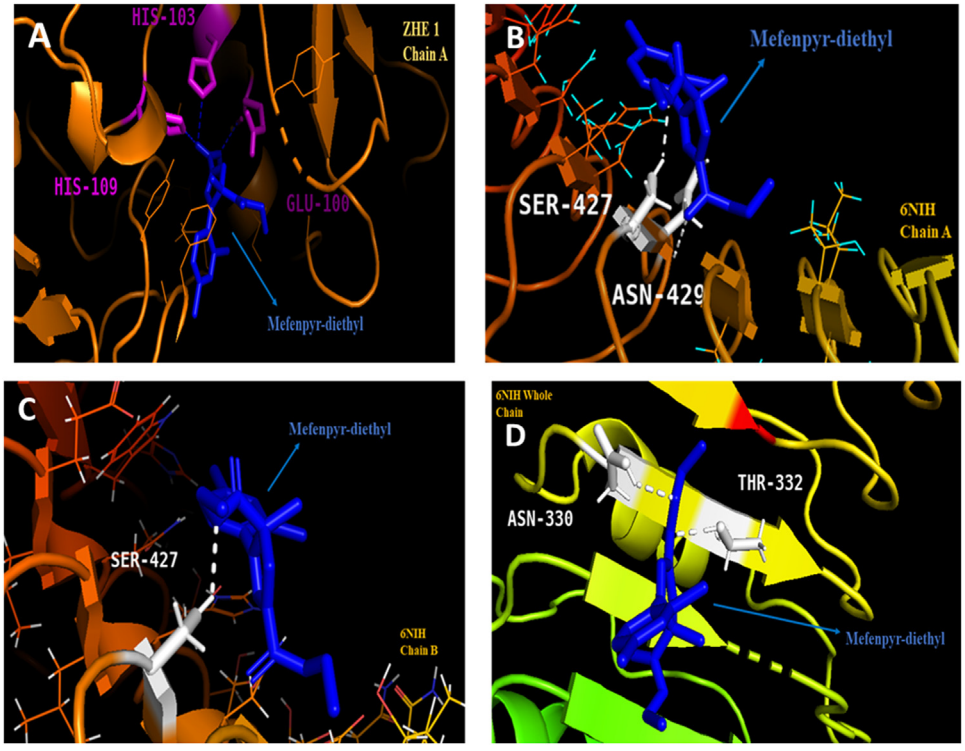



**Fig. 7.** Molecular interaction between Mefenpyr-diethyl with different protein receptors (A) ZHE1 (3lqb), (B) 6N1H chain A (C) 6N1H chain B (D) 6N1H whole chain.

tor (6N1H) (Gu et al., 2021) and hatching enzyme (ZHE1 (3lqb) (Okada et al., 2010) in zebrafish, which has been well characterized was docked with MEF (Fig. 7) and FEN (Fig. 8). ZHE2 is another hatching enzyme, but was not considered because it was rarely expressed (Sano et al., 2008). Inhibition of protein was based on significant interactions between the ligands and the combination of several amino residues found at the protein active site (Figs. 7, 8, A.7, A.8, Tables 2, and A.1). Similarly, a receptor-ligand complex binding energy was calculated using the prime molecular mechanism with the generalized Born and surface area solvation (MM/GBSA) method, which calculates the free binding energies (ΔG) of protein-ligand complexes more accurately (Genheden and Ryde, 2015). It is one of the techniques that help to improve the virtual screening of toxicity results. The ΔG of MEF and FEN with 6N1H is −23.181 and −24.655, respectively, which showed that FEN is slightly more stable in the binding cavity than MEF. Both FEN and MEF were able to affect the hatching rate because the six ZHE1-cleaving sites are in N-terminal regions of the egg envelope subunit proteins, ZP2 and ZP3, but not in the internal regions, such as the ZP domains (Sano et al., 2008), thus allowing for easy interaction with chemicals. Chemicals could interfere with embryo hatching by a chelator-sensitive mechanism that involves ligation of critical histidines in the ZHE1 center (Lin et al., 2013). Histidine has been

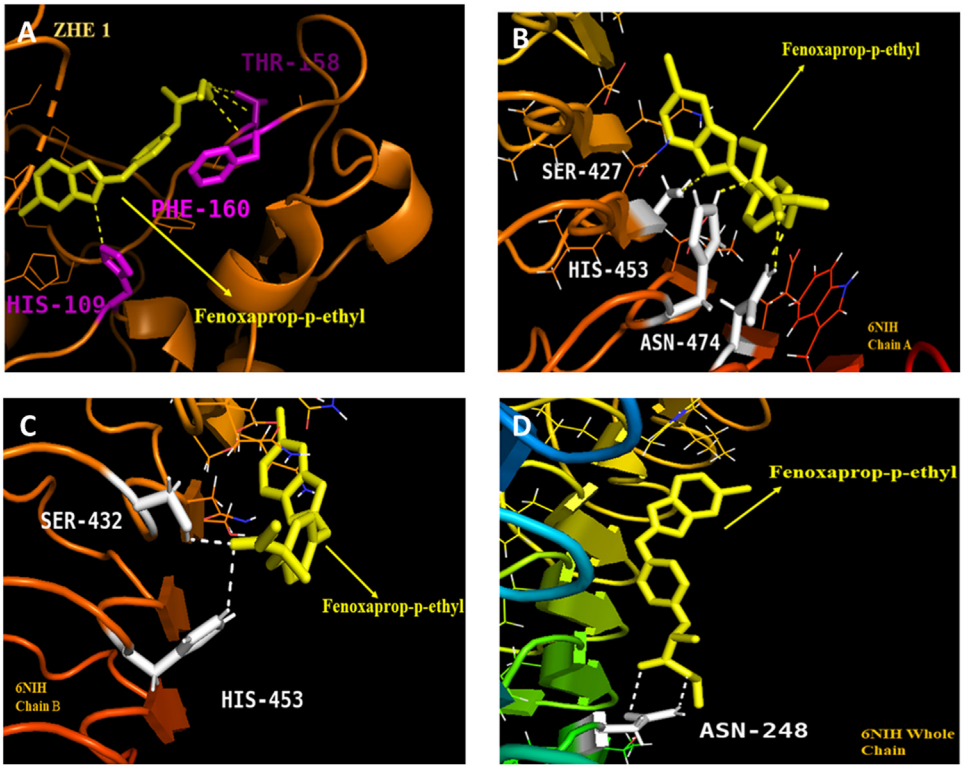



**Fig. 8.** Molecular interaction between Fenoxaprop-P-ethyl with different protein receptors (A) ZHE1 (3lqb), (B) 6N1H chain A (C) 6N1H chain B (D) 6N1H whole chain.

**Table 2**
Binding properties of MEF and FEN with ZHE1 and 6N1H.

| Chemical | Protein | Protein function | Chain | Binding energy | Residues |
|---|---|---|---|---|---|
| Mefenpyr-diethyl | ZHE1 (3lqb) | Hatching enzyme | Whole | −6.2 | His 109, His103, Glu 100 |
| | 6N1H | Sex hormone | A | −5.5 | Asn 429, Ser 427 |
| | | | B | −5.6 | Ser 427 |
| | | | Whole | −6.2 | Thr 332, Asn 330 |
| Fenoxaprop-P-ethyl | ZHE1 (3lqb) | Hatching enzyme | Whole | −6.9 | Phe 160, Thr 158, His 109 |
| | 6N1H | Sex hormone | A | −5.7 | Ser 427, Asn 474, His 453 |
| | | | B | −5.9 | Ser 432, His 453 |
| | | | Whole Chain | −7.1 | Asn 248 |

shown to have the ability to undergo various types of molecular interactions, such as cation $-\pi$ interactions, $\pi$-$\pi$ stacking interactions, hydrogen-$\pi$ interactions, coordinate bond interactions and hydrogen bond interactions (Liao et al., 2013). Its ability for multiple interactions would aid its binding with both MEF and FEN. The residues involved in the interaction between MEF and ZHE1 are two histidine residues (His109, His 103) and one glutamic acid residue (Glu100), while that of FEN and ZHE1 are Phenylalanine (Phe160), threonine (Thr158), His109 (Table 2). This confirmed ligation of histidine. These amino acids are essential because they cannot be sufficiently synthesized during certain physiological periods of growth or recovery from stress (De Koning, 2013). The distal histidine, His109 works as a proton donor to one oxygen atom and proton acceptor from the second oxygen atom of peroxide, which might result in the polarization of O-O bond, causing nucleophilic attack at the heme moiety leading to heterocyclic cleavage (Singh et al., 2021). The residues found in this study are critical to the activity of this chemical modification, which could cause mutagenesis (Kurihara et al., 1996). ROS may be generated because of the metabolism of MEF and FEN by cytochrome P450s, monooxygenases that catalyze oxidation by adding one atom from molecular oxygen into the substrate by an electron transport pathway (Pamanji et al., 2016).

Delayed hatching has been explained previously using molecular docking of Profenofos with ZHE1 as being due to the conjugation of toxicant and hatching enzyme (Pamanji et al., 2016). It was shown that the chemical binds to three amino acids, His 99, His 109 and Arg182, through hydrogen bonding, thus inhibiting hatching of zebrafish eggs. We, therefore, suggest that the binding of MEF and FEN to His 109 is likely responsible for the delayed hatching observed in this study (Fig. 2). An additional histidine (His 103) in MEF might be responsible for unsuccessful hatching at concentrations > 3 mg/L in MEF. In other studies, chemical exposures have been implicated in unsuccessful hatching (Pamanji et al., 2016).

The residues for the interaction of FEN (Asn 248) and MEF (Thr 332 and Asn 330) with 6N1H showed that MEF binds with these substrates, while FEN binds only with one when considering the whole structure. However, the binding energy of FEN with 6N1H is less than that of MEF, which suggests that FEN is more potent at interfering with sex hormone receptors than MEF. This observation is the same as that observed with ZHE1. However, as much as binding energy is important to determine the binding affinity, which translates to toxicity since binding to a site

**Table 3**
ADME property prediction.

| Compounds | GI absorption | BBB permeant | Pgp substrate | CYP1A 2 inhibitor | CYP2C19 inhibitor | CYP2C9 inhibitor | CYP2D6 inhibitor | CYP3A4 inhibitor |
|---|---|---|---|---|---|---|---|---|
| Mefenpyr-diethyl | High | Yes | - | + | + | + | | + |
| Fenoxapro-p-ethyl | High | Yes | - | + | + | + | - | - |

+active -inactive.

will prevent its functionality, the binding location also matters. Thus, MEF binding with both Thr 332 and Asn 330 will inhibit activities of the site, while FEN will only inhibit functionality of Asn 248. Threonine (Thr 332) is one of the essential amino acids, which the body cannot easily synthesize, so its blockage by chemicals will cause significant toxic effects. In contrast, Asparagine is a dispensable amino acid and can easily be synthesized from essential amino acids by the body. Therefore, MEF and FEN will have a severe toxic effect on zebrafish reproduction since they both bind to sex hormone receptors, but MEF might have more effect since it affects essential amino acids.

MEF and FEN behave similarly since they cross the blood-brain barrier (BBB), but were not predicted to be P-glycoprotein (P-gp) substrates (Table 3). The ability to cross BBB showed that they have the potential to cause toxic effects in humans and other vertebrates if accumulated. They are also inhibitors of CYP1A2, CYP2C19 AND CYP2C9. However, MEF could also inhibit CYP3A4. This additional enzyme accounts for 40 to 45% of all phase 1 metabolism and up to 70% of gastrointestinal CYP activity. Generally, CYP enzymes are relevant because they are responsible for phase 1-dependent metabolism of many endogenous chemicals (Thelen and Dressman, 2009).

## 4. Conclusions

Mefenpyr-diethyl (MEF) and Fenoxaprop-P-ethyl (FEN) are chemicals commonly formulated and applied for weed control. While FEN is the active herbicidal ingredient, MEF prevents the negative effect of FEN on crops. FEN was found to have slightly less toxic potency than MEF in the early life stages of zebrafish in both acute and chronic studies. In both MEF and FEN treatment groups, hatching was delayed; however, hatching rates were significantly reduced for MEF-treated groups at concentrations ≥ 3 mg/L, while no significant differences were observed for that concentration of FEN after 96 hpf. The combination of FEN and MEF also resulted in a significant difference at 0.1 mg/L, but changed when the concentration increased because of the contribution of FEN. Mortality followed the same pattern as hatching, with MEF being more potent, and the $LC_{50}$ of MEF and FEN were 2.93 and 10.85 mg/L, respectively. The risk of MEF to zebrafish larvae was deemed to be moderate to greaat, and its efficacy greater than FEN. Results of the chronic toxicity test confirmed the observation of the acute study, with the time to death of all organisms in the MEF-treated group at 312 h, the FEN-treated group at 336 h and the mixture at 408 h. Exposure to these chemicals resulted in malformations in embryos and larvae, singly and as a mixture. Some examples of malformations are pericardial edema, spinal curvature, tail malformation and yolk sac edema. SOD and GST showed that oxidative stress occurred in the organisms due to the chemicals, and molecular docking showed that the chemicals bind with both the hatching enzyme and sex hormone. FEN has a higher binding affinity to both hatching enzyme and sex hormone, but MEF has more residues, therefore, binds with more amino acids than FEN. Conclusively, the alleged not-so-toxic safener, MEF, caused some toxicities and lethality, and its use should be monitored and regulated.

## Funding

The research was supported by a Discovery Grant from the Natural Science and Engineering Research Council of Canada (Project # 326415-07) and a project titled “Next generation solutions to ensure healthy water resources for future generations” funded by the Global Water Futures program, Canada First Research Excellence Fund. Additional information is available at www.globalwaterfutures.ca. Prof. Giesy was supported by the Canada Research Chair program, a Distinguished Visiting Professorship in the Department of Environmental Sciences, Baylor University in Waco, TX, USA. This project was also supported by Toxicology Devolved Scholarship, University of Saskatchewan, Canada and Tertiary Education Trust Fund (TETFUND) Nigeria.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This work was approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

## Data availability

Data will be made available on request.

## Acknowledgment

We appreciate the efforts of our student assistants and laboratory technicians who helped make this project work. We also acknowledge the support of the George and Arlene Loewen, and Ivan and Margaret Toutloff Families.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2023.100334.

## References

Bai, W., Zhang, Z., Tian, W., He, X., Ma, Y., Zhao, Y., Chai, Z., 2010. Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mechanism. J. Nanoparticle Res. 12, 1645–1654.
Boswell, C.W., Ciruna, B., 2017. Understanding idiopathic scoliosis: a new zebrafish school of thought. Trends Genet. 33 (3), 183–196.
Blahova, J., Cocilovo, C., Plhalova, L., Svobodova, Z., Faggio, C., 2020. Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (Danio rerio). Environ. Toxicol. Pharmacol. 77, 103370.
Cao, Z., Huang, Y., Xiao, J., Cao, H., Peng, Y., Chen ... &, Z., Lu, H., 2020. Exposure to diclofop-methyl induces cardiac developmental toxicity in zebrafish embryos. Environ. Pollut. 259, 113926.
Cevik, F., Tutar, M., 2008. Effect of Fenoxaprop-P-ethyl on natural plankton of the Seyhan dam: a microcosm study. Bull. Environ. Contam. Toxicol. 80, 247–250.
Chen, T.H., Wang, Y.H., Wu, Y.H., 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat. Toxicol. 102 (3–4), 162–166.
De Koning, T.J., 2013. Amino acid synthesis deficiencies. Handb. Clin. Neurol. 113, 1775–1783.
Femi-Oloye, O., Oloye, F., Jones, P., Giesy, J., 2023. Sorption behaviour and toxicity of an herbicide safener “cyprosulfamide. Sci. Total Environ. 859, 160077.
Fu, Y., Zhang, D., Kang, T., Guo, Y.Y., Chen, W.G., Gao, S., Ye, F., 2019. Fragment splicing-based design, synthesis and safener activity of novel substituted phenyl oxazole derivatives. Bioorg. Med. Chem. Lett. 29 (4), 570–576.
Genheden, S., Ryde, U., 2010. How to obtain statistically converged MM/GBSA results. J. Comput. Chem. 31 (4), 837–846.
Genheden, S., Ryde, U., 2015. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. Expert Opin. Drug Discov. 10 (5), 449–461.
Gu, W., Li, X., Du, M., Ren, Z., Li, Q., Li, Y., 2021. Identification and regulation of ecotoxicity of polychlorinated naphthalenes to aquatic food Chain (green algae-Daphnia magna-fish). Aquat. Toxicol. 233, 105774.

Hill Jr, R.L., Janz, D.M, 2003. Developmental estrogenic exposure in zebrafish (Danio rerio): I. Effects on sex ratio and breeding success. Aquat. Toxicol. 63 (4), 417–429.
Jia, L., Gao, S., Zhang, Y.Y., Zhao, L.X., Fu, Y., Ye, F., 2021. Fragmenlt recombination design, synthesis, and safener activity of novel ester-substituted pyrazole derivatives. J. Agric. Food Chem. 69 (30), 8366–8379.
Kavitha, P., Rao, J.V., 2009. Sub-lethal effects of profenofos on tissue-specific antioxidative responses in a Euryhyaline fish, Oreochromis mossambicus. Ecotoxicol. Environ. Saf. 72 (6), 1727–1733.
Kurihara, H., Nonaka, T., Mitsui, Y., Ohgi, K., Irie, M., Nakamura, K.T., 1996. The crystal structure of ribonuclease Rh from Rhizopus niveus at 2.0Å resolution. J. Mol. Biol. 255 (2), 310–320.
Lewis, K.A., Tzilivakis, J., Warner, D.J., Green, A., 2016. An international database for pesticide risk assessments and management. Hum. Ecol. Risk Assess. Int. J. 22 (4), 1050–1064.
Li, X., Zhou, S., Qian, Y., Xu, Z., Yu, Y., Xu ... &, Y., Zhang, Y., 2018. The assessment of the eco-toxicological effect of gabapentin on early development of zebrafish and its antioxidant system. RSC Adv. 8 (40), 22777–22784.
Liao, S.M., Du, Q.S., Meng, J.Z., Pang, Z.W., Huang, R.B., 2013. The multiple roles of histidine in protein interactions. Chem. Cent. J. 7 (1), 1–12.
Lin, S., Zhao, Y., Ji, Z., Ear, J., Chang, C.H., Zhang ... &, H., Nel, A.E., 2013. Zebrafish high-throughput screening to study the impact of dissolvable metal oxide nanoparticles on the hatching enzyme, ZHE1. Small 9 (9–10), 1776–1785.
Liu, S., Deng, X., Bai, L., 2021. Developmental toxicity and transcriptome analysis of zebrafish (Danio rerio) embryos following exposure to chiral herbicide safener benoxacor. Sci. Total Environ. 761, 143273.
McFadden, M.E., Hladik, M.L., 2021. Cyprosulfamide: analysis of the herbicide safener and two of its degradates in surface water and groundwater from the midwestern United States. ACS Agric. Sci. Technol. 1 (4), 355–361.
National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 10937610, Mefenpyr-diethyl. Retrieved February 2, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Mefenpyr-diethyl.
OECD, 1992. Test No 210: fish, early-life stage toxicity test. OECD Guidelines for the Testing of Chemicals, Section 2.
Okada, A., Sano, K., Nagata, K., Yasumasu, S., Ohtsuka, J., Yamamura ... &, A., Tanokura, M., 2010. Crystal structure of zebrafish hatching enzyme 1 from the zebrafish Danio rerio. J. Mol. Biol. 402 (5), 865–878.
Oloye, F.F., Femi-Oloye, O.P., Challis, J.K., Jones, P.D., Giesy, J.P., 2021. Dissipation, fate, and toxicity of crop protection chemical safeners in aquatic environments. Rev. Environ. Contam. Toxicol. 258, 27–53 Volume.
Pamanji, R., Yashwanth, B., Rao, J.V., 2016. Profenofos induced biochemical alterations and in silico modelling of hatching enzyme, ZHE1 in zebrafish (Danio rerio) embryos. Environ. Toxicol. Pharmacol. 45, 123–131.
Sano, K., Inohaya, K., Kawaguchi, M., Yoshizaki, N., Iuchi, I., Yasumasu, S., 2008. Purification and characterization of zebrafish hatching enzyme–an evolutionary aspect of the mechanism of egg envelope digestion. FEBS J. 275 (23), 5934–5946.
Shi, X., Du, Y., Lam, P.K., Wu, R.S., Zhou, B., 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicol. Appl. Pharmacol. 230 (1), 23–32.
Singh, P.K., Sharma, P., Bhushan, A., Kaur, P., Sharma, S., Singh, T.P., 2021. Structure of a ternary complex of lactoperoxidase with iodide and hydrogen peroxide at 1.77Å resolution. J. Inorg. Biochem. 220, 111461.
Thelen, K., Dressman, J.B., 2009. Cytochrome P450-mediated metabolism in the human gut wall. J. Pharm. Pharmacol. 61 (5), 541–558.
Thomas, J., Janz, D., 2016. Embryo microinjection of selenomethionine reduces hatchability and modifies oxidant responsive gene expression in zebrafish. Sci. Rep. 6, 26520.
Woodward, E.E., Hladik, M.L., Kolpin, D.W., 2018. Occurrence of dichloroacetamide herbicide safeners and co-applied herbicides in midwestern US streams. Environ. Sci. Technol. Lett. 5 (1), 3–8.
Wu, M., Xu, H., Shen, Y., Qiu, W., Yang, M., 2011. Oxidative stress in zebrafish embryos induced by short-term exposure to bisphenol A, nonylphenol, and their mixture. Environ. Toxicol. Chem. 30 (10), 2335–2341.
Yang, X., Liu, Y., Li, J., Chen, M., Peng, D., Liang ... &, Y., Jiang, G., 2016. Exposure to Bisphenol AF disrupts sex hormone levels and vitellogenin expression in zebrafish. Environ. Toxicol. 31 (3), 285–294.
Zhang, W., Yan, J., Huang, Y., Wang, Z., Cheng, B., Ma ... &, J., Lu, H., 2021. Benoxacor caused developmental and cardiac toxicity in zebrafish larvae. Ecotoxicol. Environ. Saf. 224, 112696.
Zhao, F., Li, H., Cao, F., Chen, X., Liang, Y., Qiu, L., 2019. Short-term developmental toxicity and potential mechanisms of the herbicide metamifop to zebrafish (Danio rerio) embryos. Chemosphere 236, 124590.