# Individual and Combined Effects of Selected Emerging Safeners: Mefenpyr di-ethyl and Cyprosulfamide and their Co-Herbicides, Fenoxaprop-P-ethyl and Isoxaflutole on *Daphnia magna* and *Danio rerio*

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By

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**Abstract**

Herbicides and safeners have been formulated together to help protect crop plants from the injurious effects of herbicides while maintaining the ability of the herbicides to selectively remove targeted weeds. These groups of compounds became important as agriculture increased to sustain the world's ever-increasing population. Selected emerging safeners (SESs), Mefenpyr di-ethyl (MEF) and Cyprosulfamide (CPS) and their co-herbicides (Fenoxaprop-p-ethyl (FEN) and Isoxaflutole (ISO)) used during pre- and post-emergence of cereals and grains were used for this study. The mobility of safeners varied in the environment in relation to their chemical properties, such as octanol-water solubility partition coefficient. Herbicides and safeners have been found in the aquatic environment because they could dissipate from the point of application through leaching, surface runoff, and volatilization. The sorption of safeners such as CPS to soil was found to be governed by soil pH. Hence, their ability to leach varies for soils depending on their acidity/alkalinity. Safeners are classified as inert for regulatory purposes because they mostly act by upregulating detoxifying enzymes. Despite their presence in surface water and their mobility, there is limited or no data regarding their toxicity to non-target organisms in aquatic environments. Thus, the study focused on evaluating and assessing the toxicity of the SESs on non-target organisms such as *Daphnia magna*-a cladoceran invertebrate, and Zebrafish (*D. rerio*)- an aquatic vertebrate animal model.

Specifically, the primary objectives of this study were to 1) evaluate and further expand our knowledge on the toxicity of selected safeners for which little toxicological knowledge regarding non-target species like daphnids and fish exist; 2) to evaluate these chemicals singly and in a mixture in an in vivo approach that helps to assess acute, sublethal and chronic effects on the selected model aquatic animals; 3) use simulation models of molecular docking to understand further the interaction of SESs and their herbicides with respect to their binding affinity and predict toxicity on receptors such as growth receptor (4XNN)and hatching

enzymes (ZHE1). To address the information gap regarding toxicity of selected safeners, in vivo studies and biochemical assays were done to understand possible reactions in the system of the animals after exposure to serial concentrations of SESs in acute and chronic water-borne exposures. Results from the study showed sublethal and lethal effects on both organisms exposed to MEF and FEN singly and mixed at environmentally relevant concentrations. Lower concentrations of these chemicals caused deformities and inhibited hatching rate, while such effects were not observed for CPS and ISO at the same concentrations. MEF was classified as moderate to high-risk level according to ADMET software. On the contrary, CPS had low to moderate risk levels for both *D. magna* and *D. rerio*. A biphasic plot indicating a hormetic reaction was obtained for reproduction in *D. magna*, suggesting that MEF might be an endocrine-disrupting chemical and induce stress. Lower concentrations (< 3 mg/L) of MEF caused deformations and inhibited the hatching rate in *D. rerio* embryos. Mixture studies showed infra-additive reactions on endpoints, including survival and hatching rate. Biochemical activities showed a downregulation in GST in some of the chemicals and an inhibition in SOD activities, the organism is mounting an antioxidant response to maintain homeostasis. Molecular docking scores suggested that MEF, CPS, ISO and FEN all binds to the studied receptors (growth (4XNN) and hatching (ZHE1)). Therefore, MEF and CPS have potentials to affect the activities of the selected receptors. Likewise, toxicity estimation software (TEST) predicted MEF and FEN to be developmental toxicants, and that FEN was predicted to have mutagenic potential using consensus method to calculate the end point. While MEF was more potent singly, its toxicity was reduced when in a mixture with another toxic compound (FEN), and this is beneficial to the organism. In the same vein, CPS, which has low potency, also suppressed the toxicity of ISO. The key findings from this study were that even if SESs contributed to mitigating the effects of herbicides on the animal models, there are

sublethal effects associated with it, and the adverse outcome is still a cause for concern. Also, safeners categorized as inert should be re-evaluated to account for all their toxic potential.

**Acknowledgements**

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## Dedication

This work is dedicated to my best friend and spouse, Femi; you are just incredible. To Othniel and Kezia, you are adorable, and this is for you. To my Dad, Pastor Paul, your legacy lives on.

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## List of Abbreviations

| | |
|---|---|
| µg | microgram |
| µg/L | microgram per liter |
| ANOVA | analysis of variance |
| $A_t$ | Adsorption per time |
| BCF | bio concentration factor |
| CEC | cation exchange capacity |
| CECs | contaminant of emerging concerns |
| CPS | cyprosulfamide |
| CPS/L | cyprosulfamide concentration in a liter |
| Cyp | cytochrome P450 |
| D. magna | *D. magna* |
| D. rerio | *D. rerio* |
| Dpf | day post-fertilization |
| Dt | desorption per time |
| Dt50 | half life |
| EC50 | concentration of chemical causing 50% effect |
| EDC | endocrine disrupting compound |
| ELS | early life stage |
| EPA | environmental protection agency |

| | |
|---|---|
| EPI | estimation program interface |
| ERA | environmental risk assessment |
| FC | field capacity |
| FEN | fenoxaprop-p-ethyl |
| FEN/L | fenoxaprop-p-ethyl concentration in a liter |
| GAC | granulated activated charcoal |
| HESI | heated electrospray ionization |
| IC | inorganic carbon |
| $IC_{50}$ | concentration of chemical causing 50% inhibition |
| ISO | isoxaflutole |
| ISO/L | isoxaflutole concentration in a liter |
| K | kelvin |
| $K_F$ | Freundlich solid-water distribution coefficients |
| Kg/yr | kilogram per year |
| Koa | octanol air partition coefficient |
| Koc | organic carbon normalized distribution coefficient |
| Kow | octanol water partition coefficient |
| Kp | partitioning coefficient |
| LC50 | concentration of chemical causing 50% mortality |
| LCMS | liquid chromatography mass spectrometer |

| | |
|---|---|
| LD50 | dose of chemical causing 50% mortality |
| LOEC | lowest observed effect concentration |
| M | molar |
| MEF | mefenpyr di-ethyl |
| MEF/L | mefenpyr di-ethyl concentration in a liter |
| mg/L | milligram per liter |
| ng/L | nanogram per liter |
| MM/GBSA | molecular mechanics with generalised Born and surface area solvation |
| NOEC | no observed effect concentration |
| $^{o}$C | degree Celsius |
| OC | organic carbon |
| OECD | Organisation for Economic Co-operation and Development |
| PDB | protein data bank |
| pka | acid dissociating constant |
| ppb | part per billion |
| PPDB | pesticide properties database |
| Rpm | revolution per minute |
| SDS | safety data sheet |
| SESs | selected emerging safeners |
| SMILES | simplified molecular information and line entry system |

| | |
|---|---|
| T.E.S.T | toxicity estimation software tool |
| TOXNET | TOXicology Data Network |
| UHPLC | ultra-high performance liquid chromatography |
| USEPA | united state environmental protection agency |
| UV | ultraviolet |
| ZHE1 | *D. rerio* hatching enzyme |
| V. fischeri | Vibrio fischeri |

## Note to Readers

This thesis is organized to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for manuscript style thesis. Chapter 1 is the general introduction of the thesis topic which include a literature review that has been published in the *Review of Environmental Toxicology*. Chapter 2 -5 are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 6 contains the overall discussions and conclusion of this thesis. Chapter 2 has been published in the *Science of the Total Environment.* Chapter 3 is prepared to be submitted to the *Journal of Environmental Science: Advance.* Chapter 4 is in preparation for submission to *Environmental pollution.* Chapter 5 has been published in the *Journal of Hazardous materials advances*. Authors' contributions are provided in each chapter while references containing full citations of published papers are provided following the concluding chapter of the thesis. As a result of the manuscript style format, there are some repetitions of materials in the introduction and materials and methods sections across the different chapters of this thesis. The tables, figures, supporting information, and references cited in each chapter have been reformatted here to be consistent with the thesis style requirements of the University of Saskatchewan. Supporting information associated with research chapters are presented in the "Appendices" section at the end of this thesis. The supplemental files can be found in the associated publications.

# 1 Chapter 1: General Introduction

## 1.1 Preface

While there is a huge body of literature on the use and application of chemical safeners on crops, little research has been done to understand their potential effects on non-target animal species in relation to their physico-chemical properties. This chapter reviews common safeners, their common herbicides and the crops they protect while considering safeners' application, sorption and mobility. This chapter aimed to explain the fate of these class of chemicals from formulation, point of application and dissipation routes to their potential toxicity and effects, for a comprehensive understanding of their activities in the aquatic environments. These activities could subsequently help to link these chemicals' fate, mobility, and activities to the potential effects on non-target species. There is a little work on the presence of safeners in the environment and review on their presence in the aquatic environment will be covered. Also, their potential toxicity will be elucidated in this chapter. The chapter links how safeners will dissipate from point of application to when it will be available in the environment and thus available to aquatic organisms. This chapter sets the stage for subsequent chapters which will discuss specifics to each chemical used in this project.

The content of chapter 1 was reprinted (adapted) from FF Oloye, OP Femi-Oloye, JK Challis, PD Jones, JP Giesy (2021) Reviews of Environmental Contamination and Toxicology Volume 258, 27-53, doi: 10.1007/398_2021_70. (Copyright 2021). The content is modified and rearranged to meet the requirement for thesis submission. The section 1.2 to 1.8 in this chapter represents the content of the published manuscript showing important aspects of literature review of the compounds of interest including their fate and mobility while section 1.9 to 1.12 is added to this chapter to accommodate important aspect contributing to the details and work done in this thesis.Author's contributions:

Oluwabunmi P. Femi-Oloye (University of Saskatchewan)- Conceptualization, Methodology, Formal analysis, Investigation, Writing- original draft, Review and Editing, visualization

Dr. Femi Oloye (University of Saskatchewan, Saskatchewan Health Authority, Roy Romanow Lab) – Conceptualization, Writing- original draft, Methodology, Software, Formal Analysis, Investigation, Review and Editing

Dr. Jonathan Challis (University of Saskatchewan) – Review and Editing

Dr. Paul Jones (University of Saskatchewan)– Investigation, Review and Editing

Dr. John Giesy (University of Saskatchewan; Baylor State University) – Conceptualization, Methodology, Resources, Investigation, Review and Editing, Project Administration, Funding Acquisition

## 1.2 Introduction

The increased use of reduced tillage systems to minimize erosion and alterations of soil structures, weed resistance, monocropping and other factors have led to continued, large-scale use of herbicides in agriculture globally (Farenhorst et al., 2001). Knowledge of potential hazards, fate and possible dissipation of this class of chemicals and their formulations (including safeners) to ground and surface waters is important. Rates of usage of herbicides to control weeds in crops continue to increase. Used along with herbicides is a group of chemicals called safeners, which are used either in, pre- or post-application of herbicides, to protect monocotyledonous plants, which are plants that have seeds with one cotyledon, such as sugarcane, corn and rice from toxic effects of herbicides used to control weeds in these crops.

Safeners act when applied onto crop seeds through either soil treatment, seed treatment, foliar spray or as a mixture with herbicide to prevent, reduce, or suppress the adverse effects (e.g. phyto-toxicity) of herbicides to crops, by physiological or molecular mechanisms, without reducing efficiency of suppressing targeted weeds (Davies and Casley, 1999; Hatzios and Burgos, 2004; Sivey et al., 2015; Bolyard et. al., 2017; Acharya and Weidhaas, 2018). Chemical safeners can thus improve tolerance of herbicides by crops (Behringer et al., 2011).

As a result of chemical safeners being classified as “inert” components of herbicide formulations, there is less information and research regarding their fates in the environment, effects on non-target species, and possible mechanisms of action (Sivey et al., 2015; Acharya and Weidhaas, 2018). Safeners are designed to alter biochemistries in crop plants in order to impart protection from herbicide-induced damage, which suggests that they might have biological activity in non-target organisms at ecologically relevant concentrations (Sivey et al., 2015).

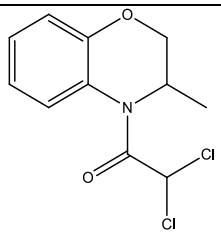
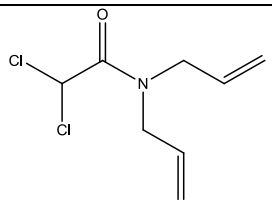
Dichloroacetamide safeners have the propensity to be transformed by reduction to herbicide-like products (Sivey and Robert, 2012). Dichlormid has been reported to degrade through dechlorination, dealkylation, oxidation, and hydrolysis (Abu-Qare, 1992). Efforts have been made to understand modes and mechanisms of action of safeners on target plants (Sivey et al., 2015; Behringer et al., 2011; Abu-Qare and Duncan, 2002); however, many of these mechanisms remain unclear. Same efforts have not been directed towards their fate, mobility and effects on organisms in various environments, including the aquatic environment.

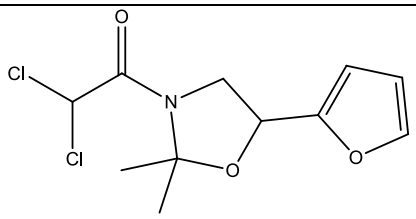
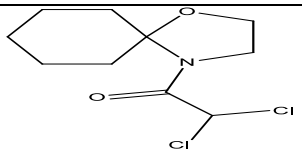
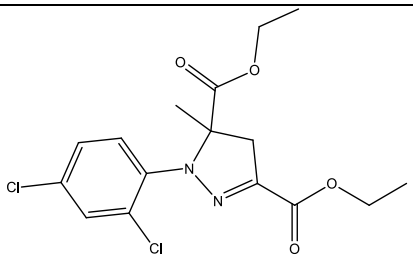
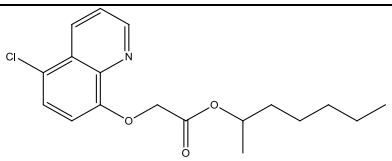
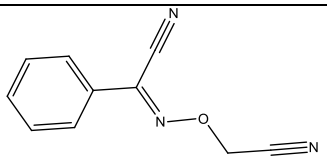
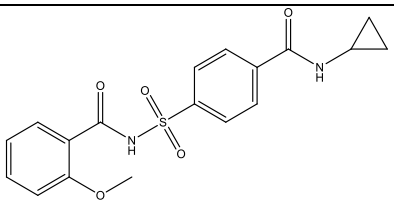
Safeners are part of the ingredients in formulations of commercial, weed control products (Acharya and Weidhaas, 2018). For example, dichloroacetamide safeners contribute up to ~5 % by weight of the herbicide formulation (Xu et al., 2020, and the references therein). This class of chemical are in continual usage. For example, the application of dichloroacetamide safeners in 2017 in the US was estimated to be more than two million kg/yr, which exceeded the application of the active ingredients of many common herbicides (Bolyard et al., 2017; Woodward et al., 2018; Xu et al., 2020). Safeners are applied in the same manner as herbicides, either sprayed as a mixture with active ingredient herbicides or as separate seed treatments (Davies et al., 1999). Their wide usage and mode of applications suggest that these chemicals will enter the environment like those of active herbicide ingredients and in proportions at which they were applied in the field. In 2004, Syngenta Crop Protection Canada submitted a field data report on application of cloquintocet-mexyl safener for protection of wheat (USEPA, 2007) in Alberta, Manitoba, and Saskatchewan, further suggesting the potential for presence of safeners in aquatic environments outside the USA.

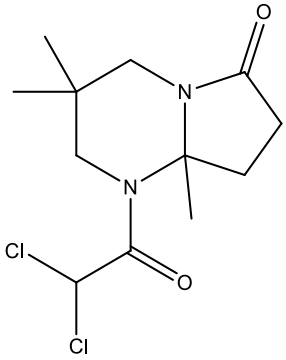
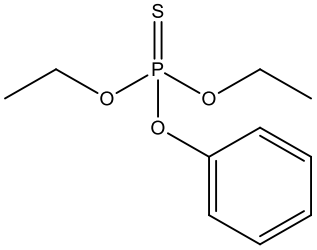
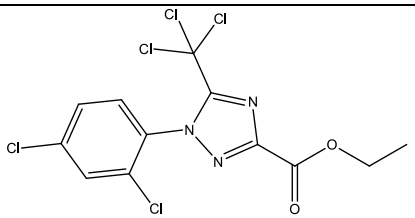
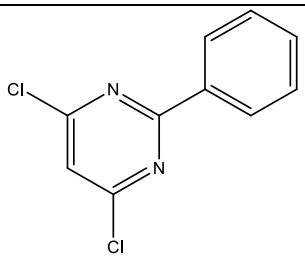
Herbicide safeners have diverse applications when it comes to protection of agriculturally important crops (see Table 1). Winter wheat and barley were protected from adverse effect of the herbicide pinoxaden (Axial) by converting its active ingredient into inactive metabolites by the safener cloquintocet-mexyl. However, this safener was ineffective

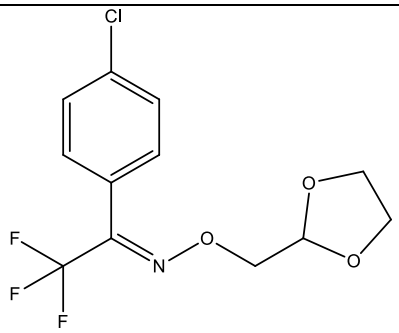
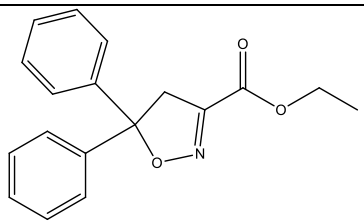
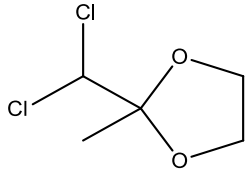
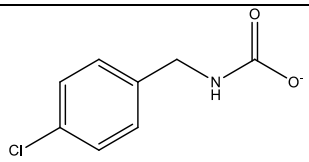
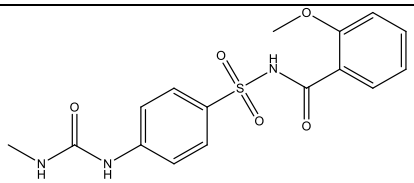
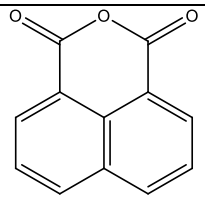
for protecting wild oats or perennial ryegrass from adverse effects of pinoxaden herbicide (Brosnan et al., 2016). Effects of the herbicide carfentrazone-ethyl on spring wheat could be suppressed by simultaneous exposure to sulfonylurea or flucarbazone-sodium (Howatt, 2006). In this case, these two herbicides, were used as safeners, not herbicides, because they were not effective against weeds, but were used to protect against effects of another herbicide. To protect from effects of herbicides, flax seeds were coated with either BASF's Insure Pulse or Vitaflo (Staff, 2019). Also, safeners have been used to protect hard, red spring wheat from injury from the herbicide, fenoxaprop (Staff, 2019). Dichlormid was also an effective safener when added to S-Ethyl dipropylthiocarbamate (EPTC) and other thiocarbamates in preventing the onset of herbicide harm to maize plants (Abu-Qare, 1992). Thus, safeners are used to protect different crops against injurious effects of herbicides.

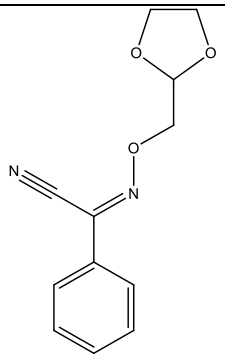
Table 1: Name and structure of herbicide safeners.

| Safeners | Structure | Common Herbicide | Crops |
|---|---|---|---|
| Benoxacor |  | Metalochlor | Corn, Soyabeans and Sorghum |
| Dichlormid |  | Chloroacetanilide, Thiocarbamate | Corn |

| | | | |
|---|---|---|---|
| Furilazole |  | Isoxaflutole | Corn, Rice |
| AD-67 |  | Acetochlor, Butachlor, EPTC | Corn |
| Mefenpyr-diethyl |  | Fenoxaprop-P-ethyl, Iodosulfuron, ACCase inhibitors Sulfonylureas | Corn, Wheat, Rye, Barley, Triticale |
| Cloquintocet-mexyl |  | Clodinafop-propargyl, Pinoxaden | Winter wheat, Barley wild oats, perennial ryegrass |
| Cyometrinil |  | Metalochlor | Sorghum |
| Cyprosulfamide |  | Thiencarbazone-methyl, Isoxaflutole, | Corn |

| | | | |
|---|---|---|---|
| | | Tembotrione,<br>Iodo sulfran,<br>Nicosulfuron | |
| Dicyclonon |  | Metazachlor<br>Metalochlor | Corn |
| Dietholate |  | Clomazone | Cotton |
| Fenchlorazole-ethyl |  | Fenoxaprop-ethyl,<br>Fenoxaprop-P-ethyl | Wheat |
| Fenclorim |  | Pretilachlor | Rice |

| | | | |
|---|---|---|---|
| Fluxofenim |  | Metolachlor,<br>S-metolachlor | Sorghum |
| Isoxadifen- ethyl |  | Foramsulfuron,<br>Tembotrione,<br>ACCase inhibitors<br>Sulfonylureas | Corn,<br>Rice |
| Jiecaowan |  | | Corn |
| Mephenate |  | | Corn |
| Metcamifen |  | | Corn |
| Naphthalic anhydride |  | Thiocarbamates | Corn |

| | | | |
|---|---|---|---|
| Oxabetrinil |  | Chloroacetanilides | Sorghum |

While some safeners are believed to be crop-specific, there is evidence that MEF and CPS can protect different types of crops (Duhoux et al., 2017) and/or inhibit other herbicides through various modes of action (Ahrens et al., 2013). CPS has been reported to protect all varieties of corn from pre-emergent applications of ISO (Ahrens et al., 2013) and tembotrione herbicides. Corn and rice are the most common cereal crops protected with safeners against chloroacetanilide, sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides (Table 1) (Davies and Caseley, 1999; Davies 2001; Guo et al., 2020). Winter cereal crops, such as wheat also use safeners to protect against effects of post-emergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides on target crop plants (Hatzios and Burgos, 2004). Examples of safeners that protect cereal crops from post-emergence applications of sulfonyl urea herbicides are isoxadifen-ethyl and MEF (Behringer et al., 2011), which can also work to protect *Arabidopsis thaliana* leaves (Behringer et al., 2011).

As with herbicides, safeners can dissipate in several ways, including leaching, adsorption, volatilization, biotic and abiotic degradation (Figure 1) (Abu-Qare and Duncan, 2002), hydrolysis and for some photolysis (Hertkorn et al., 2010). Dissipations of both herbicides and safeners are functions of their physico-chemical properties that govern behaviours in soil and water (Diez and Barrado, 2010). Potential environmental mobilities of safeners are functions of their aqueous solubility and sorption to solid particles (Acharya and Weidhaas, 2018). Environmental partitioning such as sorption and leaching from soils, uptake by plants, and accumulation in various environmental compartments can be estimated by use of fugacity based on the octanol-water partitioning coefficient (Log $K_{OW}$) (Acharya and Weidhaas, 2018; Zhang et al., 2016). Therefore, determining Log $K_{OW}$ will give fundamental information on sorption in soil and the potential availability of safeners in the environment.

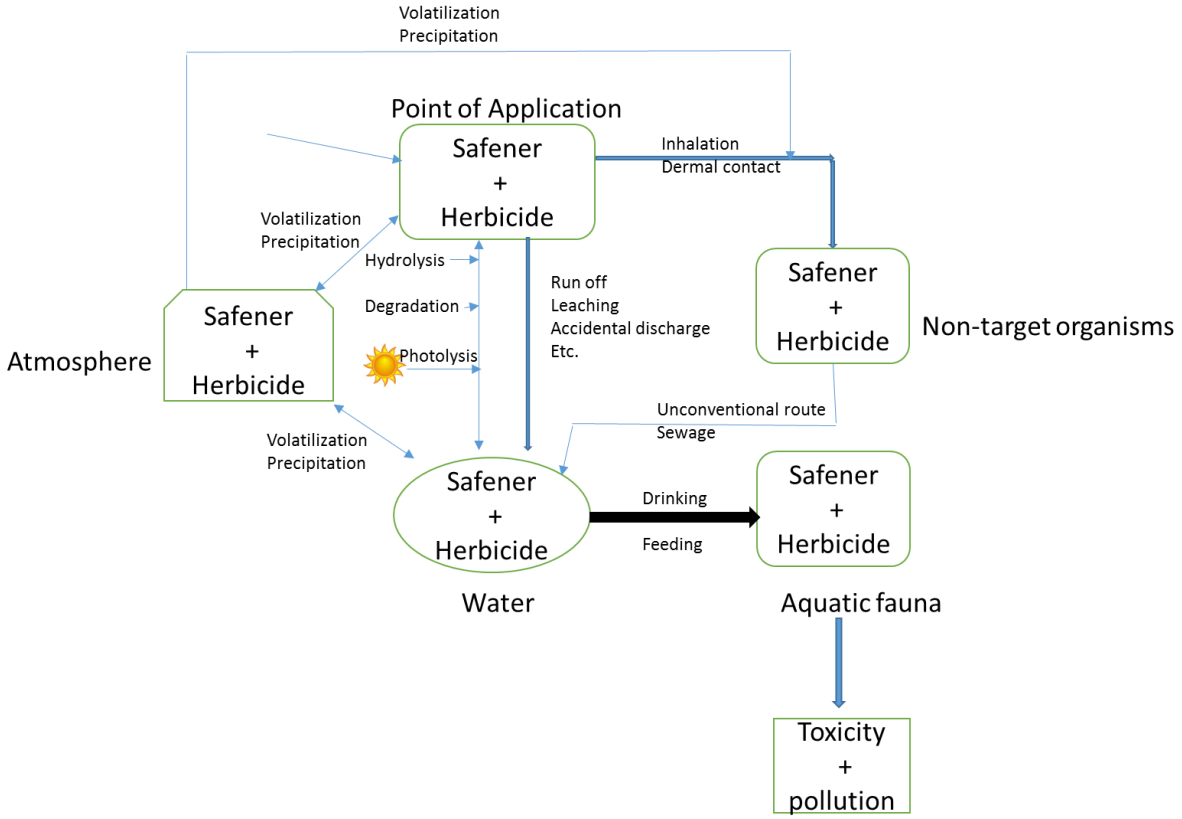



Figure 1: Possible transportation routes and ways of safeners exposure to the aquatic environment.

Despite the various sorption mechanisms and degradation pathways which chemical safeners undergo, occurrence in surface waters has been observed, and there remains a gap in our understanding of the exposure risks posed by these chemicals. Furilazole, benoxacor, dichlormid and Ad-67 were detected in Midwestern US rivers between Spring and Summer 2016, with the maximum concentrations ranging from 42 to 190 ng/L (Woodward et al., 2018). In surface waters, chemical safeners are capable of producing a broad range of responses in non-target organisms (USEPA, 2006), similar to their often structurally-related herbicide counterparts (Acharya and Weidhaas, 2018). Most of the safeners, including benoxacor and dichlormid have *di minimis* mammalian toxicity and a high potential for bioaccumulation (Levis et al., 2016; PPBD, 2020).

There are several factors to consider when assessing our understanding of the fate and behaviour of safeners in the environment. The modes of application of safeners and herbicides and how they enter aquatic environments can affect pathways and rates of transport and dissipation. Additionally, chemistries of safeners might be different when applied together with herbicides than when used alone (Bolyard et al., 2017 and Acharya and Wiedhass, 2018; Su et al., 2019).

Finally, safeners can be transformed, and their transformation products should also be considered in the assessment of fate, transport, and ultimate hazard to non-target organisms. Also, routes and rates of dissipation of herbicides and safeners in the same formulation may differ. Hence, potential hazardous effects of all types of safeners (Table 1) need to be reviewed to understand the safety of this class of chemical. In this review all classes of known safeners will be discussed with respect to their fate, mobilities and eco-toxicity. Where experimental data were not available, software and data from manufacturer safety data sheets were used. Some experimental data from our research groups were also used to corroborate some of the existing data.

Safeners work by selectively inducing specific enzymes in the crops, which help the plants to metabolize and detoxify the herbicide. However, safeners can also have unintended effects on non-target organisms, including aquatic organisms such as *D. magna* and *D. rerio* (Many works on the toxicity of the active ingredients of herbicides on non-target organisms such as *D. magna* (Hasenbein et al., 2017; Samadi et al., 2022) and *D. rerio* (Yang et al., 2016; Strähle et al., 2012) were available in the literature. However, the potential toxicity of safeners to non-target organisms, such as aquatic organisms, has not been well documented in literature.

Daphnia is a genus of small (the body is usually 0. 2 – 6.0 mm long) planktonic crustaceans and a member of Phylum Arthropoda (Figure A) (Ebert, 2022); it is otherwise

called water fleas. The two most common daphnids are *D. pulex,* and *D. magna*. *D. magna* is a small freshwater crustacean often used in toxicological and ecological studies due to its short life span, rapid reproduction, and ease of culture (Mittmann et al., 2014). It is also very sensitive to environmental pollutants and stress; thus, it is an excellent candidate for studying new or existing chemicals with little information. The toxicity of most chemicals could be estimated with computer-aided software using *D. magna* as the targeted organism. The translucent and transparent body with internal organs (such as the heart) that can be seen helps to monitor the physiological effect of various chemicals. *D. magna* is readily available and can be found in various aquatic environments ranging from acidic swamps to freshwater lakes and ponds, thereby one of the first organisms to be affected by the presence of xenobiotics that enter aquatic environments. *D. magna* serves as a food source for other organisms in the aquatic environment (such as fish and amphibians) (Hasenbein et al., 2017). Thus, a negative effect on it indirectly affects other higher organisms. Similarly, bioaccumulation and bio-concentrations of chemicals or pollutants would indirectly get to higher animals.

Figure A: *D. magna* adapted from Wikipedia.com

*D. rerio* (are small, tropical fish that have become popular model organisms in toxicology research due to their genetic similarities to humans and their transparency during early developmental stages, allowing for easy observation of organ development and toxicity effects (Roper and Tanguay, 2018). *D. rerio* embryos can develop outside the mother and are cheap to maintain (Yang et al., 2016). The transparent embryo of *D. rerio* also makes it an excellent candidate for studying various environmental stressors and pollutants. It allows the analysis of multiple endpoints ranging from acute and developmental toxicity determination to complex functional genetic and physiological analysis (Strähle et al., 2012). The translucent embryo provides an opportunity to monitor the effect of pollutants on the development of internal organs under a microscope (Figure B). *D. rerio* is very sensitive to stressors; thus, the abnormalities caused by chemicals can easily be observed from the transparent embryos.

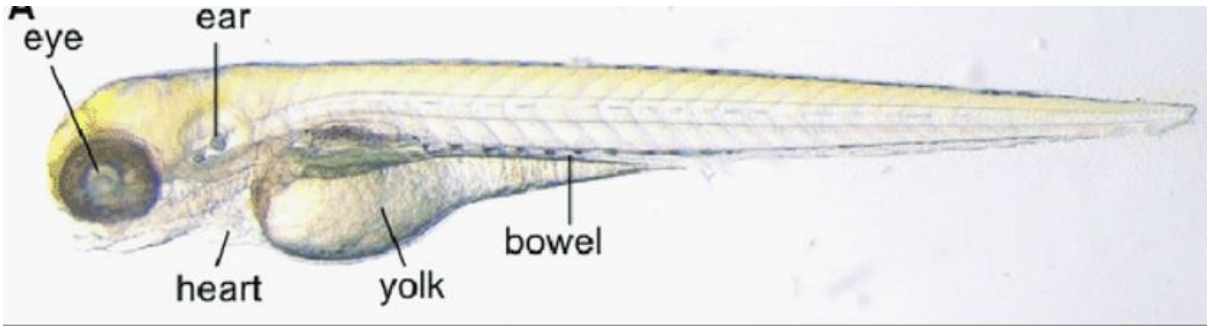



Figure B*: D. rerio at 96 h post fertilization (adapted from Dahme et al., 2009)*

This work was based on the current environmental quality criteria for safeners under the current regulatory frameworks which supports environmental risk assessments (ERA) through endpoints that have direct ecological and/or regulatory relevance such as survival, reproduction, growth, and development. Additionally, the mechanisms of toxicity, the factors influencing the toxicity of safeners and herbicides, and the implications for aquatic ecosystems were discussed to provide a comprehensive understanding of the potential risks associated with

these chemicals. Understanding the properties and potential risks associated with safeners, herbicides, and their mixture is crucial for their responsible use in agriculture and for developing appropriate regulations and management practices to minimize their adverse effects.

## 1.3 Leaching

Leaching is one of the primary mechanisms through which safeners and herbicides dissipate from points of application. Aqueous solubility is one important factor that determines the temporal and spatial mobility of safeners and herbicides (Acharya and Weidhaas, 2018). Rates of mobility of safeners and herbicides determine their ability to protect target crops as well as the potential for exposure of non-target organisms, such as aquatic organisms. This means that if safeners and herbicides dissipates at different rates, safening will not be optimized on crops (Nelson and Penner, 2007). Interestingly, a recent study showed that safeners such as benoxacor and the herbicide (S-metolachlor) moved through the soil at a similar rate (Acharya et al., 2020). Unfortunately, not all chemicals move at the same rate in soil and plants. For example, the herbicide, ISO leached faster than furilazole and thus required the use of a polymeric carrier to ensure that ISO and herbicides safeners moved at the same manner (Nelson and Penner, 2007). Rates at which chemicals move depend on multiple factors, including their physical and chemical states. Hence, herbicides' properties cannot necessarily be used to predict leaching potential of safeners. Leaching is different from other similar transportation processes, such as surface runoff and erosion, because it involves the downward movement of substances.

When new herbicides are registered, efforts are made by researchers and manufacturers to understand their potential to leach. However, little or no effort is made to understand the leaching of accompanying safeners in the academic community. Indaziflam, an emerging herbicide, was tested under simulated rain and was observed not to leach beyond 30 cm under

any conditions tested (Jhala et al., 2012). This observation may not hold true for the corresponding safener and could differ under realistic field conditions, where there is a possibility that interactions between safener and herbicide can affect mobility (Bolyard et al., 2017 and Acharya and Wiedhass, 2018). Octanol-water partition coefficients can be used to estimate mobilities of chemicals. Smaller Log $K_{OW}$ values indicate that the chemicals will have less potential for sediment and soil sorption, thus greater mobility. For example, the Log $K_{OW}$ values for benoxacor and furilazole were 2.23±0.16 and 1.96±0.22, respectively (Acharya and Weidhaas, 2018), suggesting that furilazole may leach more readily than benoxacor, assuming hydrophobic-type interactions are dominating sorption, even though there isn't much difference between the logKow.

Prediction software can be used to estimate chemical mobilities based on predicted Log $K_{OW}$. These include EPI suite (Sivey and Robert, 2012; Sivey et al., 2015; Bolyard et al., 2017), Marvin Sketch, TOXNET (TOXicology Data NETwork) (ChemIDplus) (Acharya and Weidhaas, 2018). The KOWWIN program in EPI Suite software overestimated Log $K_{OW}$ (Table 2) compared to our experimentally measured values and the select data present in the literature of which 25 and 8% were overestimation for benoxacor and furilazole, respectively (Acharya and Weidhaas, 2018). Similar overestimations by EPI Suite of 24% for benoxacor and 19% for furilazole were observed in the present study. The reason for overestimation of the EPI suite and the experiment could be attributed to a method error.

The frequency and amount of rainfall affect leaching, as well as the time between rain events and application of safeners and herbicides. Greater rainfall can result in leaching of herbicides, which might result in damage of, for example, citrus tree roots, poor weed control, and groundwater contamination (Jhala et al., (2012). The presence of both herbicides and safeners in surface waters (Woodward et al., 2018) suggests we need a better understanding of leaching behaviours of these chemicals and potentially how the use and management of these

herbicide/safener formulations could be optimized to minimize leaching to the environment (Chnirheb et al., 2012). Long- and short-time leaching experiments in laboratory and field trials are essential to determine leaching of both herbicides and safeners, when applied together. A recent study showed that both benoxacor and S-metolachlor leached at the same rate, which was suggested to be influenced by soil texture (Acharya et al., 2020). Leaching of chemicals to aquatic environments is problematic because some chemicals can bio-accumulate and bio-concentrate in either sediments or living organisms within the aquatic system. For example, pre-emergence application of the herbicide bromacil has been banned from use in Florida, USA, because it has been observed to leach and contaminate ground water (Jhala and Singh 2012; Jhala et al. 2012).

The most commonly used methods for determining potential for leaching in field studies involve chemical concentrations measured in soil, water from tile drains and suction cups and dye tracer experiments (Sarmah et al., 2004). Similarly, laboratory experiments such as soil columns are common methods for qualitative and quantitative measurements of leaching of chemicals (OECD, 2004).

Table 2: Log $K_{OW}$, Log Koa and solubility of safeners determined using EPI suite computer simulation and PubChem

| Safeners | Log $K_{OW}$ | Log $K_{OW}$ | Solubility | Solubility | Solubility | Solubility | Logkoa |
|---|---|---|---|---|---|---|---|
| | Experimental data-based match | KOWWIN v1.68 estimate (EPI Suite) | Experimental data-based match (@ 293 K) | PubChem | WSKOW v1.42 @ 298 K | WSFragment v1.01 estimate @ 298 K | KOAWIN v1.10 estimate |
| | | | mg/L | M | mg/L | mg/L | |
| Benoxacor | 2.70 (2.25)* | 2.38 | 20.00 | 0.000077 | 102.70 | 750.18 | 8.21 |
| Dichlormid | 1.84 (0.80)* | 2.28 | 5000.00 | 0.02 | 1067.00 | 2662.20 | 6.71 |
| Furilazole | 2.12 (0.72)* | 2.84 | 197 | 70800 | 254 | 7121 | 10.55 |
| Mefenpyr-diethyl | 3.83 (3.78)* | 4.82 | 20 | 0.000054 | 2.40 | 4.45 | 11.15 |
| Cloquintocet-mexyl | 5.03 | 5.28 | 0.59 | | 0.3816 | 1.9566 | 12.51 |
| Cyometrinil | - | 2.52 | 95 | | 196.8 | 467.66 | 3.974 |
| Cyprosulfamide | - (0.55)* | 2.30 | - | | 47.7 | 13.94 | 14.44 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dicyclonon | - | 1.98 | - | | 273.1 | 13826 | 9.074 |
| Dietholate | 3.46 | 3.91 | | | 27.53 | 17.652 | 5.973 |
| Fenchlorazole-ethyl | | 4.54 | 13 | 0.000002 | 0.5799 | 0.3055 | 8.916 |
| Fenclorim | 4.17 | 2.99 | 2.5 | 0.000011 | 8.883 | 19.424 | 7.529 |
| Fluxofenim | 2.90 | 2.73 | 30 | 0.000097 | 35.96 | 1.6024 | 6.701 |
| Isoxadifen- ethyl | 2.01[+] | 5.66 | - | | 0.1917 | 0.082283 | 10.516 |
| Jiecaowan | - | 1.22 | - | | 5383 | 46569 | - |
| Mephenate | 2.01 | 1.82 | - | | 992.7 | 2188.8 | 8.022 |
| Metcamifen | - | 1.47 | - | | 283.7 | 29.346 | 17.738 |
| Naphthalic anhydride | 3.24[+] | 3.24 | - | | 5.878 | 592.16 | 7.837 |
| Oxabetrinil | 2.76 | 2.78 | 20 | | 130.1 | 376.13 | 8.365 |

( *) determined by the authors in the laboratory; [+] from PubChem

## 1.4 Sorption

There is limited experimental data describing safener sorption to soil and sediment systems. Therefore, predicted physico-chemical properties, the few data available and data on herbicides will be used as a guide for understanding sorption of safeners. Estimates of chemical mobilities in various environments and rates of dissipation must include an understanding of sorption and desorption properties (OECD 2000). Fate and behaviour (e.g., transportation, or retention) of herbicides in soils are controlled by various factors, including rates of sorption and desorption (Mamy and Barriuso, 2007), soil properties, and various processes of transformation, all of which can determine the bioavailable fraction in the environment (Davies and Jabeen, 2003). Sorption of chemicals onto soil particles and their relative affinities to those soils can ultimately determine the potential for other types of dissipation.

At a molecular level, sorption is driven and controlled by diffusion, which is a relatively slow and first-order process. Thus, sorption and desorption are often rate-limiting steps in overall dissipation in soils. Desorption rates of chemicals can be used as an estimate of overall dissipation rates and of potential for non-point sources of contamination, for example in ground waters (Boukili et al., 2018). A chemical that remains strongly sorbed to the soil will not readily reach ground or surface waters through leaching or runoff. For example, the Freundlich constant for benoxacor and furilazole are 6.4 and 3.4 (mg/g) x (mg/L) (1/n) in granulated activated charcoal (GAC) (Achaya et al., 2020). This suggested that benoxacor sorbed more readily to GAC than furilazole. Similarly, the Freundlich constants for furilazole and dichlormid had been reported to be 0.79 – 3.5 and 0.25 – 0.65 (mg/g) x (mg/L) (1/n), respectively (Carr, 1990; Subba-Rao, 1990). Hence, dichlormid, furilazole and benoxacor did not sorb to any great extent on agricultural soil (Carr, 1990; Subba-Rao, 1990; Acharya et al., 2020). Consequently, the sorption of benoxacor and furilazole were found to be reversible (40-

90%) (Acharya et al., 2020). Therefore, this class of safeners can be readily leached through the soil.

Characteristics such as pH, texture, cation exchange capacity, crystalline lattice structure, organic matter content, amorphous iron oxides, inorganic matter complexes, temperature, and moisture (Harris and Warren, 1964; Gennari et al., 1998; Boukili et al., 2018; Wiersma et al., 2003) could potentially have large effects on sorption capacities of soils. Most herbicides and all safeners reported here have Log $K_{OW}$ values > 1, suggesting a potential to sorb to organic components of soil matrices (Cumming and Rücker, 2017). Both soil organic matter and clay are significant constituents affecting the sorption of MEF in soils (Boukili et al., 2018). Similarly, only organic carbon content and adsorption parameter (n) were found to influence adsorption of benoxacor and furilazole (Acharya et al., 2020). Thus, soil properties such as organic carbon content and pH might influence adsorption of safeners.

However, some herbicides, such as glyphosate have greater affinity for inorganic minerals in soils (Gennari et al., 1998). Acidic soils will generally enhance herbicide sorption (Gennari et al., 1998). Retention of chemicals by soil depends on the properties of both the soil and chemical. In some cases, retention of chemicals by soils increases/decreases, while in some other cases, it remained constant with changes in soil properties (Mamy and Barriuso, 2007). Therefore, each herbicide or safeners needs to be tested to know their sorption capacity under prevailing environmental conditions, ideally through experimentation but also by software prediction, as necessary. A detailed method for quantifying sorption has been developed (OECD, 2000) and can be used directly or with little modification for most chemicals.

Studies of sorption of active ingredients of herbicide formulations are common but reports on sorption of herbicide safeners to soil are limited. Hence, predictions of these

properties, including Log $K_{OW}$, aqueous solubilities, and Log Koa, as estimated from the programs (KOAWIN v1.10) within EPI suite, are given (Table 2). These properties can be used to predict potential mobilities of safeners as their movements in soils, in part, are a function of their aqueous solubility and Log $K_{OW}$ (Acharya and Weidhaas, 2018) (Table 2). The aqueous solubilities vary between 0.19 mg/L for isoxadifen-ethyl and 1070 mg/L for dichlormid. Solubility data varies depending on the source of the data, for example PubChem lists solubility of dichlormid as 5000 mg/L, WSKOW v1.42 lists it as 1070 mg/L and WSFragment v1.01 estimate it as 2662 mg/L. Safeners such as isoxadifen-ethyl, cloquintocet-mexyl, MEF, fenchlorazole-ethyl and naphthalic anhydride are less soluble or not readily soluble in aqueous medium, while dichlormid, furilazole, cytometrinail, CPS are readily soluble in water and are thus more likely to be mobile in soil environments. For example, Isoxadifen- ethyl and fenchlorazole-ethyl (Table 3) have relatively low water solubilities, therefore predicted to be less mobile in soils. Safeners with larger Log $K_{OW}$ values are less mobile. Isoxadifen-ethyl, fenchloazole-ethyl, cloqyuintocet-mexyl and MEF have Log $K_{OW}$ values of 5.66, 4.54, 5.28 and 4.82, respectively, and are less mobile than CPS, benoxacor, dicylonon, cyometrinil and furilazole with Log $K_{OW}$ values of 2.30, 2.38, 1.98, 2.52 and 2.84, respectively. Predicted Koc values (Table 3), which correlate with Log $K_{OW}$ (Table 2) in terms of mobility and sorption capacity of the chemicals to organic carbon content in soils are provided here. Assuming hydrophobic interactions are governing sorption, mobility will be inversely proportional to Koc. Thus, determining solubility, Log $K_{OW}$, and $K_{OC}$ properties provide fundamental information on the potential for sorption of chemical safeners to soils. Relationships exist between solubilities and adsorption capacity in one form, and adsorption capacity and molecular size in another (Acharya et al., 2020). Furilazole was determined to have lower adsorption capacity compared to benoxacor because the latter is not very soluble, while furilazole was moderately soluble. Considering their molecular structures, the smaller size of

benoxacor might be the driving force for its higher adsorption capacity compared to furilazole. Therefore, both solubility and molecular size might be factors to be consider in sorption of safeners studies.

Table 3: Sorption capacity of safeners to organic component of the soil

| Safeners | $K_{oc}$ PPDB | $K_{oc}$ MCI method | $K_{oc}$ $K_{ow}$ method | Remarks |
|---|---|---|---|---|
| Benoxacor | 109.0 | 133.2 | 267.5 | Moderately mobile |
| Dichlormid | 40 | 120.6 | 78.6 | Mobile |
| Furilazole | 199 | 252 | 91.2 | Moderately mobile |
| Mefenpyr-diethyl | 634 | 1501 | 739.6 | Non-mobile |
| Cloquintocet-mexyl | 9856 | 26130 | 4985 | Non-mobile |
| Cyometrinil | - | 709.9 | 1269 | Slightly mobile |
| Cyprosulfamide | - | 10.41 | 39.68 | Mobile |
| Dicyclonon | - | 89.86 | 84.25 | Mobile |
| Dietholate | - | - | 1543 | Non-mobile |
| Fenchlorazole-ethyl | - | 18780 | 4031 | Non-mobile |
| Fenclorim | 3655 | 3344 | 4267 | Slightly mobile |
| Fluxofenim | - | 6179 | 222.8 | Moderately mobile |
| Isoxadifen- ethyl | - | 81660 | 9777 | Non-mobile |
| Jiecaowan | - | 14.69 | 26.23 | Non-mobile |
| Mephenate | - | - | 34.54 | Non-mobile |
| Metcamifen | - | - | 233.1 | Slightly mobile |
| Naphthalic anhydride | - | 15.16 | 181.4 | Mobile |
| Oxabetrinil | - | 368.9 | 459.9 | Mobile |

## 1.5 Volatilisation

Information on volatilisation of chemicals used as herbicides is required by regulatory bodies in many countries (Allen et al., 2004; Boivin and Poulsen 2017). This is because losses of these chemicals from soils, plants, and water bodies can accumulate in the atmosphere and result in long-range atmospheric transport (Dekeyser et al., 2015). Physico-chemical parameters, such as Henry's law constant, soil/air and water/air distribution coefficients and vapour pressure can be used to predict volatilisation from water, soil and plant matrices (Allen et al., 2004) (Table 2 and 4). Vapour pressure is the best parameter to estimate volatilisation of chemicals from plants, while water/air and soil/air distribution coefficients are the best parameters to predict volatilisation of chemicals from water and soil, respectively (Allen et al., 2004). Compounds with vapour pressures less than $10^{-3}$ to $10^{-4}$ Pa or Henry's law constant less than $5x10^{-5}$ atm-$m^3$/mol will demonstrate negligible volatility (Allen et al., 2004), therefore safeners such as cyometrinil, and fenclorim, with Henry's law constants of $8.59x10^{-4}$ and $1.07x10^{-5}$ atm-$m^3$/mol, respectively may be relatively volatile. In contrast, metcamifen with a Henry's law constant of $1.32x10^{-18}$ atm-$m^3$/mol would be considered non-volatile (Table 4).

Greater water content of soils normally results in greater vapour pressures of chemicals, which can therefore result in volatilisation (Sarmah et al., 2004). Water can facilitate movement of safeners from soil surfaces after displacement from sorption sites. Other activities occurring within the plant, soil, or water can hasten volatilisation. For example cohesion, transpiration, capillary action and translocation in plants may cause the chemicals to move to parts of the plant, where volatilisation may be aided (Ramanjaneyulu, 2016). The data presented in Table 5 show that the half-life for volatilisation from a river can be different from a lake. The half-life, as a result of volatilization in river and lake of cyometril was estimated to be <1 and 6 days, respectively, while CPS's half-life was estimated as $2.7x10^9$ and $2.9x10^{10}$ days, for river

and lake, respectively. The proposed differences in volatilisation of cyometril and CPS could be attributed to the differences in their Henry law's constant. However, mineral components and other factors such as turbulent mixing in rivers might enhance the removal of the safeners.

Other parameters that affect volatility, which are frequently used in volatility experiments, include agricultural practice (rate of application of the chemical), competing processes (uptake by plants and degradation) and environmental conditions (wind speed, air humidity, soil moisture, and temperature) (Deskeyser et al., 2015). The higher the temperature and relative humidity, the higher the volatilisation of chemicals. Therefore, the rate of volatilisation of the same chemical in different climates will be differ. Volatilization rates can be quantified by direct or indirect measurement. The indirect measurement involves conducting a complete mass balance for all relevant environmental compartments (e.g., water, soil, sediments, and plants) and determining rates of volatilization by difference. This indirect approach will often include modelling components such as inverse modelling by the use of the dispersion model (Atmospheric modelling system, ADS) and parameterised PEARL (Pesticide Emission Assessment at Regional and Local scales) model simulation (Deskeyser et al., 2015). The direct measurement can be done by measuring the concentration of air in the treated area over a period while accounting for potential losses due to degradation. The common methods for these measurements are aerodynamic profile (ADP), energy balance (EB), relaxed eddy accumulation (REA) and plume dispersion (PD) (Dekeyser et al., 2015). Herbicides and the corresponding safeners will be present in the atmosphere of the treated sites to varying degrees based on rates of volatilisation.

Table 4: Volatilization from water as estimated using EPI suite.

| Safeners | Henry Law's constant | Half-life (Lake) | Half-life (River) |
|---|---|---|---|
| | atm-$m^3$ / mol | days | days |
| Benoxacor | 7.58E-008 | 5669 | 519 |
| Dichlormid | 3.29E-007 | 1173 | 107 |
| Furilazole | 9.2E-011 | 4842000 | 442200 |
| Mefenpyr-diethyl | 1.16E-009 | 443200 | 40630 |
| Cloquintocet-mexyl | 8.1E-010 | 602100 | 55190 |
| Cyometrinil | 0.000859 | 6 | <1 |
| Cyprosulfamide | 1.78E-014 | 2.893E+010 | 2.652E+009 |
| Dicyclonon | 1.97E-009 | 231300 | 21200 |
| Dietholate | 7.51E-005 | 11.77 | <1 |
| Fenchlorazole-ethyl | 1.03E-006 | 508 | 46 |
| Fenclorim | 1.07E-005 | 43.25 | 3.484 |
| Fluxofenim | 3.87E-006 | 128 | 11.17 |
| Isoxadifen- ethyl | 3.41E-007 | 1348 | 123 |
| Jiecaowan | 4.45E-008 | 7872 | 721.2 |
| Mephenate | 2.38E-008 | 15240 | 1397 |
| Metcamifen | 1.32E-018 | 3.843E+014 | 3.523E+013 |
| Naphthalic anhydride | 6.19E-007 | 610.8 | 55.54 |
| Oxabetrinil | 6.08E-008 | 6676 | 611.5 |

Table 5: Biodegradation of safeners: Probability of Rapid Biodegradation (BIOWIN v4.10)

| Safeners | Biowin1 (Linear Model) | Biowin2 (Non-Linear Model) | $DT_{50}$ (typical)* | $DT_{50}$ (lab@ 293 K) * |
|---|---|---|---|---|
| Benoxacor | 0.7430 | 0.6339 | 50 | - |
| Dichlormid | 0.6359 | 0.2768 | - | - |
| Furilazole | 0.0712 | 0.0008 | 29 | 65 |
| Mefenpyr-diethyl | 0.3694 | 0.5283 | 17.5 | - |
| Cloquintocet-mexyl | 0.8198 | 0.9878 | 5 | 1.75 |
| Cyometrinil | 1.4015 | 1.0000 | - | - |
| Cyprosulfamide | 0.9114 | 0.9329 | - | - |
| Dicyclonon | 0.4376 | 0.0510 | | |
| Dietholate | 1.0723 | 1.0000 | | |
| Fenchlorazole-ethyl | -0.1882 | 0.0000 | 2.4 | 2.5 |
| Fenclorim | 0.4036 | 0.0817 | 26 | |
| Fluxofenim | -0.7974 | 0.0000 | | |
| Isoxadifen-ethyl | 0.8534 | 0.9916 | | |
| Jiecaowan | -0.1409 | 0.0003 | | |
| Mephenate | 0.5563 | 0.3468 | | |
| Metcamifen | 0.7065 | 0.5242 | | |

| | | | | |
|---|---|---|---|---|
| Naphthalic anhydride | 0.6532 | 0.5485 | | |
| Oxabetrinil | 0.3774 | 0.3307 | | |

*PPDB - Pesticide Properties Database: University of Hertfordshire

## 1.6 Photolysis

Photolysis of safeners is important because safeners are exposed directly to sunlight either in soil, on plants or crops, during transportation, and in water, if they are reaching surface waters (Balmer and Goss, 2000). Photolysis of chemicals is possible inside leaves and thus the mechanisms of such processes need to be studied (Schroer et al., 2019). Photodegradation could occur directly as a result of absorption of photons by a chemical or indirectly as result of photon absorption by other constituents (photosensitizing species) in the environmental compartment (e.g., water, soil), resulting in energy transfer to the target chemical and subsequent degradation (Remucal, 2014; Karpuzcu et al., 2016; Lin et al., 2019). The photochemical behaviour of chemicals can be affected by their chemical speciation and light absorption capacity (Challis et al., 2013; Remucal, 2014; Karpuzcu et al., 2016). For example, in photolysis, p*K*a values of a chemical and the pH of the environment are important parameters because they control the chemical speciation and dictate the chemical light absorption (Calvayrac et. al.,2013; Challis et al., 2013, 2014).

The p*K*a for benoxacor and furilazole are 12.99 and 16.44, respectively, and thus, they will be fully protonated at environmentally relevant pH values (Acharya and Weidhaas, 2018). For a chemical to have both protonated and deprotonated species, it must have a p*K*a in the pH range relevant in the environment (i.e., pH 4-9). When this is the case, chemical speciation can have a large impact on rates of photolysis, making the study of pH dependence in photolysis experiments very important (Calvayrac et. al.,2013; Challis et al., 2013; Remucal, 2014). Most

safeners are characterized by at least one aromatic ring, conjugated π system, with 1 or more heteroatoms (such as nitrogen, sulphur) and some functional group. All these aforementioned parameters facilitate direct absorption of solar radiation (Challis et al., 2014). However, Achaya and Weidhaas (2018) observed that both benoxacor and furilazole did not photodegrade (rate coefficient k = -0.0031 $h^{-1}$) to any extent under UV light (Halogen S302C lamp (Thorlabs, Newton, NJ) emitting 265 mW with a maximum output = 200 $W \cdot cm^{-2}$ (λ = 250 – 300 nm)). Nevertheless, Kral *et al.,* (2019) noted that benoxacor underwent direct photolysis ($t_{1/2}$ ~ 10 min) through photo-initiated ring closure when exposed to simulated natural sunlight (1000 W Newport Xe arc lamp, λ = 300-400 nm, output = $8.8 \times 10^{-3}$ W $cm^{-2}$). The difference in these observations might be because of the experimental setup or the energy and wavelength output of the UV source.

Dichlormid underwent degradation in both water and methanol at 254 nm, (313 kcal $mol^{-1}$) but did not transform under environmentally relevant UV light >290 nm due to the lower energy (Abu-Qare and Duncan 2002). Since the UV wavelength of 254 nm is more energetic than natural sunlight, such results are not directly applicable to environmental photolysis. Photodegradation rate constants determined in the lab at < 290 nm usually bear little meaning to environmental photolysis rates; however, they might be of importance in a place where UV light is being employed for water treatment. Compounds that cannot absorb light at > 290 nm can still undergo indirect photolysis via photo excited species such as dissolved nitrates, carbonates, iron, and dissolved organic matter, which is often abundant in natural waters, soil, and sediments. Direct photolysis quantum yields can also be measured for chemicals in order to predict photodegradation rates under any light conditions (Challis et al., 2014). Quantum yields are a characteristic property of a chemical and provide information about how efficiently a chemical degrades upon absorption of light (Remucal, 2014; Challis et al., 2014; Lu et al., 2015; Lin et. al., 2019). For example, benoxacor has a quantum yield of 0.14, which suggests

that benoxacor is a highly photo-efficient organic micro-pollutant (Kral, 2018). Hence, for photodegradation monitoring to be meaningful, efforts should be made to determine quantum yields and rate constants as a function of p*K*a and consider exposures under both simulated and natural sunlight.

Photodegradation of the herbicide metolachlor was enhanced in the presence of benoxacor on a quartz surface and, to a lesser extent, in water, but not on a soil-simulated (kaolinite) surface (Su et al., 2019). This suggests that the reaction media and chemical mixtures can play a role in photolysis. However, an earlier study showed that dichlormid did not significantly affect s-ethyl dipropylthiocarbamate (EPTC) photodegradation at 254 nm or at > 290 nm in water or methanol (Abu-Qare and Duncan, 2002). Hence more study is needed to fully understand the role safeners play in the photodegradation of herbicides and potentially the role that herbicides play in the photodegradation of safeners.

In the report of Abu-Qare and Duncan (2002), the role of singlet oxygen, hydroxyl and peroxide radicals were highlighted. Singlet oxygen is a nucleophile and thus can react with any electron-rich moiety (McNeill and Canonica, 2016; Katagi, 2018). For example, the furan moiety in the safener furilazole is believed to be reactive with singlet oxygen and was indicated as the major reason for the indirect photolysis of furilazole (Kral, 2018; Kral et al., 2019). Other components present in natural waters can facilitate electron transfer, which will aid photodegradation of the chemicals, and might be facilitating degradation of safeners that do not absorb light above 290 nm. These indirect mechanisms are complex and more difficult to predict than direct photolysis due to the multiple ways in which some chemicals can interact with photosensitizers present in natural waters (nitrate, carbonate, dissolved organic matter) (Challis et al., 2014; Remucal, 2014; McNeill and Canonica, 2016).

Carbonate species are believed to contribute to photodegradation of sulphur containing compounds in natural water (Huang and Mabury, 2000) as a result of abstraction of electrons

from sulphur. This might play an important role in indirect photolysis of safeners such as CPS containing sulphur moieties. Hydroxyl radicals are capable of oxidizing aromatic ring and alkyl chains (Zepp et. al., 1987; Katagi, 2018); therefore, this is an important oxidizing agent to consider for photolysis in natural waters. For example, indirect photolysis of furilazole and dichlormid in the presence of $^{\bullet}OH$ radicals resulted in 30%, and 20% increased decay (Kral et al., 2019). Both furilazole and dichlormid exhibited 80-90% transformation in the presence of nitrate (which is known to mediate production of $^{\bullet}OH$) during indirect photolysis. Photodegradation products are sometimes assessed when studying photolysis kinetics and can be essential to characterize since in certain scenarios they can be more toxic than their parent chemicals. MEF yielded four transformation products when irradiated under sunlight with a first-order rate constant of 0.580 $h^{-1}$ (El Boukili et al., 2015). Two of these products were a result of hydrolysis (mefenpyrethyl and mefenpyr) (Hertkorn et al., 2010), suggesting that hydrolysis might be an important loss pathway in addition to photolysis (Chovelon et al., 2005).

Variability in kinetic concentration data for safeners has been reported by both Abu-Qare and Duncan (2002) and Acharya and Weidhaas (2018). Kinetic concentration data is important for allowing comparisons across studies and understanding variability in the methods, especially as it relates to rate constants and quantum yields, which can have large uncertainties when compared across studies (Challis et al., 2014). Natural sunlight exposure experiments will provide the most realistic estimation of photolysis rates at the specific latitude and sunlight conditions. However, most researchers conduct these types of photolysis experiments under simulated sunlight using laboratory photo-reactors, which can limit their applicability to environmental systems. Additionally, light attenuation in natural systems can be significant so the result of photolysis based on test tube exposures can only be applied to near surface photolysis (Lu et al., 2015). Despite the reports of dichlormid, benoxacor, furilazole (Kral, 2018), and MEF (El Boukili et al., 2015) photolytic fate, more work is still

needed on other safeners since photochemical behaviour varies greatly from chemical to chemical (Boreen et al., 2004; Chnirheb et al., 2012).

## 1.7 Chemical reduction and nucleophilic substitution

Electron-rich compounds such as hydrogen sulphide may be able to reduce safeners. For example, graphite (black carbon) can aid the transfer of electrons from hydrogen sulphide to dichloroacetamide safeners through nucleophilic substitution pathways (Xu et al., 2020). This process can lead to transformation of dichloracetamide safeners. Specifically, hydrogen sulphide has been implicated to facilitate the conversion of dichlormid, benoxacor and AD-67 (Xu et al., 2020). Reductive dechlorination was responsible for dichloroacetamide safener degradation when reduced by Fe (II) - amended goethite in anaerobic abiotic system. (Sivey and Roberts, 2012). Chemical such as iron, manganese and hydrogen sulphide are naturally occurring in the subsurface environment (Ricko et al., 2020).

Different chemicals generally have different abilities to reduced safeners. For example, the half-life for dichlormid was 10 h by graphite-hydrogen sulphide (Xu et al., 2020) and 50 h by Fe (II) – amended goethite (Sivey and Roberts, 2012). Also, the increase in molar ratio of Fe (II) to Mn (IV) oxide, increased the rate of transformation of benoxacor and furilazole (Ricko et al., 2020). This mixture of Fe (II) and Mn (IV) oxide did not cause transformation of dichlormid after six hours of reaction. This suggested that individual safeners react differently.

However, the herbicide (S- metalachor) and three surfactants (Triton X-100, sodium dodecyl sulphate (SDS) and Myristryltrimethylammonium bromide (MyTAB)) did not influence rates of transformation of dichloroacetamide safeners, even in the presence of $Cr(H_2O)_6^{2+}$ (Ricko et al., 2020). This could be an indication that the herbicide (active ingredient) and accompany safeners may not naturally react.

Finally, since safeners can be transformed, their transformation products also need to be considered in the assessment of fate, transport, and ultimate hazard to non-target organisms. For instance, after harvest of rice, no FEN or isoxadifen-ethyl was detected in straw or grain from plants, which were previously treated with a formulation containing both chemicals. However, the metabolite of FEN, fenoxaprop-P was detected (Lucini and Molinari et al., 2010; 2011). Isoxadifen-ethyl dissipated completely from the target, while FEN was metabolised to a single product, which was still present as a residue.

## 1.8 Biological degradation

Microorganisms, such as bacteria in the soil, can also degrade organic chemicals present in their environment (McGuinness and Dowling (2009); Fenner et al., 2013; Joutey et al., 2013). Soil microorganisms will often treat organic chemicals such as herbicides and safeners as xenobiotics, and thus they can develop adaptive strategies which might result in chemical transformation. Biological degradation of pesticides has not received much attention because most pesticides are not quick to degrade (Parsek et al., 1995; Rama Krishna and Philip 2011; Fenner et al., 2013). Microorganisms initiate chemical degradation by splitting the parent chemicals, then hydrolysing it (Nakamiya et al., 2007). However, soil microorganism might find dichloroacetamide safeners more difficult to degrade/metabolise because of the additional chlorine atom compared to chloroacetamide herbicides (Xu et al., 2020).

Predictions on biodegradation of safeners using EPI suite are reported here (Table 5). Safeners such as benoxacor are moderately biodegradable based on Biowin model predictions of 0.7430 and 0.6339, for linear and nonlinear models, while fenchlorazole-ethyl have biodegradation probabilities of -0.1882 and 0.000, from linear and non-linear models. In general, a half-life ($DT_{50}$) of a chemical in the soil below 50 days is non-persistent, between 50 and 70 is moderately persistent, $\geq 70 \leq 100$ days is persistent. Benoxacor, furilazole, MEF cloquinocet-mexyl, fenchlorazole-ethyl and fenclorim have measured $DT_{50}$ values of 50, 29,

17.5, 5, 2.4 and 26 days, indicating that they are non-persistent, consistent with most of the safeners reported in Table 5.

## 1.9 Safeners in the environment

While safeners have been used to reduce the negative effect of herbicides for a long time and information about herbicides toxicity is common in literatures. There is limited information on the presence of safeners in the environment. Nevertheless, results have shown that safeners will move through the environment to surface waters like herbicides do (Acharya et al., 2020). Dichloroacetamide safeners were found in seven Midwestern U.S streams (five in Iowa and two in Illinois) in samples collected between spring 2016 to summer 2017 (Woodward et al., 2017). The maximum concentrations ranged from 42 to 190 ng/L. Another study was conducted to determine the level of CPS in ground water and surface samples collected around Midwestern U.S where there is history of safeners usage, and the maximum ranged found was 22.0 to 5185.9 ng/L in surface water (McFadden, 2021). Thus, safeners are available in the in the environment.

### 1.9.1 Safeners of interest

CPS and MEF belong to the acylsulfonamide and pyrazoline class of safeners, and they are relatively new safeners that have not been scientifically examined thoroughly, regarding environmental fate and biological effects on non-target species. CPS's application is typically through spray formulations during pre-emergence or early post-emergence stage of the planting season in the early spring, a time when heavy rains and large amounts of runoff often occur in the midwest where corn is extensively grown (Andersen et al., 2012; Dashevskaya et al., 2013).

MEF is a foliar acting safener of the pyrazoline group used with several herbicides on cereal grain crops (Dias et al., 2021) and its application is during post emergence of the target crops. These compounds have been found in surface waters where they may pose threats to

non-target organisms (Mcfadden and Hladik, 2021). Studies have shown that some safeners are able to transform into products with increased biological activity that may pose human and environmental health risks, under environmentally relevant conditions (Abu-Qare et al., 2002; Woodward et al., 2018; Kral et al., 2019; Mcfadden and Hladik, 2021). CPS has been found to transform into the degradates, *N*-cyclopropyl-4-sulfamoylbenzamide and cyprosulfamide desmethyl (McFadden and Hladik. 2021).

## 1.10 Toxicity

Chemical risk assessment is one way from which concerns raised by continuous use and production of chemicals for agricultural purposes could be resolved (Joly et al., 2013). Potential impact of toxic substances including pesticides needs to be assessed by evaluating the responses of living organisms to these substances. Several studies evaluating such effects have been published to better inform the public and help researchers in further toxicity studies. A Microtox® study of the effects of the safener benoxacor on bacteria (*V. fischeri*) has shown that the chemical was more toxic considering the concentration capable of inhibiting half ($IC_{50}$) of the bioluminescence of *V. fischeri* ($IC_{50}$ of 93 mg/L) than atrazine ($IC_{50}$ of 197 mg/L) (Joly et al., 2013). Benoxacor has been reported to decrease growth of the freshwater algae, *S. capricornutum* (Day and Hodge, 1996). Benoxacor is classified as having high toxic potency with an effective concentration ($EC_{50}$), exhibiting a maximal response in half of the algae population ($EC_{50}$ of 0.63 mg/L) of fresh water algae (*S. subspicatus*) (Table 6) (European Chemical Agency, 2020). Its lethal concentration ($LC_{50}$) that kills half of the freshwater fish species, *Ictalurus puntatus* is 1.4 mg/L, and the ($LC_{50}$) for *Lepomis macrochirus* is 6.5 mg/L, this is considered to be a moderately toxic potency to aquatic species (US EPA, 2020; Xu et al., 2020; PPBD, 2020). Furilazole has lesser toxic potency with an $LC_{50}$ of 4.6 mg/L for fresh water fish and $EC_{50}$ of 85.2 mg/L for algae compared to benoxacor (US EPA, 2020; PPBD, 2020; SDS, 2020), while the $LC_{50}$ of fluxofenim to rainbow trout was reported to be 0.86 mg/L

(SDS, 2020). During chronic exposure to benoxacor, no observed effect concentrations (NOEC) of 0.31, and 0.35 mg/L was reported for *Pimephales promelas* (freshwater fish) and *D. magna* (freshwater invertebrates), respectively (European Chemical Agency, 2020). Benoxacor also caused a significantly reduced condition index (0.016 mg/L) of rainbow trout (European Chemical Agency, 2020). Benoxacor might be moderately toxic to birds, honeybees, earthworms, and most aquatic organisms (Table 6) (European Chemical Agency, 2020; PPBD, 2020). MEF and CPS were stated as harmful to aquatic life with long lasting effects (SDS, 2020). Similarly, Isoxadifen- ethyl, fenchlorazole-ethyl and cloquintocet-mexyl were described as very toxic to aquatic life with long lasting effects and fenchlorazole-ethyl may cause cancer (SDS, 2020). Alternatively, dichlormid might be relatively non-toxic to birds, fish, and aquatic invertebrates (considering their lethal concentration in Table 6 (PPBD, 2020)). As an example of potential hazards, the safener, naphthalic anhydride, can cause allergic skin and eye irritation in humans (National Center for Biotechnology Information, 2020; SDS, 2020), depending on the concentration of exposure.

Maximum concentrations found in surface water for CPS was 5185.9 ng/L and for the degradates, *N*-cyclopropyl-4-sulfamoylbenzamide and cyprosulfamide desmethyl, it was 616.9 ng/L and 22 ng/L respectively (McFadden and Hladik. 2021). Concentrations of these safeners that are considered toxic are high compared to the amount measured in the environment (Woodward et al., 2018; McFadden and Hladik. 2021). Therefore, there could be possibilities of lethal toxicities of this class of chemical if there were spillage to the environment. Nevetheless, more work is needed on the effects of all groups of safeners to understand their various effects.

There are reports that possible synergistic effects exist between safeners and herbicides when used together (Bolyard et al., 2017; Acharya & Weidhaas 2018). However, an earlier study showed that mixtures of benoxacor and s-metolachlor did not alter the toxicity of s-

metolachlor to any extent (Joly et. al., 2013). The $IC_{50}$ of s-metolachlor before the addition of benoxacor was 178 mg/L, and the $IC_{50}$ of the mixture was 174 mg/L (Joly et. al., 2013). More work is needed using other organisms and combinations of different safeners and herbicides to fully understand mixture toxicity of these chemicals.

Table 6: Ecotoxicity of safeners on selected organisms

| Safeners | Fish | D-Magna | Algae | Honeybees | Earth worm | Birds- | BCF* |
|---|---|---|---|---|---|---|---|
| | 96 h $LC_{50}$ | 48 h $EC_{50}$ | 72 h $EC_{50}$ | 72 h $LD_{50}$ | 14 days $LC_{50}$ | $LD_{50}$ | (BCFBAF v3.01) |
| | mg / L | mg / L | mg/L | µg / bee | mg / kg | mg / kg | |
| Benoxacor | 6.5 | 4.8 | 0.63 | 100 | 1000 | 2000 | 47.15 |
| Dichlormid | 141 | 161 | - | - | - | >5200 | 6.34 |
| Furilazole | >6.2 | >26 | >85 | >100 | - | >2000 | 6.48 |
| Mefenpyr-diethyl | 4.2 | 53 | 1.65 | >700 | >1000 | >2000 | 30.83 |
| Cloquintocet-mexyl | >14 | >100 | 0.53 | >100 | 1000 | >2000 | 88.47 |
| Cyometrinil | >5.6 | - | - | - | - | - | 25.84 |
| Cyprosulfamide | >106 | >102 | >99.7 | - | - | - | 16.15 |
| Dicyclonon | - | - | - | - | - | - | 6.85 |
| Dietholate | - | - | - | - | - | - | 92.09 |
| Fenchlorazole-ethyl | 0.08 | 1.8 | - | >300 | - | >2400 | 1028 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Fenclorim | 0.6 | 2.2 | 20.9 | 20 | 62.5 | 500 | 701.3 |
| Fluxofenim | 0.86 | 0.22 | - | - | - | 2000 | 78.69 |
| Isoxadifen- ethyl | 0.34 | 0.51 | 1.26 | - | - | - | 88.43 |
| Jiecaowan | - | - | - | - | - | - | 2.389 |
| Mephenate | - | - | - | - | - | - | 7.042 |
| Metcamifen | - | - | - | - | - | - | 2.718 |
| Naphthalic anhydride | - | - | - | - | - | - | 132.6 |
| Oxabetrinil | 7.1 | 8.5 | 10.7 | >20 | - | 2500 | 36.07 |

*BCF = bioconcentration factor

### 1.10.1 Enzyme activity analysis

Chemicals such as safeners which are not expected to induce lethality could cause other sub lethal effects including oxidative stress in aquatic organisms. This effect of chemicals on living organisms can be monitored by measuring changes in the activities of the antioxidant enzymes such as SOD, CAT and GST. SOD and CAT provides first line of defence against cellular reactive oxygen species (ROS). These enzymes are responsible for elimination of ROS induced by toxicants, in which SOD firstly disproportionate the highly reactive and potentially toxic superoxide radicals ($O_2^-$) to hydrogen peroxide ($H_2O_2$). After which, $H_2O_2$ is converted to molecular oxygen and water by CAT catalyzing (Ma et al., 2014). A decreased in activities of SOD was recorded in zebrafish exposed to benaxacor compared to control, which suggest that the zebrafish embryo protective system might have been destroyed as a result of benaxacor (Liu et al., 2022). However, the study recorded a higher expression of glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) in mixture of benoxacor and metolachlor (an herbicide and its safener) compared to benoxacor alone in *D. rerio* embryos (Liu et al., 2022).

GST belongs to the phase II detoxification enzyme, and they play significant role in metabolizing and detoxification of chemicals. They can transform a variety of hydrophobic pollutants to prevent cellular membrane from lipid peroxidation. Previous studies have shown the role of herbicide safeners in increasing herbicide detoxification by upregulating cytochrome P450 (CYP450), glutathione (GSH), glutathione S-transferases (GSTs), and ATP-binding cassette (ABC) transporter proteins (Liu et al., 2021; Yang et al., 2018).

Activities of CAT, SOD and GST have been found to be significantly altered in combined group of chemicals compared to individual groups after 14 and 21 days of exposure

to insecticide imidacloprid (IMI), the herbicide acetochlor (ACT), and the fungicide tebuconazole (TBZ) in zebrafish (Chang et al., 2020). Similarly, benoxacor and *S*-metolachlor mixture effect on GST and SOD were different from their individual effects (Liu et al., 2022). Thus, monitoring the activities of safeners singly and in combination with herbicides will give a better understanding of their toxicity.

### 1.10.2 Toxicity prediction software

Since, there are limited information about the toxicity of safeners, it become prudent to use toxicity prediction software to gain insight about safeners' possible toxicity. Several toxicity software have been used in literatures to evaluate the toxicity of different chemicals against different organisms (Hamadache et al., 2014). Insilico predictive methods are becoming popular because of the need to reduce and replace the use of animals in predicting biological activity of chemicals. This is reasonable for many reasons, including, economic considerations, reduction of time constraints, and pressure of public opinion. Prediction softwares come with advantages and their demerits. However, the need to take into account the active ingredients and metabolites together for determination of environmental fate and toxicity of a chemical compounds (Carles et al., 2018) has been emphasized.

Prediction software used in this study include the toxicity estimation software tool (T.E.S.T), Adsorption, Distribution, Metabolism, Elimination and Toxicity (ADMET) predictor, US EPA Estimation Program Interface (EPI) Suite$^{TM}$ and molecular docking. These are used to get information that predicts how these chemicals could behave in living organisms. The structures and properties of safeners and herbicides used in this study were imputed into these software to predict their fate and toxicities, so that the results can be compared with experimental results. Testing everything experimentally is laborious and time consuming, thus using predictive software is essential. Toxicity prediction to determine lethal dose (LD50) of 62 herbicides on rats have been done using quantitative structure -activity relationship in a

single study (Hamadache et al., 2014). This would take a long time if the research were to be done in the laboratory alone.

## 1.11 Molecular docking

Molecular docking has emerged as a powerful tool to explore the mechanisms of the protein-herbicide interactions. This approach can model the interaction between a small molecule and a protein at the atomic level. It enables the characterization of the behavior of small molecules in the binding site of target proteins and provides insight into fundamental biochemical processes. (McConkey et al., 2002; Meng et al, 2011). It has been described as a method that allow receptor or protein to bind with ligands to form a stable complex when they are in favoured orientation (Agarwal and Mehrotra, 2016; Kumar and Mishra, 2016). The main aim of molecular docking is to computationally simulate the molecular identification process and accomplish an optimized conformation so that the free energy of the overall system is minimized. This help to achieve the three connected goals of docking which are pose prediction, virtual screening (or analysed the interactive mode) and binding affinity estimation (Fan et al., 2019; Guedes et al., 2014). Molecular docking has therefore played a critical role in drug design and discovery process (Fan et al., 2019). It is now used in other fields to understands interactions between two compounds. The basic tools of a docking include a search algorithm and an energy scoring function for generating and evaluating ligand poses (Guedes et al., 2014). Crystal structure of the protein can be obtained from protein data bank, which make the docking relatively simple and cheap (Fan et al., 2019). Nevertheless, docking methods employ rough approximations because of entropic effects, which make the results of docking not be the only factor to be consider in making conclusion on the interaction of a ligands and a receptor. Be as it may, in case of small ligands (such as safeners) with few bonds, there is possibility of obtaining satisfactory results when docked against protein binding pockets in which flexibility does not play significant factor (Guedes et al., 2014).

Endocrine disrupting compounds such as polychlorinated biphenyls plasticizers, and pesticides have been docked against human estrogen receptor α and found to bind in the steroid binding cavity, interacting with at least one of the two hydrophilic ends of the steroid binding site (Celik et al., 2008). Molecular docking showed that novel substituted thiazide/thiazole compounds compete with chlorsulfuron in the acetolactate synthase active site, causing the herbicide ineffective in maize (Fu et al., 2019). Thus, using software to predict possible interaction between chemicals of interest and receptor is becoming popular. The approach is used in this study to predict toxic effects that will result from the interactions in the binding sites between the safener and herbicides. The safeners and herbicides used in this project will be docked with certain protein of interests (growth protein in daphnids and hatching protein in *D. rerio*) to observe their binding scores, distances and affinities which can be used to understand and predict the chemicals' ability to cause an effect depending on the strength of the bond at an active site.

## 1.12 Summary

Despite having biological activities, chemical safeners, which are antidotes for the effects of herbicides on target plants, have, for regulatory purposes, been classified as inert. Just as for herbicides with which they are formulated or applied with, safeners can dissipate and be transported to aquatic environments and have been detected in surface waters. However, fates, and potential mobilities of safeners in the environment as well as possible adverse effects on non-target organisms, which are affected by estimates of exposure, including magnitude and duration of exposure in addition to toxic potencies have received little attention to date. Some safeners (such as benoxacor) have low aqueous solubility, are quite volatile and, based on their chemical properties, have potential for leaching to groundwater. Here, several possible routes, mechanisms, and rates of dissipation of safeners used with herbicides, including surface runoff, sorption/ desorption, photodegradation, leaching and biological transformation, were reviewed.

Most of the safeners can be readily leached because of their high solubilities. Laboratory experiments done in the absence of natural materials, such as soil and water could not be easily used to predict what might be occurring during and after the application of safeners in the environment. Most of the safeners are not persistent in soil systems. Hazards presented by safeners were also investigated by comparing measured or predicted exposures to aquatic organisms with threshold toxic potencies for effects. It was determined that various safeners exhibit different potentials for exposure in aquatic environments and have a range of toxic potencies among organisms. Most safeners have low mammalian toxicity and moderate potential for bioaccumulation. They are moderately toxic to birds, honeybees, earthworms and most aquatic organisms.

Considering the presence of safeners in the environment more work is needed to understand their dissipation mechanisms. Baseline information is needed on safeners from various nations of the world so that the exposure of this class of chemical can be used in risk assessment. Furthermore, various degradation products from each of the commonly used safeners should be studied. Also, aggressive efforts should be targeted towards understanding the toxicity of safeners on different organisms. The fate and toxicity of some recently synthesized safeners such as the closely related sulphonamide safeners, metcamifen and CPS and their effects on living organisms need to be evaluated as this could help to determine their effects on impacted ecosystems and inform proper use and management of these important agrochemicals.

## 1.13 Objectives and hypotheses

Considering the unpredictable behaviour of chemicals depending on various factors, exploring, and understanding the toxicity of safeners and their co-herbicides is important to developing their chemical hazard assessment. Typically, several toxicity testing have been done in times past on chemicals, one chemical at a time to better understand their effects,

interaction, and bioavailability in environmental media and across food chains. Also, the study of mixture in toxicology is increasing and this is important because chemicals are often found together in the environment. However, many of these studies are limited to chemicals termed priority contaminants of concerns and not to those who are termed inert especially safeners, hence, most studies are focused on supposed toxic chemical. Most safeners' evaluation was carried out on the crops (Dias et al., 2021) with which they are to protect from herbicides' while limited or no attention was given to their effects on non-target aquatic organisms considering that the possibility of safeners washing away into the aquatic environment. Hence, there is a need to determine risk/hazard thresholds and understand effects of these supposed inert safeners, while estimating apical toxicity thresholds of SESs singly and in combination with their co-herbicides. Using acute and chronic biological assays and simulation software studies to understand the link to adverse effects in both invertebrate and vertebrate animal models will help to evaluate the capability of these chemicals in regulatory ecotoxicology.

Therefore, the overall objective of this dissertation was to determine and evaluate effects of short-term and long-term embryonic exposure assays to derive sublethal and lethal effects of CPS, MEF and ISO and FEN individually and in binary mixtures respectively, using a cladoceran invertebrate (*D. magna*) and early life stages (ELS) of *D. rerio* (- a representative vertebrate model. This research utilized techniques such as evaluation of effects of the two chemical safeners and their co-herbicides on apical endpoints in *D. magna* and ELS of *D. rerio*. It also utilized the use of simulation software with the goal to linking predicted and observed perturbations to adverse apical outcomes and effects and to derive approximate protective apical thresholds. This study serves as proof in the use of short- and long-term embryonic assays in deriving thresholds to support regulatory decision-making. There is repetition in some objectives, and this is to separate each chapter according to their specific objectives. The primary objectives and testable hypotheses for each chapter were as follow:

*Objectives 1: To understand gaps in literature pertaining to CPS and MEF which are emerging safeners commonly used to protect grains from ISO and FEN respectively and the general knowledge of safeners in the environment (Chapter 2).*

*Objective 2: To understand the sorption behavior and toxicity of the herbicide safener, CPS using several toxicity software and an in vivo approach. (Chapter 3).*

1. $H_o$: There are no statistical differences in apical responses resulting from acute (survival rates) and chronic (reproduction parameters) exposure across treatment groups after exposure of *D. magna* to CPS from <24 hours to 21 days

2. $H_o$: There are no statistical difference in the leaching/partitioning capacity of cyprosulfamide in selected Saskatchewan soils

*Objective 3: To establish the toxicity of MEF to D. magna using experimental and computational studies for acute and chronic exposures (Chapter 4).* The computational studies referred to in this chapter used several toxicity software and simulation studies to predict toxicity of MEF coupled with the little information from literature. This is done to predict toxicity using non-animal testing method.

1. $H_o$: There are no statistical differences in apical responses resulting from acute

(including survival and mortality) and chronic (reproduction) exposure across treatment groups after exposure of *D. magna* to MEF from <24 hours to 21 days

2. $H_o$: There are no differences between toxicity thresholds estimated from experimental and prediction software tools used in determining and predicting toxicity in *D. magna* exposed to or supposedly exposed to MEF.

*Objective 4: To understand and characterize the individual and combined effects of the herbicide, Isoxaflutole and its plant safeners, cyprosulfamide to ELS of D. rerio from 24 hours to 21 days. (Chapter 5)*

1. $H_o$: There are no statistical differences in apical responses resulting from acute (including survival and heart rate (96 hours)) and chronic (growth) exposure across treatment groups after exposure of embryonic *D. rerio* to CPS or ISO or a binary mixture of CPS and ISO from <24 hours to 21 days (swim up stage)
2. $H_o$: There are no statistical differences in the expression of GST and SOD activities across treatment groups after exposures of embryonic *D. rerio* to CPS, ISO and a binary mixture of CPS and ISO from 96 hours to 21 days
3. $H_o$: There are no differences in the docking score of CPS and ISO after using toxicity software to determine binding affinity of these chemicals to ZHE1 receptor which is related to endpoints observed in this study

*Objective 5: to characterize the effects of individual and combined effect of MEF safener and its co-herbicide, FEN, to ELS of D. rerio to swim up stage and to use toxicity predictive software to determine binding affinity of these chemicals to hatching, ZHE1 receptor (Chapter 6).*

1. $H_o$: There are no statistical differences in apical responses resulting from acute (including survival rates and heart rates) and chronic (growth) exposures across treatment groups after exposure of embryonic *D. rerio* to MEF, FEN and a binary mixture of MEF and FEN from <24 hours to 21 days
2. $H_o$: There are no statistical differences in the expression of GST and SOD activities across treatment groups after exposures of embryonic *D. rerio* to MEF, FEN and a binary mixture of MEF and FEN from 96 hours to 21 days (swim up stage)

3. $H_o$: There are no differences in the docking score of MEF and FEN after using toxicity software to determine binding affinity of these chemicals to hatching enzyme receptor.

## 1.14 Scope and Limitations

This dissertation implemented several separate ELS exposure experiments using two representative animal models (*D. magna* and *D. rerio* embryos). There were logistical limitations which resulted to difference in the start time and study durations. Daphnids were cultured in-house, and *D. rerio* embryos were obtained from *D. rerio* culture in the Collaborative Science Research Building (CSRB)). Nevertheless, acute toxicity tests used to assess hatchability and mortality were conducted at 4dpf for all exposures. Chronic toxicity tests assessing survival, growth and reproduction for each model were designed to last for 21 days and experiments were terminated before 21 days depending on observations and trend of effects, thereby causing a range of apical responses to be measured/observed at different time points whenever it was logistically possible. Some observations were also opportunistic and qualitative, and thus, statistical tests were not possible. Finally, this research did not aim to develop and establish new statistical methods and workflows. Instead, it used well established toxicity methods, software, and workflows recommended by respectable groups and organizations and which are well cited in the literature. This dissertation leveraged the use of these existing tools and applied these to datasets derived from *D. magna* and ELS of *D. rerio* exposures.

# 2 Chapter 2: Toxicity and Sorption Behavior of the Herbicide Safener Cyprosulfamide

## 2.1 Preface

Building upon the results of chapter 1, this chapter leveraged on the review of the safener, CPS, taking into consideration its sorption behaviour to selected soils and understanding its link with its toxicity. In this chapter a representative cladoceran invertebrate, *D. magna (<24 hours)* was used to evaluate the toxicity of this safener to evaluate effects on apical endpoints including, survival, growth, and reproduction. This chapter determine the sorption properties, octanol-water partition co-efficient, acute, and chronic effects of CPS by looking into its affinity and mobility, for and in soil. Results were then compared to its physicochemical properties and sorption behaviour to create a link between these properties and observed effects. *D. magna* was now exposed to varied concentrations of CPS to determine its effects on apical endpoint. The results from the sorption studies gave information on whether CPS can get into surface water and affect aquatic organisms and it helped to design the experiment having understood the properties of CPS.

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## 2.2 Abstract

Cyprosulfamide (CPS) is an herbicide safener that works against the injurious effects of herbicides such as isoxaflutole (ISO), dicamba, nicosulfuron, tembotrione, thiencarbazone-methyl. However, its sorption behaviour in soils and toxicity to aquatic organisms have not been thoroughly examined. This study determined the octanol-water partition coefficient, sorption properties, acute and chronic, toxic effects, and potency of CPS to the cladoceran water flea, *D. magna*. The influence of soil properties such as organic carbon content, cation exchange capacity, pH, and field capacity on adsorption and desorption properties were also examined. The Log Kow (0.55) of CPS was less than that of some other safeners, such as benoxacor or furilazole, which have been found in aquatic environments. Sorption of CPS to the soil was driven by pH with greater sorption at lesser pHs. Other characteristics such as cation exchange capacity (CEC), organic carbon content, and field capacity seem not to correlate with the distribution coefficient directly. CPS generally has a low affinity for soil and is thus mobile and prone to transport to surrounding surface waters. No lethality was observed at the highest concentration (120 mg/L) tested for acute toxicity to *D. magna*; hence the $LC_{50}$ will be greater than 120 mg/L. During chronic exposures, CPS caused adverse effects at a concentration of 120 mg/L on the number of neonates and brood size. The death rate for the chronic study was a function of concentration and increased with days of exposure. CPS is unlikely to cause lethality to *D. magna* at relevant environmental concentrations.

Keywords: Sorption, Lethal toxicity, Octanol-water partition coefficient, Cyprosulfamide safener, Acute test.

## 2.3 Introduction

Herbicides are essential to agriculture worldwide because of the increased use of reduced tillage systems, especially in North America. Hence, the potential migration of this class of chemicals and their formulations, including adjuvants and safeners, to groundwater and surface waters needs to be evaluated for potential effects on non-target organisms (McFadden & Hladik, 2021; Oloye et al., 2021). Mobilities of chemicals in the environment and routes of dissipation can best be estimated from measured adsorption and desorption (OECD, 2000). Fates and behaviours of herbicides in the soil are controlled by various factors, such as transportation, retention, which is controlled by adsorption and desorption (Mamy & Barriuso, 2007), and transformation, all of which affect their efficacies and potential availability (Davies & Jabeen, 2003). Desorption of chemicals can be used to estimate the potential for release rate and the fate of chemicals in soils (El Boukili et al., 2018). Therefore, sorption and desorption isotherms can be used to approximate non-point source contamination of ground and drinking waters. Safeners have been observed in surface waters in agricultural regions of North America (McFadden & Hladik, 2021). For instance, the herbicide safeners furilazole, benoxacor, dichlormid and Ad-67 were detected during Spring and Summer in the Midwestern rivers of the USA at maximum concentrations ranging from 4 to 190 ng/L (Woodward et al., 2018). Concentrations of CPS in the same Midwestern river ranged from 0.022 to 5.186 mg/L (McFadden & Hladik, 2021).

CPS belongs to the acylsulfonamide class, a relatively new class of safeners. It is an example of a class that has received minimal scientific examination regarding its fate effects and transport in the environment. CPS is the first of the acylsulfonamide safeners to be registered for

use by the Environmental Protection Agency (EPA) and was first commercialized in 2008 for use on corn (McFadden & Hladik, 2021). CPS is usually applied through spray formulations during the pre-emergence or early post-emergence stages of the planting season in the early spring (McFadden & Hladik, 2021).

CPS is one of the latest safeners used in providing additional safety when using thiencarbazone-methyl (Lewis et al., 2016), and it has successfully been used to minimize the adverse effects of ISO, tembotrione, dicamba, nicosulfuron and other herbicides (Authority, 2018; Jablonkai, 2013). It is generally used to protect crops from the injurious effects of herbicides (Oloye et al., 2021). It can also protect plants from salinity stress and induce vigorous growth, includings the formation of new tillers and early flowering (Dashevskaya et al., 2013), so this safener is becoming popular. However, to the best of our knowledge, the sorption chemistry and toxicity of CPS have not been studied previously. Therefore, the toxicity of CPS was studied on apical endpoints in *D. magna*. *D. magna* is a model organism for determining chemical safety, and is commonly used as a system for ecotoxicological testing worldwide (Jordão et al., 2016). Hence, sorption chemistry of CPS considering soil of varied characteristics and toxicity of CPS on *D. magna* were studied.

While the literature is replete with reports of sorption of herbicides in soils, there are few reports of chemical behaviours of adjuvants such as herbicide safeners in soils. The potential mobility of safeners in soils and aquatic environments is a function of their aqueous solubilities (Acharya & Weidhaas, 2018). Octanol-water partition coefficients (*Kow*) provide a fundamental, integrative approximation of sorption to the soil as well as accumulation into aquatic organisms. They can be used to calculate the soil water partitioning coefficient (*Kp*) and organic carbon partitioning coefficients (*Koc*). The *Kow* can also be used to predict minimal toxicity.

CPS is predicted to be mobile (Oloye et al., 2021) and has been observed in surface waters of the Midwestern United States of America (USA) at maximum concentrations ranging from 0.022 to 5.186 mg/L (McFadden & Hladik, 2021). The log *Kow* of CPS, which is 0.55, suggests that this safener will have little potential to partition to soils and be soluble in water, thus mobile in soils and aquatic environments. By comparison of log *Kow* values with value for other herbicide safeners, benoxacor and furilazole are 2.23±0.16 and 1.96±0.22, respectively(Acharya & Weidhaas, 2018), this suggests that furilazole will be desorbed more readily than benoxacor. Similarly, cation exchange capacity and surface area have been highlighted as important in the adsorption of chemicals to soil (Doherty & Warren, 1969).

## 2.4 Materials and Methods

All chemicals were purchased from Sigma Aldrich, Toronto, Canada, at 98% purity. Four soils were collected from the surface layer (0-20 cm) of agricultural sites with no history of applications of herbicide safeners. Soils were air-dried under laboratory conditions, homogenized, and sieved through 2 mm mesh, after which samples were stored in methanol cleaned glass bottles. A part of the sample was characterized for organic carbon, inorganic carbon, soil texture, pH, field capacity, and cation exchange capacity following previously published methods (Ololade et al., 2014; Ololade et al., 2016).

### 2.4.1 Preparation of Test substance

CPS (400 ppb) was dissolved in 0.01 M $CaCl_2$ in ultrapure water, and the stock solution was kept at 4 ºC in the dark to avoid degradation before application to soil samples (OECD, 2000). Solvent concentration for toxicity study was kept at 0.01% acetone (v/v) throughout the experiment and the solvent as control. A semi-static approach with up to 98% water renewal per

day was adopted to retain stable concentrations of the test compounds in the exposure plates and petri dishes.

### 2.4.2 Determination of octanol-water partition coefficient (Kow) and acid dissociation constant (pKa)

The method employed for the determination of Kow was as published previously (Oloye et al., 2021), where a known volume (15 mL) of the CPS solution was brought to the desired pH with HCl or NaOH. The solution was added to an equal amount of octanol in a 50 mL polypropylene centrifuge tube. The mixture was shaken for approximately 2 h at room temperature and was then centrifuged at 3000 rpm for 20 min, and the two layers were separated. Aliquots of each phase were put into LC vials and spiked with atrazine D5 as the internal standard. The solutions were then injected into the HPLC to determine the amount of CPS in each phase (Oloye et al., 2021). Then, the Kow was calculated using equation x

$$Kow = \frac{Concentration\ of\ test\ substance\ in\ the\ octanol\ phase}{Concentration\ of\ test\ substance\ in\ the\ aqueous\ phase} \quad \text{..... Eq x}$$

The acid dissociation constant (pKa) was determined by titrating 0.01 mol HCl with a known concentration of CPS. Then the pH plot against the HCl volume was plotted, and the pKa was calculated from the pKb using equation xx. To confirm the pKa, the Marvin suite software was used to determine the pKa considering the microspecies distribution of CPS against pH.

$$pKa = 14 - pKb \quad \text{..... Eq xx}$$

### 2.4.3 Adsorption kinetics

A known weight (10 g) of soil samples was added to a 50 mL polypropylene tube containing 10 µg/L solutions of CPS (soil to water ratio was 1:5), and another tube contained the same test substances in 0.01 M $CaCl_2$, but without soil (control). A blank run was conducted with

the soil sample alone in an aqueous solution of 0.01 M $CaCl_2$ but without adding CPS. All experiments were performed in triplicate. Samples were collected sequentially over a 48-h period of mixing (0, 4, 8, 12, 24, and 48 h) on a thermostated shaker. At the defined time intervals, the mixture was centrifuged for 30 min to separate phases, then a small aliquot of the aqueous phase was immediately analyzed for test substances. The plot of adsorption against time was used to estimate the equilibration time for each soil. The $K_d$ (eq 1 &2) value at equilibrium was estimated from the concentration of test substances in aqueous and solid phases. The organic carbon normalized adsorption coefficient $K_{oc}$ (eq 3) was calculated from $K_d$.

$$K_d = \frac{C_s}{C_{aq}} \quad \text{…… Equation 1}$$

$$K_d = \frac{A_{eq}}{100 - A_{eq}} \frac{V_o}{M_{soil}} \quad \text{…… Equation 2}$$

$$Koc = K_d \frac{100}{\%oc} \quad \text{…… Equation 3}$$

$$Ms = (Mo - Ma) \frac{V_o}{V_a} \quad \text{…… Equation 4}$$

$$A_t = \frac{M_s}{M_o} 100\% \quad \text{…….. Equation 5}$$

Where $K_d$ is the distribution coefficient, Ms and Cs are the mass and concentration of the test substance in the soil, Va, Ma, and Caq are the volume of the aliquot, and mass of the test substances in the aliquot, and concentration in the aqueous phase. Ms is the mass of the test substance in the solid phase (eq 4) and $A_t$ is the percentage adsorption (eq 5). Mo is the original mass of the test substance, and Msoil is the mass of soil (g).

### 2.4.4 Desorption kinetics

This study was conducted immediately after the adsorption kinetics study. The mixture of soil and CPS was centrifuged, and the aqueous phase was removed as much as possible. The amount of aqueous solution removed was replaced with an equal amount of 0.01 M $CaCl_2$ solution without the test chemical. The mixture was agitated on a shaker, and a small portion of the aqueous phase was removed after centrifugation at the earlier defined time interval. The experiment continued until desorption equilibrium was reached. At each time the aliquot was taken, the same volume of 0.01 M $CaCl_2$ solution was added to maintain the initial ratio. The percentage desorption was calculated, then the percentage desorption (Dt) and adsorption (At) against time were plotted to estimate the reversibility of the adsorption process.

#### *2.4.4.1 Isotherm*

Five test substance concentrations (10, 20, 30, 40, 50 µg/L) were used, and an aliquot of the aqueous phase was taken for analysis after 12 h, which was the time predetermined that the equilibrium state would be reached. The equilibrium concentrations in the solution were determined, and the amount adsorbed was calculated for each concentration.

The aqueous phase was analyzed at the desorption equilibrium (24 h) determined from the desorption kinetics. The amount of test substance left adsorbed on the soil at desorption equilibrium was plotted as a function of the equilibrium concentration of the test substance in solution. The value of $K_F$ and $n^{-1}$ was determined by regression analysis following the Freundlich adsorption equation (eq 6). Furthermore, the correlation coefficient was estimated using eq 7 or 8.

$$C_{s\,(eq)} = \frac{C_o - C_{aq}(eq)}{M_{soil}} \frac{V_o}{} \quad \ldots\ldots\ldots\ldots \textit{Equation 6}$$

The Freundlich adsorption equation is given below

$Cs(eq) = K_{F.}(C_{aq(eq)})^{1/n}$ ……. *Equation 7*

*Or* $lnC_{s(eq)} = lnK_F + n^{-1}ln\ C_{aq(eq)}$ ……. *Equation 8*

Where, $K_F$ and 1/n are the Freundlich constants and can be related to the adsorption capacity (adsorption coefficient) and adsorption intensity, respectively.

The Freundlich desorption coefficient $K_F^{des}$, and regression constant (n) was determined from fitting the desorption data to the Freundlich plot.

### 2.4.5 Toxicity study of CPS to *D. magna*

#### 2.4.5.1 Acute toxicity test

D. magna were raised in a 30-ml glass vials containing 25 ml of test solution. Neonates less than 24 h old from the third generation of a single parthenogenetic female were used for each set of experiment. Ten replicates with one neonate per vial were used for each treatment concentration and the control group. CPS concentrations used were solvent control, 5, 20, 50, 90 and 120 mg/L in aerated reconstituted water. The dead or immobile *D. magna* were recorded at 24, 48, 72 and 96 h. Solvent concentration was kept at 0.01% acetone (v/v) throughout the experiment and the solvent control contains acetone and water alone. A semi-static approach with up to 98% water renewal per day was adopted to retain stable concentrations of the test compounds in the glass vial. The temperature during the test was 21± 1 °C. The daily photoperiod was 16 h of light and 8 h of darkness.

2.4.5.2 Chronic toxicity test

Chronic tests of CPS toxicity to *D. magna* were carried out following the OECD's recommended procedures (OECD, 2018). The test was done using a similar method as the acute study, except that the water was renewed every other day, and the test lasted for 21 days. The concentrations of CPS used were 10 mg/L and 120 mg/L in reconstituted water. The lower concentration represents what could be available in the environment and the higher concentration to test if the $LC_{50}$ of CPS would be comparable to the OECD proposed CPS $LC_{50}$ for daphnia, which was greater than 102 mg/L. The animals were fed with algae (*Chlamydomonas sp* and *Pseudokirchneriella subcapitata*) supplemented with vitamin B12 and selenium at every water change to ensure the general well-being of the animals. The number of neonates and the number of deaths were assessed whenever water and food were renewed. Glass vials used for the test were covered with a glass lid at 21 ± 0.5°C, under 16 h light and 8 h dark photoperiod and the experiment was terminated at 21 d. Test substance was dissolved in 0.01% v/v acetone to give a solvent control.

2.4.6 Statistical Analysis

The effects of safeners on the number of first-brood neonates, total number of neonates, and average brood size were analysed with a multivariate analysis of variance because responses are not independent. When significant multivariate effects were found, each response variable was analysed `separately using a one-way analysis of variance. Tukey's multiple-comparison test was used to detect differences between pairs of treatments.

2.5 Results and Discussion

Soil characteristics such as pH, texture, cation exchange capacity, and organic matter content, have been reported to have significant implications on the sorption capacities of soils

(Franco et al., 2009; Oloye et al., 2021; Ong & Lion, 1991; Weber et al., 2004). The influence of dissolved organic matter, total organic carbon, CEC, and pH on the sorption of the CPS were examined (Table 7). Soil pH varied from acidic to weak alkalinity. Soil pH is expected to significantly affect the portioning of CPS between soil and water because pH governs speciation (Franco et al., 2009). The soil with more acidity has the highest distribution coefficient (Kd), while those with pH greater than 7 have low Kd values for CPS. A previously published study showed a systematic decrease in adsorbed pentachlorophenol with increasing aqueous solution pH, regardless of the nature of the soil (Ololade et al., 2016). Similarly, another study showed that adsorption was only promoted by low pH (Gennari et al., 1998). Therefore, this observation of more adsorption in soil with the lowest pH supported the idea that soil pH significantly affects the distribution coefficient.

The organic carbon normalized distribution coefficient (Koc) for soil with the least pH is greater than the soil with the highest pH with orders greater than 3 (Table 7). The difference observed in Koc, and Kd soils with different pH values show that CPS exhibits acid functionality and dissociates in acidic conditions close to its pKa (Figures 2 and 3). Thus, soil acidity plays a significant role in the chemical sorption of CPS. The pH of 4.85 of the soil is close to the pKa of 4.5 and 4.19 for CPS determined experimentally and using software, respectively, confirming that CPS will dissociates at this soil pH. Other factors such as organic carbon, inorganic carbon, class, and field capacity of the soil do not have any positive correlation with the Kd and Koc. Thus, the distribution coefficient, and organic carbon normalized distribution coefficient, were determined to be a function of soil acidity.

Table 7: *Properties of selected Saskatchewan soils and their implication on CPS*

| Soil | Kd | Kdes | Koc | pH | OC | IC | Class | CEC (meq/100g) | FC (%w/w) |
|---|---|---|---|---|---|---|---|---|---|
| SK3 | 0.8 | 2.8 | 21 | 5.95 | 3.8 | 0.1 | Loam | 15.9 | 38.9 |
| SK6 | 6.6 | 1.6 | 300 | 4.85 | 2.2 | 0.1 | Loam | 18.8 | 35.5 |
| SK7 | 1.6 | 2.5 | 94 | 7.75 | 1.7 | 0.1 | Clay | 20.8 | 42.4 |
| SK8 | 1.5 | 2.8 | 52 | 7.35 | 2.9 | 0.3 | Sandy loam | 14.6 | 28.1 |

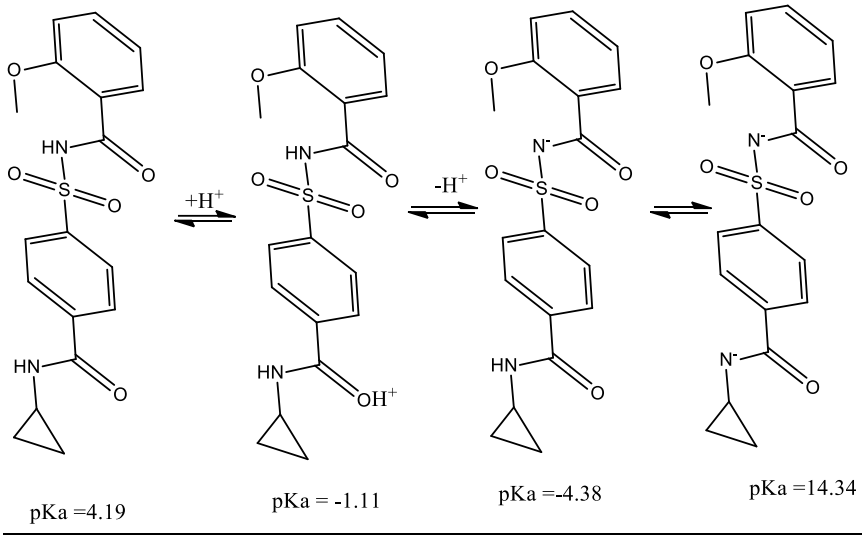


Figure 2: Possible dissociation scheme of CPS at different pH

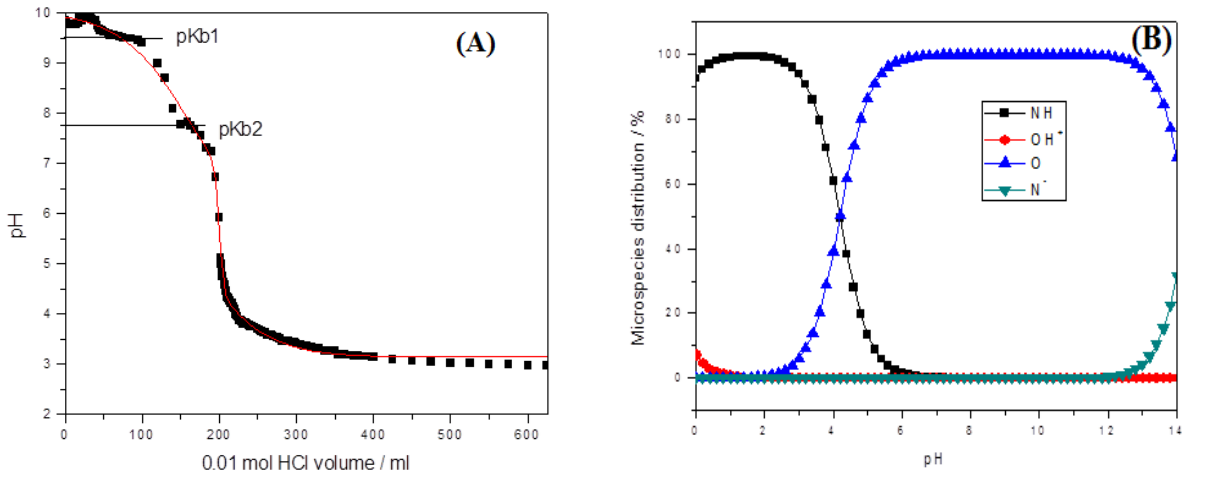


Figure 3: Determination of pKb of CPS (A) titration of 0.01 mol HCl against CPS, (B) % microspecies distribution of CPS against pH simulated using Marvin suite.

It has earlier been previously reported that CPS has the potential to be mobile in soil based on predictions from KOWWIN v1.68 implemented within the EPI suite (EPA 2021; Oloye et al., 2021). The log Kow reported from the software was 2.3, and the one determined experimentally was 0.55 (see table 8). However, both log Kow agreed that the chemical would be relatively mobile in soil (Oloye et al., 2021). It is well established that a positive relationship exists between adsorption capacity and solubilities/log Kow (Acharya et al., 2020; Acharya & Weidhaas, 2018; Oloye et al., 2021). Thus, CPS can be soluble and mobile because of its low logKow. The low logKow could be why CPS has been seen to occur in surface water but not in the groundwater (McFadden & Hladik, 2021).

Table 8*: Octanol-water partition coefficient for CPS. *Oloye et al., 2021*

| Methods | Log Kow |
|---|---|
| Literature* | 0.55 |
| Chemaxon | 1.45 |
| KOWWIN v1.68 estimate * | 2.30 |
| Consensus | 1.80 |

Strong electrical interactions in soil with pH > 7 have been proposed as a compelling factor that might aid the sorption of chemicals (Franco et al., 2009). However, soil with acidic pH sorbed more CPS (figure 4), with the highest Kd and least Kdes. In contrast, only the organic carbon content and adsorption parameter (n) influenced the adsorption of benoxacor and furilazole (Acharya et al., 2020). Differences in their sorption behaviours could be related to two chlorine atoms at the terminal end of both benaxacor and furilazole. In another report, soil organic matter and clay are significant constituents that affect the sorption of MEF in soils (El Boukili et al., 2018). Thus, the sorption of chemicals by soil depends on both soil and chemical properties. Hence, the difference in the soil properties which affected the sorption of CPS compared to other safeners is a function of their structure.

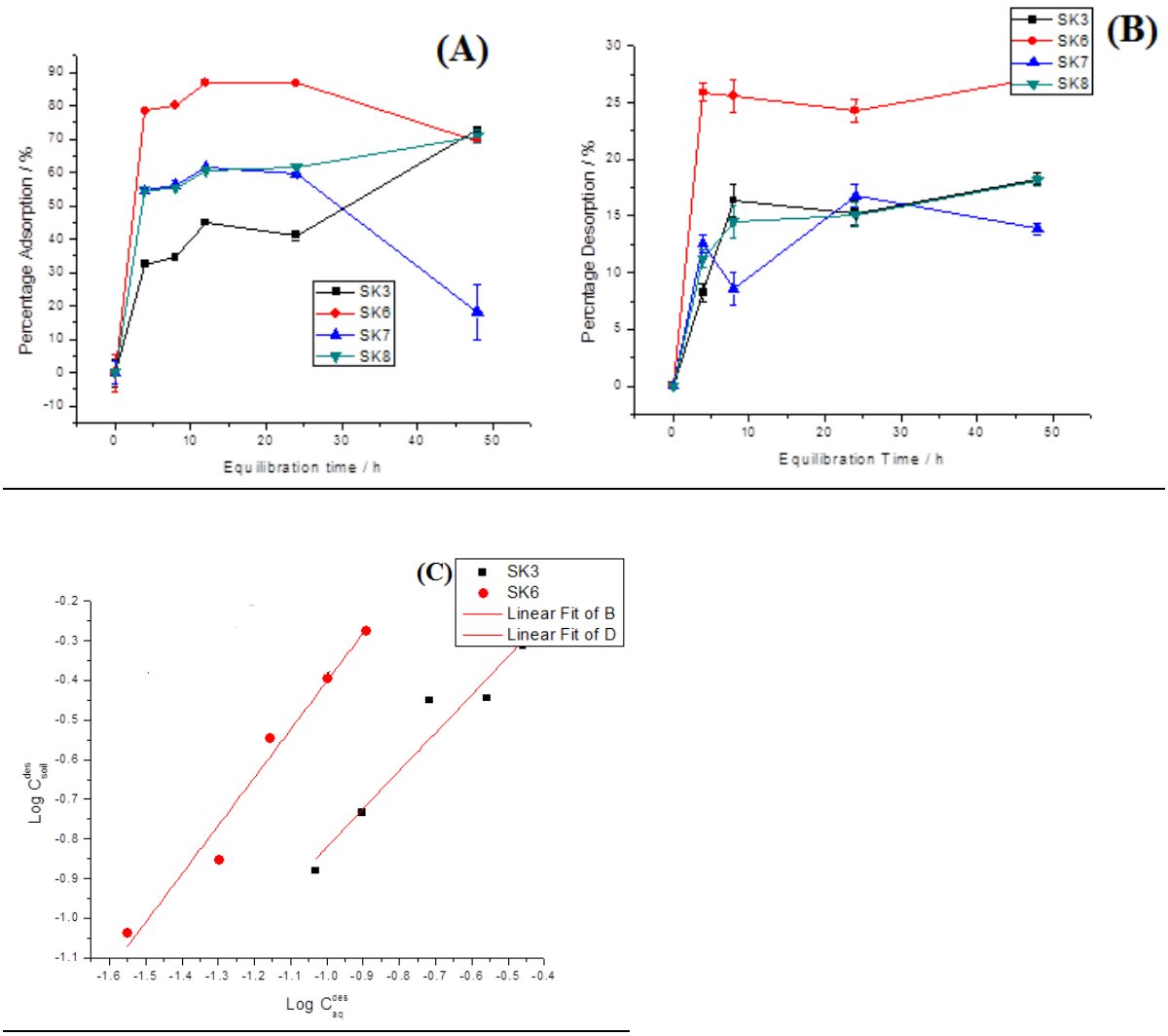


Figure 4: Kinetics of (A) % adsorption, (B) % desorption of CPS on four selected Saskatchewan soils and (C) isotherm of CPS on two of the selected soils

The desorption kinetics of CPS gave the understanding of the reversibility of CPS after sorption to the soil. $K_F$ and 1/n are the Freundlich constants and can be related to the sorption capacity (sorption coefficient) and sorption intensity of CPS, respectively (Figure 4C and table A.1). Freundlich adsorption was the only model employed because the data fit well to the model with $R^2 > 0.9$. Therefore, the soil with highest $K_F$ (6.5) had the highest sorption capacity for CPS.

The 1/n of SK6 soil was higher than SK3, indicating that sorption energy differed among the soils (Ololade et al., 2016). A value of 1/n greater than 1 represents a solvent affinity type isotherm, concave (upward curve), while 1/n represents convex; downward curve (Ololade et al., 2016). The convex curve has been shown to indicate low chemical competition from the water molecule, which would suggest a high affinity of soil for chemicals at low concentrations (Singh, 2009). Nevertheless, the concave curve showed that CPS had low interaction with soil and, therefore, low adsorption. This data was essential for computer modelling of leaching and dissolved run-off simulation. Generally, CPS has low sorption capacity and, thus, is expected to be in surface water.

### 2.5.1 Toxicity of Cyposulfamide

The potential toxicity of CPS was tested on *D. magna,* representing aquatic fauna, because it has a long history of usage as a model for toxicological bioassays. The organism is very sensitive to chemicals (Jordão et al., 2016). CPS did not have any lethal effect on *D. magna* even at a high concentration of 120 mg/L (Figure A.1). *D. magna* was mobile and active at the 96-h post exposure time point, suggesting that this chemical will not be lethal at environmentally relevant concentrations to this invertebrate in the short term.

A 21-day chronic exposure test to observe survival, reproduction, and growth rate of *D. magna* after exposure to CPS has shown that there was no lethality in the control group as expected but that both 10 mg/L and 120 mg/L had some percentage of *D. magna* mortality (Figure 5). The chronic test supported the observation of no lethality in the acute study because the 120 mg/L doses could only cause 60 % lethality of the *D. magna*.

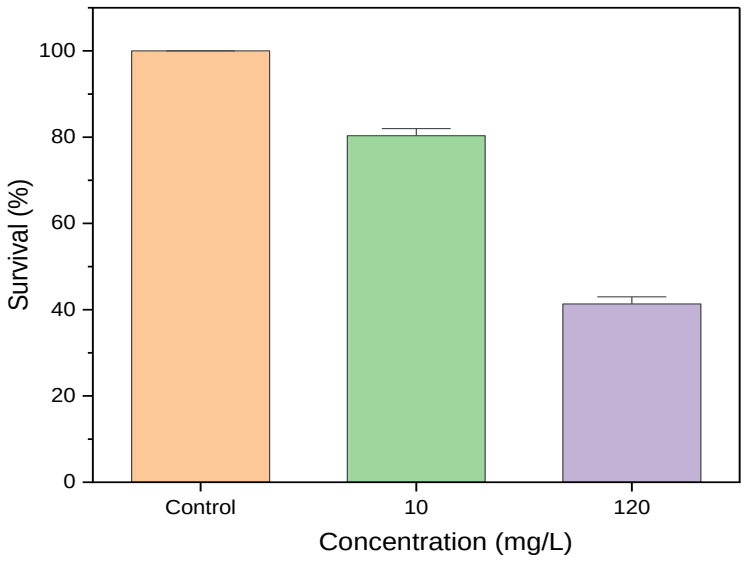


Figure 5*: Survival (%) (mean ± SD) of D. magna after 21 days of chronic exposure to CPS (n= 3 treatment, 10 organisms per treatment).*

The average brood produced by the control and treated groups showed no significant difference (Figure 6 A). Nevertheless, some observable differences occurred in brood size or number within the group treated with 120 mg/L. In the group treated with 120 mg/L, neonate numbers were reduced with increasing days of exposure. Therefore, as the day of exposure increases for high concentrations, the number of neonates per brood reduces, not noticed in low concentrations.

The chemical did not significantly affect the organism's growth (Figures A.2 and A,3). However, a slight difference was observable in the correlation factor. Control and 10 mg/L correlated more than control and 120 mg/L, indicating that for a longer time, the chemical might have an effect on the organism at higher concentrations (>120 mg/L), but such concentrations are not likely in the environment except in the case of spillage. There was no significant difference

between solvent control and negative control and therefore, the use of solvent control throughout other studies.

CPS did not affect the day *D. magna* had its first brood of neonates because both control and treated groups had neonates on day nine (Figure 6). However, the number of first brood neonates was greater in control others, suggesting that CPS affected the number of neonates.

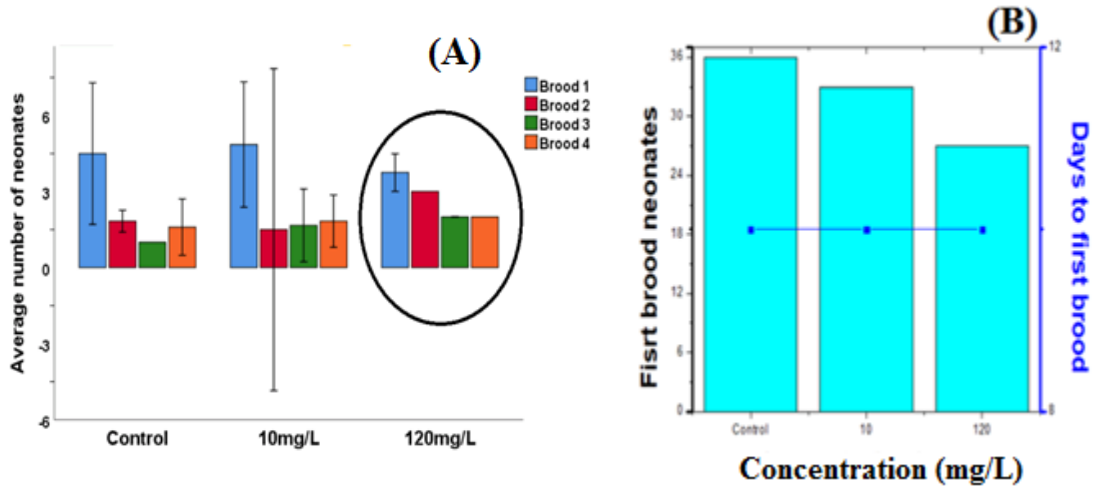


Figure 6: *Effect of CPS exposure on reproductive parameters of D. magna, (A) the average number of neonates after exposure for 21 days, (B) the number of first brood neonates and days to first brood.*

## 2.6 Conclusion

Cyprosulfamide is relatively soluble in water considering its octanol water partition coefficient. Its sorption to soil was governed by soil pH. The soil with higher acidicity has higher Freundlich sorption constant. Cyprosylfamide has low sorption capacity therefore prone to be transported to nearby surface water, The acute test on *D. magna* showed no lethal toxicity. Similarly, there was no significant difference in growth and survival observed in both control and treated groups after chronic exposure. This suggested that CPS might not be toxic to *D. magna* at

environmentally relevant concentrations. Nevertheless, some observable differences occurred in brood size or number within the group treated with 120 mg/L CPS. If no spillage, the risk level of *D. magna* to cyprosulfamide is moderate to low. Future studies could be carried out using omics techniques to evaluate the potential effect of CPS on gene expression of exposed daphnids to understand other potential mechanisms of action for this safener. It will also be interesting to determine if this safener infers protection on the animal against its co-herbicides by exposing *D. magna* to the combination of the safener and its co-herbicides.

# 3 Chapter 3: Experimental and Computational Studies on the Toxicity of Mefenpyr Diethyl Safener to *D. magna*

## 3.1 Preface

This chapter looks at what was described in chapter 2, but only with another chemical, MEF, the other safener used in this project. While there is a huge work of literature of the use of MEF as a safener in plants, little research has been done to understand and determine the toxicity of MEF to non-target organisms including *D. magna*. This chapter aimed to test the hypothesis that MEF will not have effect on apical endpoints (survival, growth, and reproduction) in *D. magna*. It also aimed at understanding the cohesion and accuracy of MEF's toxicity prediction using different computational software. Acute and chronic toxicity tests were done, where less than 24 hours *D. magna* were exposed to a varied concentration MEF in a 30 ml glass vial for 96 hours and 21 days respectively. This chapter discussed the effect of MEF on *D. magna* as it is with CPS in chapter 2, using both in vivo studies and computer simulation software. Both studies showed that MEF is toxic to *D. magna*. Chapter four discussed the result of MEF's toxicity to *D. magna*.

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## 3.2 Abstract

Mefenpyr-diethyl (MEF) is one of the safeners that is becoming popular because it protects crops from herbicides such as fenoxaprop-p-ethyl (FEN). While efforts have been made to understand the toxicities of herbicides, there are limited reports in literature about the toxicity of safeners. Therefore, the toxicity of MEF was investigated experimentally to understand the effects on *D. magna* in a 96-h acute study and a 21-day chronic study. Lethality was observed in concentrations as low as 6 mg/L and concentrations ≥ 32 mg/L, resulted in 100% mortality in the MEF treated group. The docking score of MEF showed its possibility to inhibit growth because it binds to the growth enzyme receptor (4XNN). Likewise, toxicity estimation software showed the chemical to be a developmental toxicant. It also showed that it has a bioconcentration factor of 30.83 and, thus, could easily bioaccumulate in an organism. The concentration at which half of the *D. magna* will die or become immobile was determined to be 17.9 mg/L, and T.E.S.T. calculated it to be 1.38 mg/L (consensus). Thus, MEF poses a high risk to the survival of *D. magna*. MEF inhibits reproduction, and there is a positive correlation between longevity, days to first brood, and the average number of broods per adult.

Keywords: Herbicide, Acute toxicity, chronic toxicity Mortality, Reproduction, Molecular docking.

## 3.3 Introduction

Concerns about human-induced pollution in the aquatic environment are rising. Therefore, there is a need to understand the risk of exposure of aquatic animals to new chemicals to evaluate their risk factor. Once approved for use, the rate at which pesticides are withdrawn from the market showed that the initial assessment of the active ingredients before approval is only sometimes complete and accurate. Many chemicals can alter the energy reserves of organisms even at low concentrations (Jordão et al., 2016) without directly having lethal effects on the animal. Therefore, chemicals certified to be non-toxic may be altering the lipids composition, thereby altering the energy reserves and thus contributing negatively to the organism. Compounds such as safeners which are classified as an inert component of herbicide formulations (Sivey et al., 2015), may be affecting the non-target organisms as it is typical of some chemicals. The structure of safeners show that there is possibility that it could undergo reactions because of the number of electrons around the conjugated π system. MEF is an example of a safener with at least one aromatic ring and many heteroatoms currently used to protect cereal crops and rice against the adverse effects of herbicides.

MEF is a safener that protects cereal plants under the enzyme acetyl CoA carboxylase (ACCase) and acetolactate synthase (ALS) inhibitor herbicides (Bianchi et al., 2021). MEF is used with FEN and mesosulfuron-methyl (Yuan et al., 2021). This chemical, just like any safener, reduces the toxicity of herbicides to crops without reducing their toxic efficacy in weeds. When applied to crops, MEF can shift the sigmoidal dose–response curve to tolerating higher herbicide rates, thereby permitting selective control of weeds with respect to the botanically related crops (Bartucca et al., 2016). Despite their ability to enhance crop tolerance by inducing the expression of enzymes such as glutathione transferases (GST) involved in the metabolism of herbicides, thus

accelerating their detoxification (Taylor et al., 2013), little effort has been made in literature to understand the toxicity of this class of chemical. Thus, it is important to understand the effect of MEF on two apical endpoints: acute lethality and reproduction.

*D. magna*, commonly known as water fleas, are small crustaceans widely used as model organisms in ecotoxicology studies. This species is a model for determining chemical safety and is a commonly used system for ecotoxicological testing worldwide. Like any other organism, *D. magna* needs a balanced diet for development, growth, survival, and reproduction. They are handy for evaluating the toxicity of chemicals in aquatic environments due to their sensitivity to environmental stressors and ecological importance as a food source for other aquatic organisms. They are also transparent, allowing for easy observation of internal structures and physiological processes. Therefore, they are ideal invertebrates for monitoring chemical toxicities. The effect of chemicals on *D. magna* can also be evaluated using molecular docking since there is a well-characterized protein available in Protein Data Bank. Therefore, the effect of MEF was investigated on *D. magna* using experimental and molecular studies.

## 3.4 Materials and methods

### 3.4.1 Chemicals and reagents

MEF safener was purchased from Sigma-Aldrich (Canada). All other chemicals were of analytical grade and purchased from the same vendor Exposure concentrations were confirmed using LC-MS (method described in appendix A.2 and A.3).

### 3.4.2 Acute toxicity test

*D. magna* were raised in 30-ml glass vials containing 25 ml of test solution. Neonates less than 24 h old from the third generation of a single parthenogenetic female were used for each

concentration in each set of experiments. Solvent concentration was kept at 0.01% Acetone (v/v) throughout the experiment and the solvent control contains acetone and water alone. A semi-static approach with up to 98% water renewal per day was adopted to retain stable concentrations of the test compounds in the exposure glass vials. Ten replicates with one neonate per vial were used for each treatment concentration and the control group. MEF concentration used were solvent control, 3, 6, 9, 12, 15, 32, 52, 72 and 92 mg/L. These concentrations were based on the EC50 provided for MEF on *D. magna* from the PPDB. The dead or immobile *D. magna* were recorded at 24, 48, 72 and 96 h. The temperature during the test was 22 ± 1 °C. The daily photoperiod was 16 h of light and 8 h of darkness.

### 3.4.3 Chronic toxicity

Chronic tests of CPS and MEF toxicity to *D. magna* were carried out following the OECD-recommended procedures (OECD, 2018). The test was done using a similar method as the acute study, except that the water was renewed every other day, and the test lasted for 21 days. The animals were fed with algae (*Chlamydomonas sp* and *Pseudokirchneriella subcapitata*) supplemented with vitamin B12 and selenium at every water change to ensure the general well-being of the animals. The number of neonates and deaths were assessed whenever water and food were renewed. Glass vials used for the test were covered with a glass lid at 21 ± 1°C, under 16 h light ad 8 h dark photoperiod. The experiment was terminated at 21 d.

### 3.4.4 Toxicity Estimation Software Tool (T.E.S.T)

T.E.S.T is a free software for estimating the effect of various organic pollutants on different endpoints using quantitative structure-activity relationship (QSAR). The input, such as CAS, SMILES, name, InChi, InChiKey or DTXSID, for MEF was obtained from PUBCHEM and entered into a search column. The software then calculates the endpoint based on the method of

choice. Here, consensus, hierarchical clustering, single model and nearest neighbour were chosen (USEPA 2020). The consensus method predicts toxicity and is estimated by taking an average of the predicted toxicities from each of the below QSAR methodologies. Hierarchical method predicts and estimates toxicity for a given compound by using the weighted average of the predictions from several different models. The different models are obtained by using Ward's method to divide the training set into a series of structurally similar clusters. A genetic algorithm-based technique is used to generate models for each cluster. The models are generated prior to runtime. Single-model method predicts toxicity using a multilinear regression model that is fit to the training set (using molecular descriptors as independent variables) using a genetic algorithm-based approach. The regression model is generated prior to runtime. Nearest neighbor method predicts toxicity by estimating an average of three chemicals in the training set that are most similar to the test chemical.

### 3.4.5 Molecular docking analyses

Receptors associated with embryo growth (4XNN) in *D. magna* was docked with MEF. The protein structure was obtained from Protein Data Bank (PDB). The 3D ligand structures in SDF format for MEF were obtained from PubChem Database and energy-minimized using the ViewerLite and Chem3D Ultra. These optimized structures were further stabilized using auto dock tools by adding or deleting bonds and charges and saved in pdbqt format. A grid was created for 4XNN proteins and MEF 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 4XNN with the two ligands, with minimal energy. Interaction energy values were utilized to determine the strength of the interaction between protein receptors

and MEF, with higher absolute binding values corresponding to greater binding affinity. The probable 3D models were viewed using pyMOL molecular visualization program.

### 3.5 Statistical analysis

All experiments were performed in triplicate at least, and results were expressed as means ± standard deviation (SD). The obtained data were first subjected to normality test the using Shapiro-Wilks test in Graph Pad prism. Data that passed normality test were further analyzed by one-way or two-way analysis of variance (ANOVA) following Duncan's multiple range test, and Kruskal-Wallis analyzed the one which failed normality test. A p-value of 0.05 or less was considered statistically significant. $LC_{50}$ was determined by fitting the data using a non-linear regression model with least squares fit so that Prism could calculate the best fit and a complete confidence interval. The probability of survival was generated using 1 when an event of death occurred and 0 for the censored value.

### 3.6 Results and discussion

#### 3.6.1 Acute and chronic test

Mortality rate during acute study showed that MEF induced lethality in *D. magna* at concentrations ≤ 6 mg/L (Figure 7 A), and the effective concentration at which 50% of the *D. magna* would die or become immobile was found to be 17. 9 mg/L. The ability of the organism to survive in a chronic study was tested, and no difference was found for concentration ≤ 15 mg/L (Figure 7 B). A hundred percent mortality was observed in ≥ 72 mg/L on the second day, while 100 % mortality was observed on day 7 for ≥ 52 and ≥ day 21 for ≥ 32 mg/L. Thus, the chronic study showed that under laboratory conditions, there was > 50% probability of survival for *D. magna* in < 32 mg/L MEF contaminated water. However, the cumulative effect could affect the ability of the organism to tolerate the chemical for an extended time (Pacheco et al., 2018); this

could be why 100% mortality was observed at 32 mg/L at 21 days. The ability of the compounds to bioaccumulate and bioconcentrate within living cell post a negative risk to the organism. The bioconcentration factor was high (Table 9), and hence the chemical could potentially bind to a living cell and disrupt the energy balance or prevent the normal function of the cells.

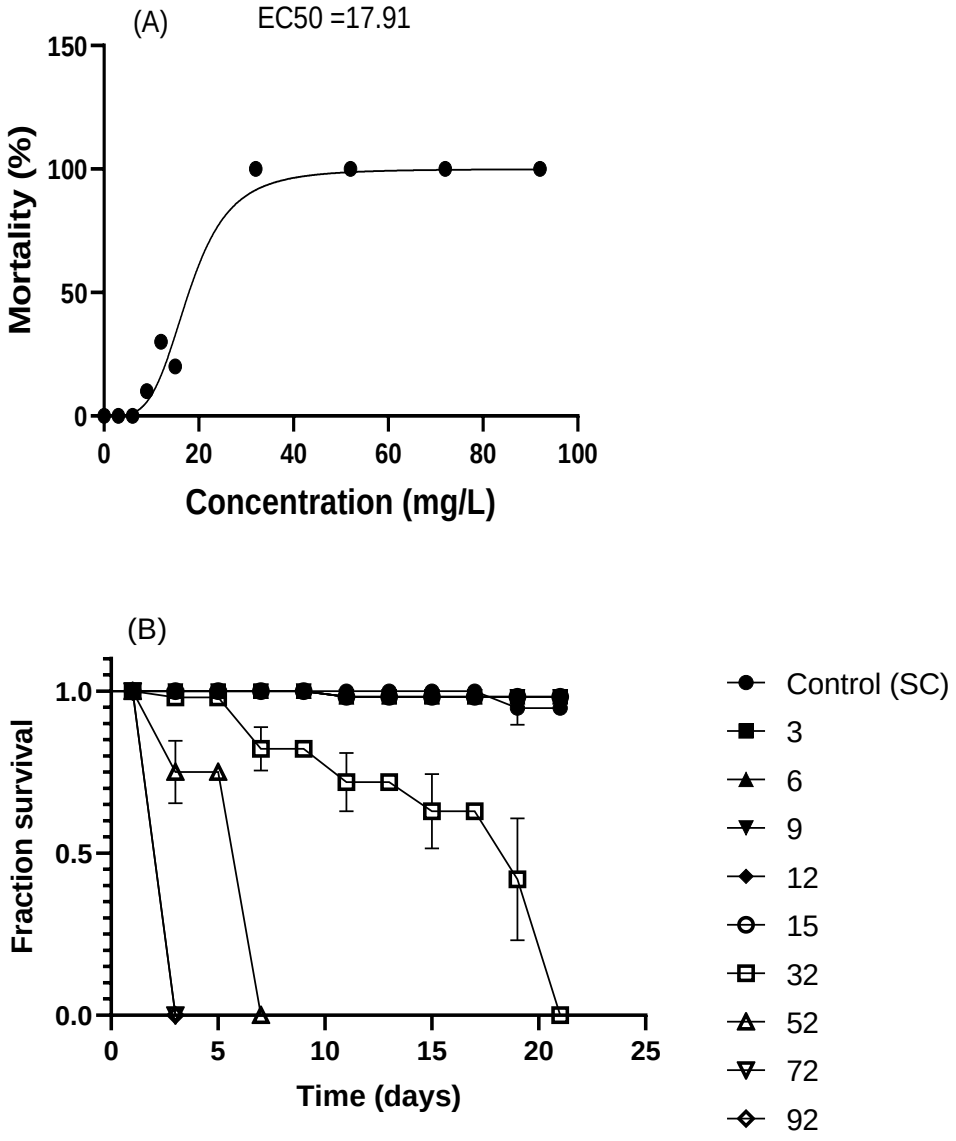


Figure 7: *Mortality (mean ± SD) rate of D. magna exposed to MEF (A) acute exposure 96 hours (B) chronic exposure 21 days (n = 4 treatment, 10 organisms per treatment). Concentrations are measured in mg/L and SC is the solvent control.*

The lethal dose (estimated) that would kill half of the tested organism was estimated from T.E.S.T to be 1.38 mg/L (Table 9); this is approximately a two-order magnitude lesser than what

was determined experimentally. This difference could be because laboratory study never takes into account all environmental conditions, which were factored into the T.E.S.T calculation. Nevertheless, the $LC_{50}$ from T.E.S.T and the experiment both agreed that the chemical is lethal to *D. magna* at concentrations less than < 20 mg/L. MEF is also a development inhibitory toxicant (Table 9); thus, it could affect growth and cause morphological alterations in the organism's body. Interestingly, the chemical is not a mutagen (Table 9) because its mutagenicity test was negative. The solubility of the chemical is relatively low (Table 9). Thus, MEF is more lipophilic than hydrophilic, hence would bind more to living cells with a longer residency time and hence, the higher possibility of causing developmental toxicity and death.

Table 9*: In silico prediction of toxicity and properties of MEF*

| End points | | Consensus | Hierarchical clustering | Single model | Nearest neighbour | Results |
|---|---|---|---|---|---|---|
| | Experimental | Prediction | Prediction | Prediction | Prediction | Prediction |
| $LC_{50}$ *D. magna* (mg/L) | N/A | 1.38 | 1.71 | 0.42 | 3.58 | Toxic |
| Bioconcentration factor (L/kg) | N/A | 66.01 | 56.57 | 76.31 | 54.85 | |
| Development toxicity | N/A | 0.87 | 0.99 | 0.75 | N/A | Development toxicant |
| Mutagenicity | N/A | 0.21 | 0.09 | 0.33 | N/A | Mutagenicity negative |
| Water solubility (mg/L) @ 25 °C | 20 | 7.08 | 13.31 | 26.27 | 1.01 | |
| Density ($g/cm^3$) | N/A | 1.34 | N/A | 1.40* | 1.29 | |

*Group contribution method and not a single model

The concentration at which there was no observable effect on reproduction compared with control was 3 mg/L, while at 6 mg/L, there was a significant effect on the average number of neonates (Figure 8A). Thus, the higher the concentration of MEF, the higher the ability of the chemical to inhibit reproduction. The number of neonates produced in the 3 mg/L and 6 mg/L treatment groups were not significantly different from the control group. However, higher concentrations (≥ 9 mg/L) significantly inhibit the number of first neonates (Figure 8B). Hormesis was observed in reproduction (Figure 8C). The plot was a non-monotonic curve, which showed that the MEF induced a biphasic effect. Hormesis is one indicator displayed by endocrine-disrupting chemicals (Vandenberg et al., 2013). Hormesis is a toxicological phenomenon whereby exposures to low doses of stress result in biological stimulation (Rix et al., 2022). Hormesis is a biphasic response characterized by inhibition of biological functions (such as reproduction) with exposure to high amounts of stress and stimulation of biological processes when the organism is exposed to low amounts of the stressor (Rix et al., 2022). This is why the total number of neonates increased at concentrations ≤ 6 mg/L but decreased at higher concentrations. In another study, low doses of antiseptics triclosan (1–10 $\mu mol\ L^{-1}$) were found to stimulate reproduction by up to 301% compared to the control, which might be due to endocrine disruption or other stress-related compensation responses (Vingskes, and Spann, 2018).

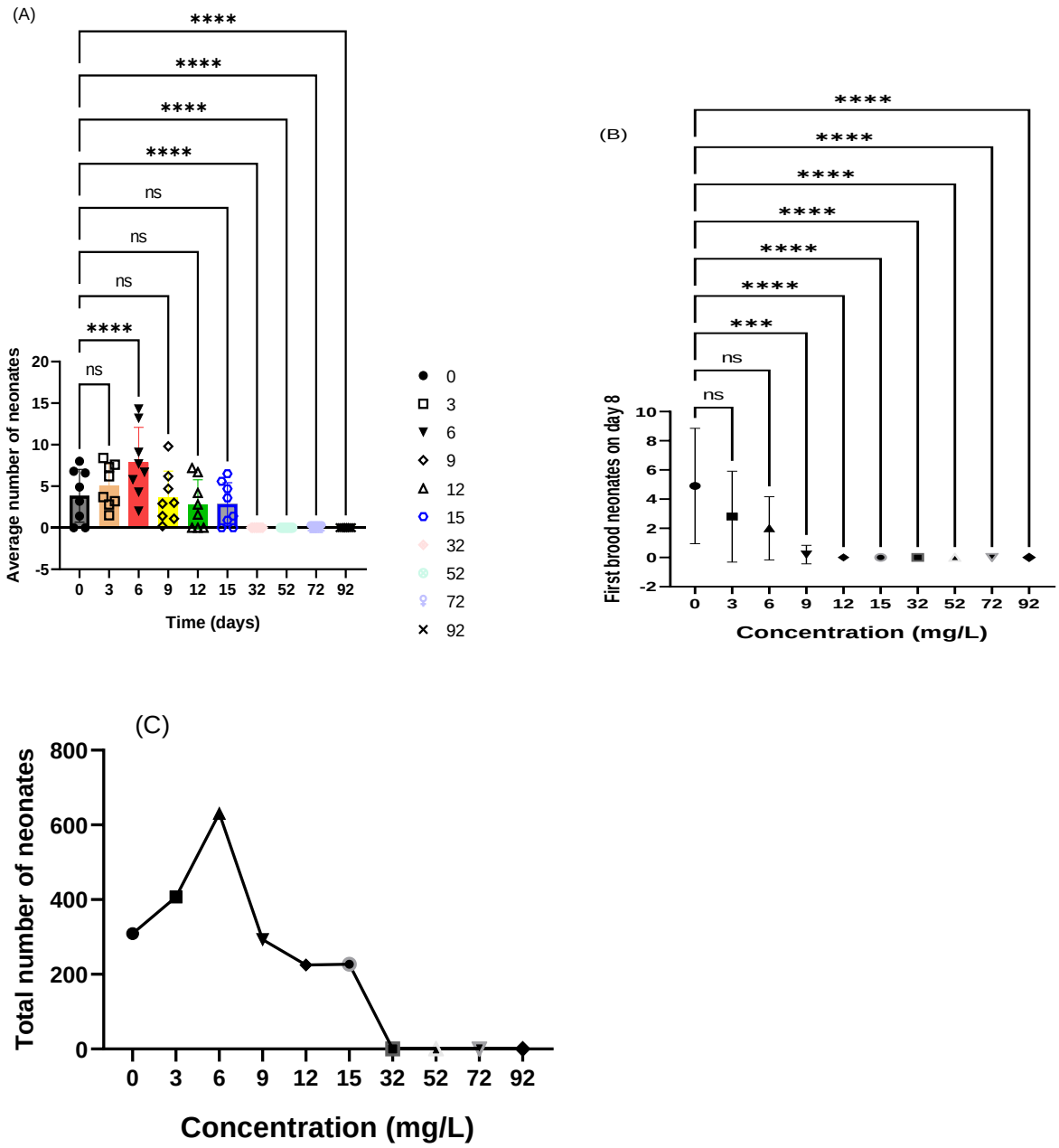


Figure 8: Reproduction profile for D. magna (a) comparing average number of neonates per concentrations, (b) Average number of first brood neonates (c) total number of neonates per concentrations. ***P < 0.005, ****P<0.0001, 0=solvent control

The longevity of *D. magna* in each group were examined to understand the ability of at least a 10% survival at the end of 21 days of chronic study. It was found that > 15 mg/L MEF affected longevity (Table 10). The higher concentration significantly delays days to the first brood (Fig. 8B and Table 10), while the average number of broods per adult also increased at low concentrations, then decreased at higher concentrations. There was a negative correlation between longevity, average number of broods per adult and days to first brood (Figure 9), confirming that MEF significantly inhibits reproduction and longevity factors. Longevity positively correlates to the average number of broods per adult and days to the first brood.

Table 10: *Chronic toxicity of MEF on D. magna treated with varied concentrations and control for 21 days.*

| Treatment (mg/L) | Longevity (day) | Days to first brood | The average number of broods per adult |
|---|---|---|---|
| 0 | 21 | 8 | 3 |
| 3 | 21 | 8 | 6 |
| 6 | 21 | 8 | 6 |
| 9 | 21 | 10 | 3 |
| 12 | 21 | 14 | 4 |
| 15 | 21 | 14 | 3 |
| 32 | 14 | - | 0 |
| 52 | 4 | - | 0 |
| 72 | 2 | - | 0 |
| 92 | 2 | - | 0 |

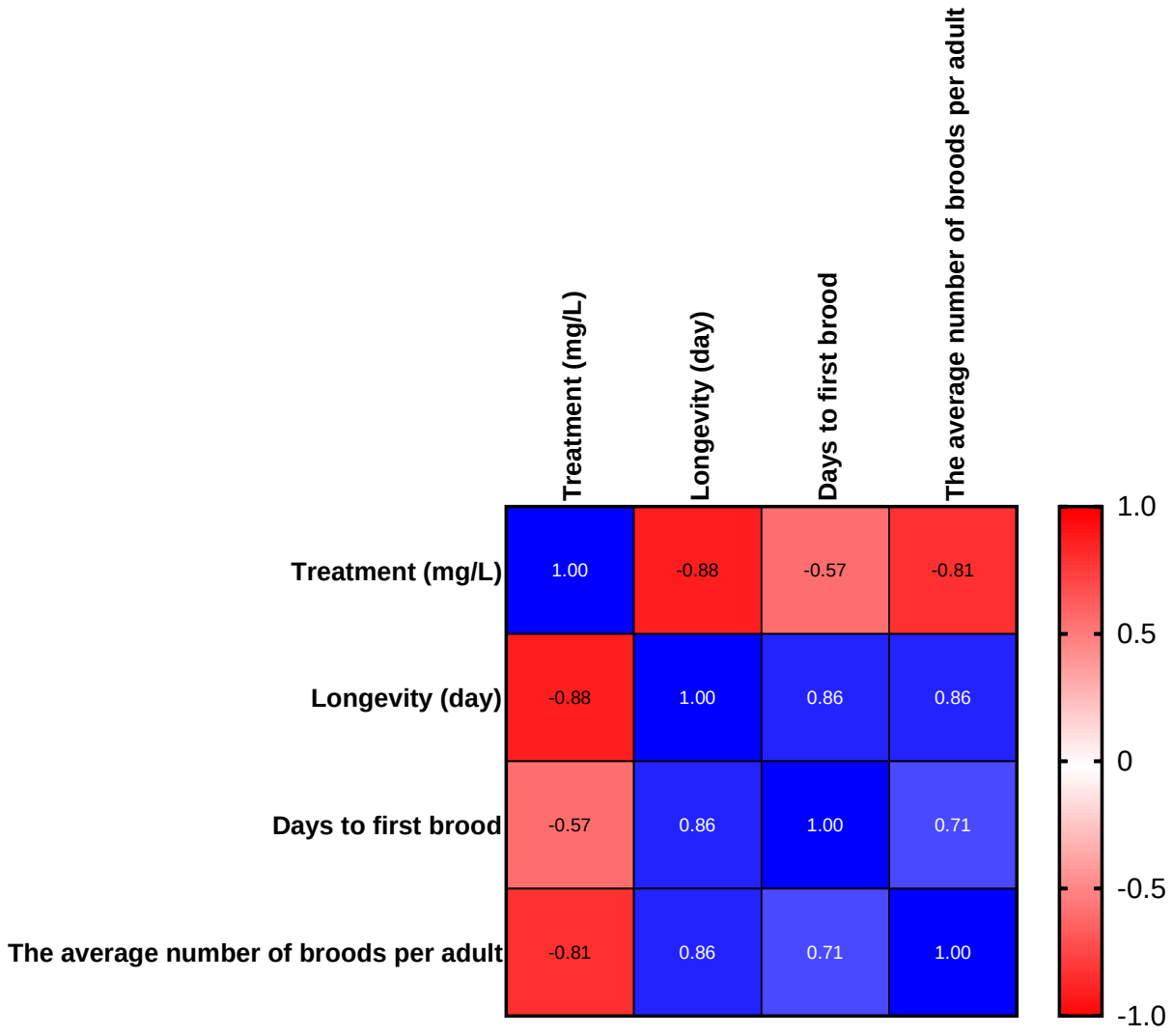


*Figure 9*: Spearman correlation profile for chronic test factors

3.6.2 Molecular docking

*D. magna* maturity is a function of growth; therefore, growth hormones are essential. Ability of a chemical to bind to the receptor could portray its ability to inhibit their functionality. MEF has the same binding score for chains A, B and the whole chain (Table 11), which confirms that it will bind in the same way if it gets in contact with the chemical from any side of the chain (Figure 10). The pictorial representation showed how the chemical binds with different amino

acids (Figure 10). Each of these amino acids performs an important function for growth, and their attachment with the chemical would make them less functional and, thus, inhibits growth.

Table 11*: Docking profile of MEF and growth hormones of D. magna.*

| Chemical | Protein | Protein function | Chain | Binding energy | Residues |
|---|---|---|---|---|---|
| Mefenpyr-diethyl | 4XNN | Growth hormones | A | -7.4 | Asn 224, Asn 126, Ser 124, Tyr 104 |
| | | | B | -7.4 | Asn 224, Asn 126, Tyr 104 |
| | | | Whole Chain | -7.4 | Asn 224, Asn 126, Ser 124, Tyr 104 |

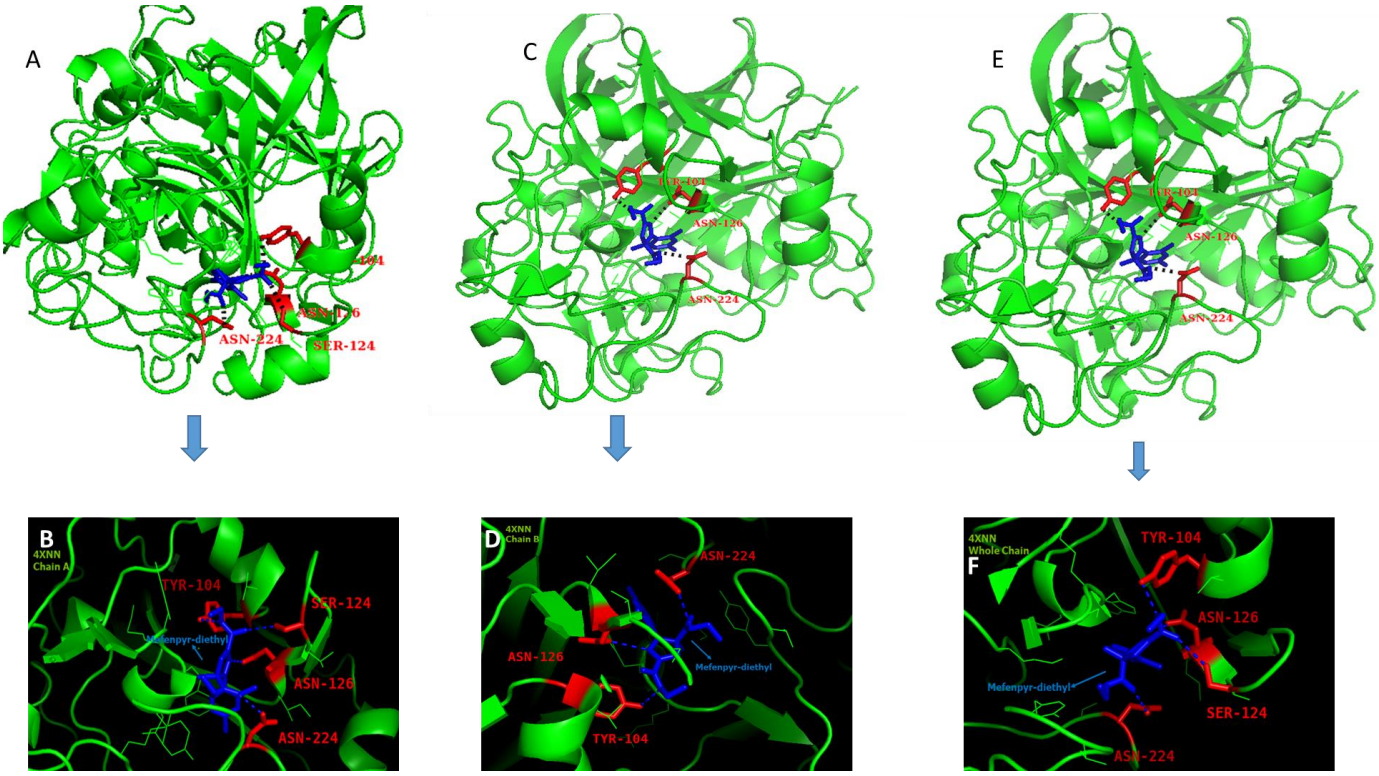


*Figure 10*: Docking profile of MEF and 4XNN (A and B) Chain A, (C and D) Chain B, (E and F) whole chain.

## 3.7 Conclusion

MEF is a safener commonly used with herbicides to reduce the effect of herbicides in corn and some cereals crops. The toxicity study of this class of chemicals showed that it is lethal to *D. magna*. It was also predicted to be a developmental toxicant but is mutagenic negative. The chemical has a high bioconcentration factor and low solubility in water, therefore, could bioaccumulate and bioconcentrate in *D. magna*. Concentrations ≥ 32 mg/L led to 100% mortality after a chronic study, and the least effective concentration (LOEC) was 6 mg/L for reproduction. MEF also inhibits growth4XNN and is a potential endocrine-disrupting chemical as it showed biphasic effects for low and high concentrations. There was a negative correlation between longevity, average number of broods per adult and days to first brood, confirming that MEF

significantly inhibits reproduction and longevity factors. Thus, MEF is lethal to *D. magna* and has non-lethal effects at low concentrations and hence should be used with caution.

# 4 Chapter 4: Individual and Combined Effects of the Herbicide, Isoxaflutole and Its Plant Safener, Cyprosulfamide, on *D. rerio*.

## 4.1 Preface

Understanding mixture toxicities to non-target organisms has become paramount considering that there are many mixtures in the environment. These chemical mixtures could be potential threats to environmental media and organisms. Because chemicals may affect organisms differently when they are exposed individually or in combination with another chemical. Following the study and result in 3 which looked at a representative invertebrate, chapter 5 looked at the effect of CPS and ISO singly and in combination in ELS of *D. rerio*- a representative vertebrate model. This chapter aimed to test the hypothesis that early embryonic life stages of *D. rerio* are not affected differently when exposed to CPS and ISO individually or with a binary mixture of CPS and ISO. Acute and chronic studies using approximately 2hpf *D. rerio* embryos, coupled with biochemical tests using plate reader were used to determine difference in effects of CPS, ISO and their mixture on ELS of *D. rerio*.

This chapter incorporates and sets the stage for the use of some biochemical tests (enzyme activity assays) and molecular docking in understanding interactions and binding affinities of the CPS and ISO to *D. rerio* and predict their effects in chapter 4 and in subsequent chapter.

The content of this chapter is in preparation for submission to the Environmental Pollution journal.

Author's contributions:

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Dr. John Giesy (University of Saskatchewan; Baylor State University) – Conceptualization, Methodology, Resources, Investigation, Review and Editing, Project Administration, Funding Acquisition

## 4.2 Abstract

For purposes of registration, studies are generally done on individual herbicides. Herbicides are often formulated with other chemicals that act as "safeners" to protect crops from unwanted effects of herbicides that control weeds. These safeners are often listed as being "inert" constituents of crop protection products, and thus, their potential effects on non-target organisms, such as aquatic organisms, are often not assessed. not However, the rate at which registered chemicals are withdrawn showed that more work needs to be done on them before their use. Isoxaflutole (ISO) and its corresponding safener, Cyprosulfamide (CPS), were studied for toxicity to *D. rerio*. Acute lethality and rate of hatching were determined during 96-hr exposure to the chemicals, singly in and combination. Effects of chronic exposures on survival, growth, and morphological deformations were assessed during 21-day exposures. The ability of the chemical to bind to hatching enzyme receptor was also examined by use of simulation models of molecular docking. Exposures to neither chemical nor binary mixtures inhibited hatching. Exposure to concentrations ≥ 20 mg CPS/L and 1.5 mg ISO/L, caused some mortality with an $LC_{50}$ of 136 mg CPS/L and 7 mg ISO/L for CPS and ISO, respectively, which indicates that ISO is more potent than CPS. CPS did not have significant effects on activities of SOD or GST, but ISO altered SOD compared to control, but not GST. The mixture significantly resulted in a down regulation of activities of SOD and GST, which suggested that there is an interaction between the two chemicals such that the mechanism of action of the mixture differs from that of the individual chemicals. The docking score showed that both chemicals exhibited similar binding energies for associations with hatching enzymes, which is consistent with the similar effects on rates of hatching of eggs.

Keywords: Acute, Chronic, Mortality, Reproduction, Endocrine Disruption, Juvenile, Molecular docking.

## Introduction

There is an increasing need to use herbicides that control weeds to ensure better yields of crops and enhance food security for the increasing global population (Femi-Oloye et al., 2020). However, herbicides also have effects on crops and non-target organisms (Zhao et al., 2019), thus a need to add adjuvants which are chemicals classified as inert in that they are not active as herbicides. These chemicals, referred to as herbicide safeners, can prevent the damaging effect of herbicides on the crops they are used to benefit (Woodward et al., 2018). Safeners are classified as inert for regulatory purposes without a complete understanding of their potential for effects on non-target organisms. This class of chemicals has various chemical properties and therefore do not all have similar effects on aquatic organisms. Hence, each safener must be comprehensively examined for toxicity against common aquatic organisms. Insufficient assessments are reasons pesticides are frequently withdrawn from the market; thus, there is a need to continuously explore the toxicities of old and new herbicides to understand their health effects better (Mesnage and Antoniu, 2021). Benaxacor, a common safener, has been reported to increase mortality and decrease heart and hatching rates in embryos (Zhang et al., 2021), while acute toxicity of CPS has no lethal toxicity on *D. magna* (Femi-Oloye et al., 2023). Another example of an inert safener having effects on non-target species is metamifop, a novel aryloxyphenoxy propionate, which has been implicated as toxic to *D. rerio* embryos by inhibiting hatching, induced abnormalities, oxidative stress, and cell apoptosis (Zhao et al., 2019).

ISO is a broad-spectrum herbicide of the isoxazole family mostly used during corn pre-emergence or pre-plant against the grass and broad-leaved weed species (Milan et al., 2015). ISO has been reported to be an emerging pollutant in drinking water sources because of the use of ISO-tolerant genetically modified crops in 2020 in the United States (Rogers et al., 2023). ISO solubility

in water is 6.2 mg/L (20 ºC at pH 5.5), and its octanol/water partition coefficient is 2.34 at 20ºC; thus, ISO is persistent, mobile and can leach from soils and accumulate in groundwater and through surface waters. Generally, the risk of leaching is believed to be high in pre-emergence herbicides (Milan et al., 2015). Therefore, ISO may be found in aquatic environments. However, the rate of application in each area will determine its possibility of being bioavailable in the environment. The half-life of ISO, which is predicted to be less than one day is another constraint that does not make its toxicity study common.

Most of the toxicity data available on ISO toxicity are on rats, rabbits, and guinea pigs, but there is some information on effects on aquatic organisms. Results of a chronic study indicated increased mass and histopathological changes in livers and tumors in thyroids of mice and rats (USEPA, 1998). ISO is classified as toxicity category IV because its acute toxicity (LD50) in rats was > 5000 mg/kg, and acute inhalation ($LC_{50}$) was 5.26 mg/L. It is classified as Toxicity Category III based on results from acute dermal toxicity in rats (LD50 > 2000 mg/kg) and primary eye irritation in rabbits, which cleared in 72 h (US-EPA, 1998). The toxicity to Bluegill sunfish (*Lepomis macrochirus*, $LC_{50}$), Rainbow trout (*Oncorhynchus mykiss*, $LC_{50}$), water flea (*D. magna*, EC50), Sheepshead minnow (*Cyprinodon variegatus*, $LC_{50}$), Eastern oyster (*Crassostrea virgica*, EC50) and Mysid (*Amerricamysis bahia*, $LC_{50}$) were 4.5, 1.7, 1.5, 6.4, 3.3 and 0.0178 mg/L, respectively, which indicates that ISO is more toxic to aquatic organisms (National Center for Biotechnology Information, 2023). Exposure of humans to this chemical has been classified as negligible (EFSA, 2017); this is because residue of ISO and its metabolite RPA 202248 in maize in the field trials was less than the individual limit of quantification of 0.01 mg/kg (EFSA, 2017). The metabolite RPA 203328 in food and feed commodities exceeds 0.01 mg/kg (EFSA, 2016).

CPS is an acyl sulfonamide compound commonly used with ISO to prevent negative effects of the herbicide. It is also used with Thiencarbazone-methyl (Santel, 2012) for cereal crops. Half of the mixture used in the herbicide ISO, SC 480 (Merlin Flexx) contains 240 g/L ISO and 240 g/L CPS (EFSA, 2017). CPS is relatively soluble and mobile compared to ISO, considering its solubility and octanol/water partition coefficient (Oloye et al., 2021; Mcfadden and Hladik, 2021). It has been found, along with its transformation products, in surface water of the Midwestern United States (Mcfadden and Hladik, 2021). Since CPS is generally applied with ISO via spray formulations during the pre- or post-emergence stage of the planting season in early spring, there is a high possibility of it being found in aquatic environments through leaching or runoff. Mechanisms of action of safeners involve inducing genes and proteins responsible for herbicide metabolism and crop detoxification, thereby lessening their effects on crops (Rosinger and Schulte, 2019). Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione *S*-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. In addition, safeners enhance the vacuolar transport of glutathione or glucose conjugates of selected herbicides (Hatzios and Burgos, 2004). Dichlormid, another type of safener, was found to reduce lipid peroxidation induced by herbicides, suggesting safeners' role as antioxidants (Bernasinska et al., 2013).

The probability of exposure to mixtures of chemicals is great, so it is important to assess the most likely mixtures. Although there could be multiple chemicals present in the environment, it is highly probable that individuals will only be exposed to a limited number of major chemicals at any given time (Shukla et al., 2017). While, in natural environments, compounds can migrate from their point of introduction to the surrounding environments and the nearer to the point of introduction, the more likely organisms will be exposed to chemicals released together. Thus,

assessing all possible combinations is unnecessary, but the canonical or most likely combinations (Oloye et al., 2021). The combined toxicological effects of a mixture of compounds can be independent, dose addition or interaction (Hernández et al., 2013); thus, mixtures of compounds can result in different toxic effects. These interactions can be supra-additive and enhance responses or infra-additive and decrease the efficacy and or potency of the mixture. It was decided to assess effects of the herbicide ISO in the presence of its formulated safener, CPS, on survival and early stages of the small model fish, *D. rerio*.

*D. rerio* are becoming more popular as a model to test toxicity because they possess high physical and genetic similarity to humans, which provides more advantages for using *D. rerio* compared to in vitro systems (Wang et al., 2023). The *D. rerio* is a tropic or sub-tropical freshwater fish belonging to the minnow family (Cyprinidae) of the order Cypriniformes and is native to South Asia.

Testing the toxicity of ISO on *D. rerio* is important because of the huge differences in reported toxicity data using the same organism. For instance, the $LC_{50}$ of 1.7 and 160 mg/L in Rainbow trout had been documented (National Center for Biotechnology Information, 2023). *D. rerio* is an ideal model for evaluating morphological abnormalities and ascertaining for high-throughput screening of chemicals with a special emphasis on particular organ or system toxicity (Zhang et al., 2021).

Molecular docking is a computational method to predict the binding interactions between a small molecule and a target protein. It is a valuable tool in drug discovery as it allows researchers to identify potential drug candidates that can bind to a specific target with high affinity and specificity. In recent years, molecular docking has also been used in toxicology studies to predict the effects of environmental contaminants on non-target organisms. Hatching enzymes are critical

for the development, survival and reproduction of *D. rerio*, and any disruption to these enzymes can adversely affect health and well-being. Safeners generally exhibit structural similarity to the herbicides that might be competitive, binding with the herbicide at the active site to increase the tolerance of treated plants (Wang et al., 2021). The binding of chemicals with the active sites of the proteindetermine their toxicity and their binding sites or residues. Safeners are hypothesized to bind to this receptor because several studies have shown that safener could delay and inhibit hatching altogether (Liu et al., 2022).

In this study, effects of CPS and ISO singly and binary mixtures on survival and growth of juvenile *D. rerio* were determined and chronic effects on and hatching hormone (3lqb) by use of in silico modelling of molecular docking. The goal was to determine whether CPS and ISO can bind to the and hatching enzyme receptor and potentially interfere with its function. This information could help inform future studies on the toxicity of CPS and ISO to non-target organisms and aid in developing safer alternatives for agricultural use.

## 4.3 Materials and methods

### 4.3.1 Chemicals and reagents

The herbicide, ISO and its safener, CPS, were purchased from Sigma-Aldrich (Canada). Commercial assay kits for determining enzyme activities of SOD and 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.

### 4.3.2 *D. rerio* maintenance and egg collection

Cultivation of adult and juvenile *D. rerio* and egg collection were performed according to previous procedures (Li et al., 2018). *D. rerio* were bred at a ratio of 2:1 (female: male), with

approximately 20 females and ten males per breeding (Li et al., 2018). The fish were maintained at the Collaborative Science Research laboratory at the University of Saskatchewan, Canada, from which eggs less than equal to 3 hours post-fertilization (hpf) were collected. Adult *D. rerio* were fed twice daily - alternating Gemma micro 500® fish food (Skretting, Vancouver, British Columbia) 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 contained 10 gallons glass aquarium 1/3 -½ 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, with produced eggs collected the following morning. The facility water was used and tested for ammonia and chlorine weekly.

4.3.3 Egg collection:

Eggs were collected the next day at approximately 9:00 AM, the breeding fish, plants, netting, and air stone 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 while several drops of methylene blue were added, and additional feces were removed with a pipet. Healthy and fertilized embryos were transferred into a clean petri dish with a diameter of 90 mm for chemical exposure.

4.3.4 Embryotoxicity test

The *D. rerio* embryo toxicity test was performed following the Organization for Economic Co-operation and Development (OECD) 236 guidelines with some modifications to assess the effects on some endpoints, including mortality and reproduction. Solvent concentration was kept at 0.01% acetone (v/v) throughout the experiment and the solvent control contains acetone and

water alone. A semi-static approach with up to 98% water renewal per day was adopted to retain stable concentrations of the test compounds in the exposure plates and petri dishes. Briefly, 20 embryos at 2 hpf were exposed in a 24 well plate to different concentrations of either CPS (solvent control, 5, 20, 40, and 90 mg/L) or ISO (solvent control, 0.01, 0.1, 0.5, 1.5, and 3 mg/L) or their mixtures of equal concentrations each of MEF and FEN (solvent control, 0.1 and 1 mg/L) until 96 hpf. Concentrations of CPS and ISO were selected based on previously determined $LC_{50}$ values of other fishes at 96 hpf and earlier acute toxicity test data (Pubchem). Rates of hatching of eggs were calculated at 72 and 96 hpf and the heartbeat rates per 30 s at 96 hpf under an inverted Zeiss (Jena, Germany) microscope. The temperature during the test was $26 \pm 1$ °C. The daily photoperiod was 16 h of light and 8 h of darkness.

### 4.3.5 Chronic toxicity - developmental toxicity test

Chronic exposures were done using static-renewal methods for 21 days, following the same method as acute but were done in Petri-dishes. About 20-30 *D. rerio* embryos were placed in petri dishes containing about 60 to 70 ml of exposure solution for each compound and concentration. Feeding and water change were done twice every day. Effects of the combination of ISO and CPS on developmental toxicity were evaluated according to previous methods (Blahova et al., 2020). Lengths of the larvae were measured on days 4 and 21. All determinations were performed in triplicate.

### 4.3.6 Assessment of deformations

Detection of deformations in the zebrafish embryos were done by a visual assessment and phenotype scoring of morphological features. Each embryo and larvae were viewed under the microscope and every form of deformations were recorded. An overall incidence of deformities was thereafter calculated by dividing the number of individuals classified as deformed by the total

number of individuals in that replicate. The results were then converted to the percentage of the population for each concentration.

### 4.3.7 Determination of oxidative stress biomarkers

Sixty embryos were randomly selected and placed into three Petri dishes for each concentration (90 mm diameter) with 60 mL of exposure solution per replicate. Over two-thirds of the exposure solution was renewed every 24 h, and the dead individuals were removed daily. The fish were then homogenized with 1ml of PBS buffer (pH 7.4) per 5-10 mg of fishes in a tissue homogenizer for 15 minutes at 20Hz and centrifuged at 12,000 g at 4°C for 15 min. The obtained supernatant was used for oxidative stress-related biomarkers (SOD and GST) and was determined by commercial assay kits.

### 4.3.8 Molecular docking analyses

Receptor associated with hatching (ZHE1 (3lqb)) was selected as target for molecular initiating events. Protein structure was obtained from the Protein Data Bank (PDB). The 3D ligand structures of CPS and ISO in SDF format were obtained from PubChem Database and 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/). These optimized structures were further stabilized using auto dock tools by adding or deleting bonds and charges and saved in pdbqt format. A grid (20X20X20) was created for ZHE1 for CPS and ISO 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 ligandswith minimal energy. Interaction energy values were utilized to determine the strength of the interaction between protein receptors and CPS and ISO, with greater absolute binding affinities corresponding

to greater binding affinity. Probable 3D models were viewed using pyMOL molecular visualization program.

#### 4.3.9 ADMET study

ISO and CPS were 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.

### 4.4 Statistical analysis

All experiments were performed in triplicate or more. Results were expressed as means ± standard deviation (SD). The obtained data were first tested to see if they conformed to normality test using the Shapiro-Wilks test in Graph Pad Prism. The assumption of homogeneity of variance was evaluated using Leven's test. Data that passed the normality test were further analysed by one-way or two-way analysis of variance (ANOVA) following Duncan's multiple-range test. The Kruskal-Wallis's test was used to analyse data that failed normality test. A p-value of 0.05 or less was considered statistically significant. $LC_{50}$ was determined by fitting the data using a non-linear regression model with least squares fit that prism calculated by the best fit and a complete confidence interval. The probability of survival was generated using 1 when an event of death occurred and 0 for the censored value.

### 4.5 Results and discussion

#### 4.5.1 Hatching rate

No significant difference was observed in the rate of hatching between the control and treated groups in CPS and ISO. The maximum concentration where there was no significant effect on rate of hatching was 90 mg CPS/L (Figure 11A) and 3 mg ISO/L (Figure 11B) for CPS and

ISO, respectively. The mixture of CPS and ISO also did not cause significant changes in the rate of hatching of eggs (Figure 11C). Thus, the inability of CPS and ISO to cause significant changes in the hatching rate might be related to their high mobility (log Kow < 2.5), which allows them to pass through the pores of the embryo without staying too long in the pore to inhibit or delay hatching.

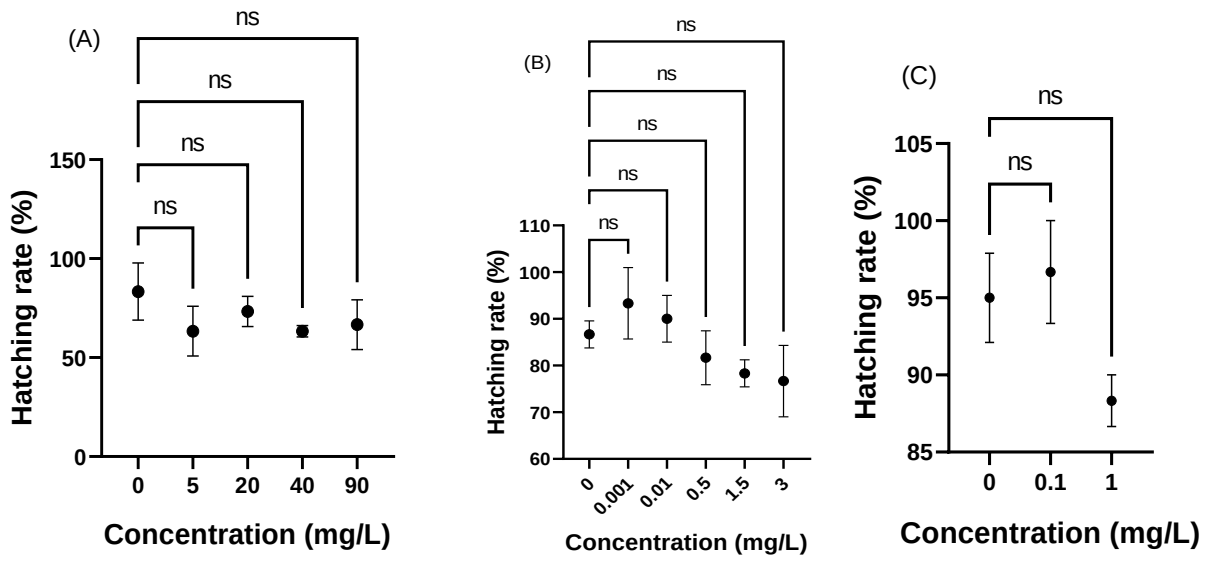



Figure 11: Number (mean ± SD) of *D. rerio* larvae hatched after exposure to (A) CPS and (B) ISO, and (C) Mixture of CPS and ISO for 96 hours. (n = 4 treatments, 20 organisms per treatment, 3 replicates/treatment)

### 4.5.2 Survival rate and morphological changes

CPS did not significantly change mortality at < 5 mg CPS/L in a 96-h acute study (Figure 12A). After 24 hpf of exposure, some significant changes in the lethality were observed between the control and > 20 mg CPS/L; this observation changed after 48 hpf, where ≤ 20 mg CPS/L did

not result in any significant changes in lethality. Therefore, *D. rerio* embryo was able to tolerate ≤ 20 mg CPS/L, above which significant changes were evident throughout the study. The lethal concentration ($LC_{50}$) for which half of the population will die decreased with days of exposure to CPS and ISO (Table 12). The $LC_{50}$ at 96 h for CPS and ISO was 82 mg CPS/L and 7 mg ISO/L, respectively. The calculated ISO $LC_{50}$ for *D. rerio* was close to the reported value for Sheepshead minnow (6.4 mg ISO/L) (National Center for Biotechnology Information, 2023).

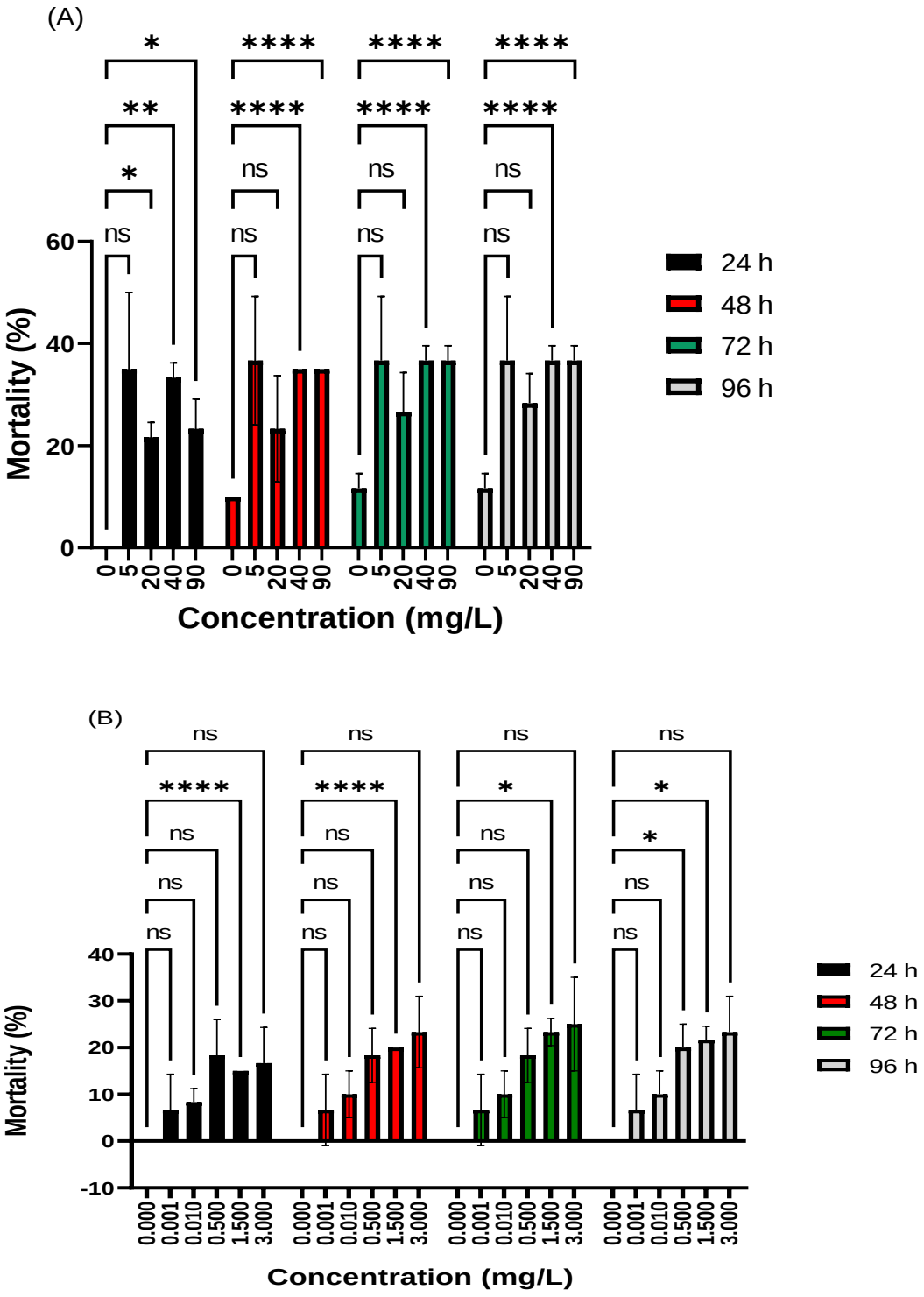



Figure 12: Mortality of *D. rerio* larvae exposed to (A) CPS (B) ISO and (C) CPS +ISO.

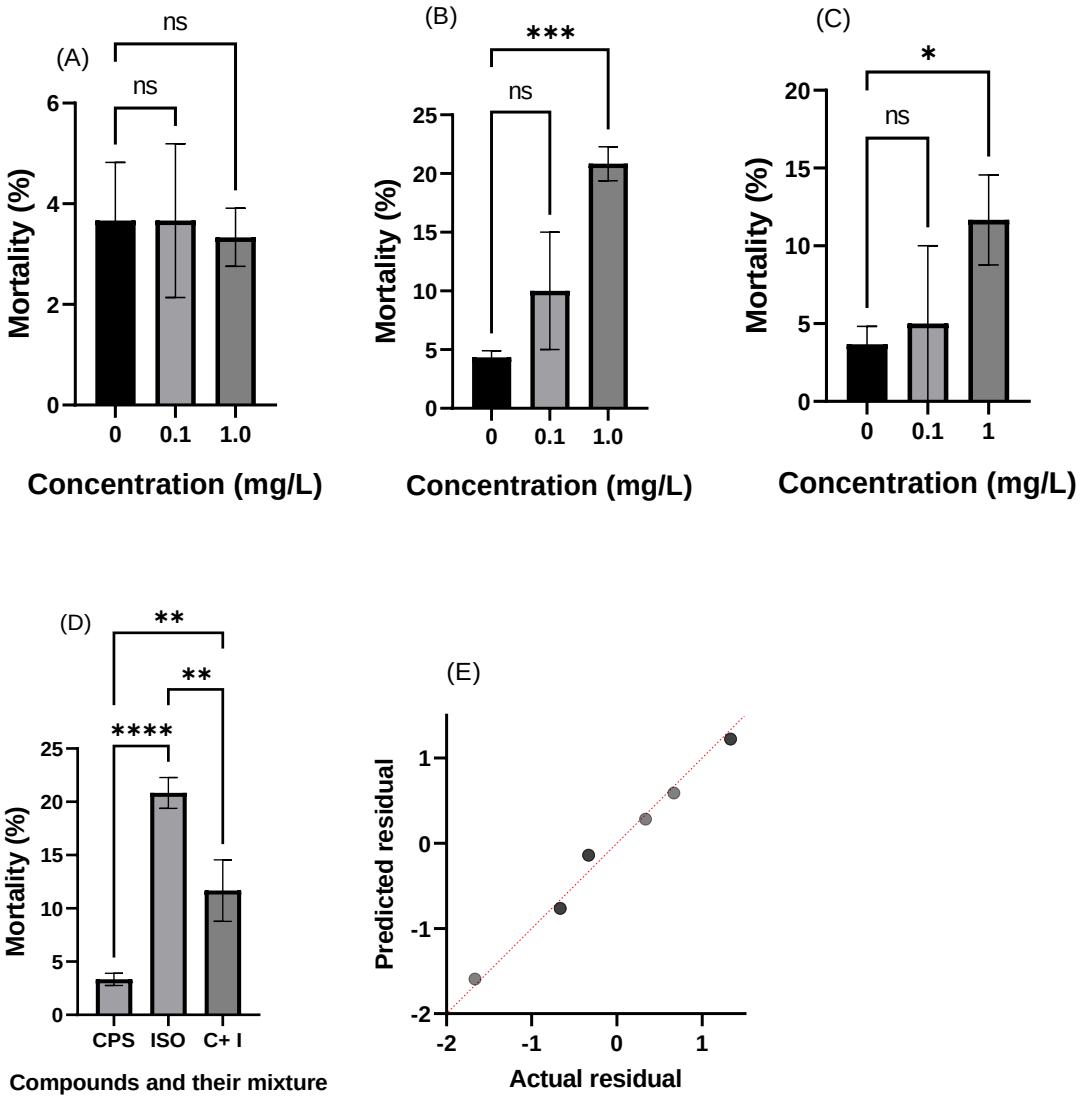
****P<0.001.**P<0.05, *P<0.05, 0=solvent control.

Table 12*:* Lethal toxicity ($LC_{50}$) of CPS and ISO

| | CPS | | | | ISO | | | |
|---|---|---|---|---|---|---|---|---|
| | $LC_{50}$ | 95% CI | Sy.X | Sum of Squares | $LC_{50}$ | 95% CI | Sy.X | Sum of Squares |
| 24 h | 136.40 | 65.78-375.8 | 18.72 | 4906 | 10.71 | 6.22-24.79 | 10.46 | 1859 |
| 48 h | 94.39 | 49.33-205.60 | 18.11 | 4593 | 7.36 | 4.67-13.38 | 10.30 | 1803 |
| 72 h | 83.96 | 43.79-179.2 | 18.30 | 4688 | 6.47 | 4.10-11.58 | 10.82 | 1992 |
| 96 h | 82.01 | 42.61-175.10 | 18.36 | 4719 | 6.97 | 4.34-13.01 | 10.95 | 2039 |

During chronic exposure to 1.0 mg CPS/L (Figure 13A), there was no significant effect on lethality compared to the control, but 1 mg ISO/L (Figure 13B) significantly affected mortality of *D. rerio*. Similarly, chronic exposure to a 1.0 mg mixture of CPS and ISO/L (Figure 13C) significantly affected mortality. However, the percentage of lethality was reduced by 8.5%. This rescue from mortality could be related to the ability of safener to manipulate the expression of xenobiotic detoxifying enzymes. In crops, the mechanism of action of safener to prevent the harmful effects of herbicides is to enhance crop tolerance by inducing the expression of proteins involved in the metabolism of herbicides, thus accelerating their detoxification (Taylor et al., 2013). All the data passed the normality test as the predicted value correlated well with the measured value (Figure 13D-F); thus, the observed significant differences from the chronic study were statistically valid. The maximum survival time upon exposure to 1.0 mg CPS/L or 1.0 mg ISO/L was 33 hpf, while that of the mixture was 408 hpf, and *D. rerio* in the control group were able to survive the entire duration of chronic exposure (Figure 14 A). The longevity introduced in

the mixture group compared to CPS and ISO single-group showed that the mixture of the two chemcals was infra-additive, which increases the probability of survival (Figure 14B).



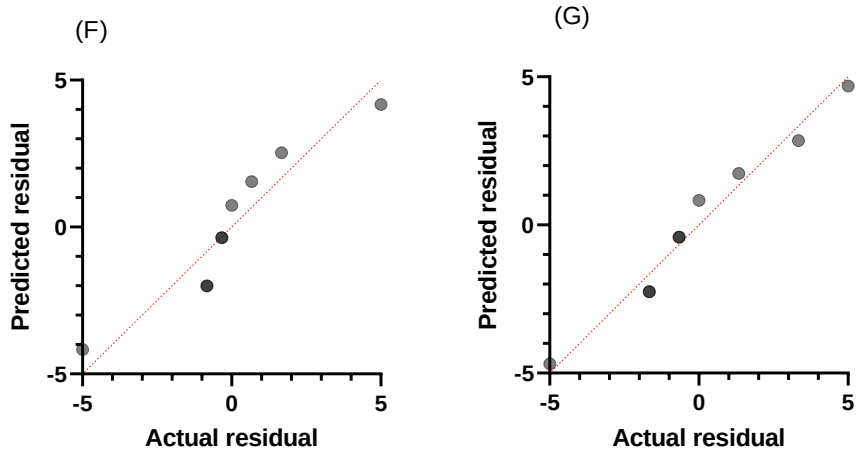



Figure 13: Chronic lethality of *D. rerio* larvae after 21 days post fertilization exposed to various concentrations of (A) CPS (B) ISO, (C) CPS and ISO, (D) effect of MEF, FEN and CPS and FEN at 1 mg/L, (E) QQ for CPS, (F)QQ for ISO, (G) QQ for CPS and ISO. *P<0.01, **P<0.005, ***P<0.001, ****P<0.0001.

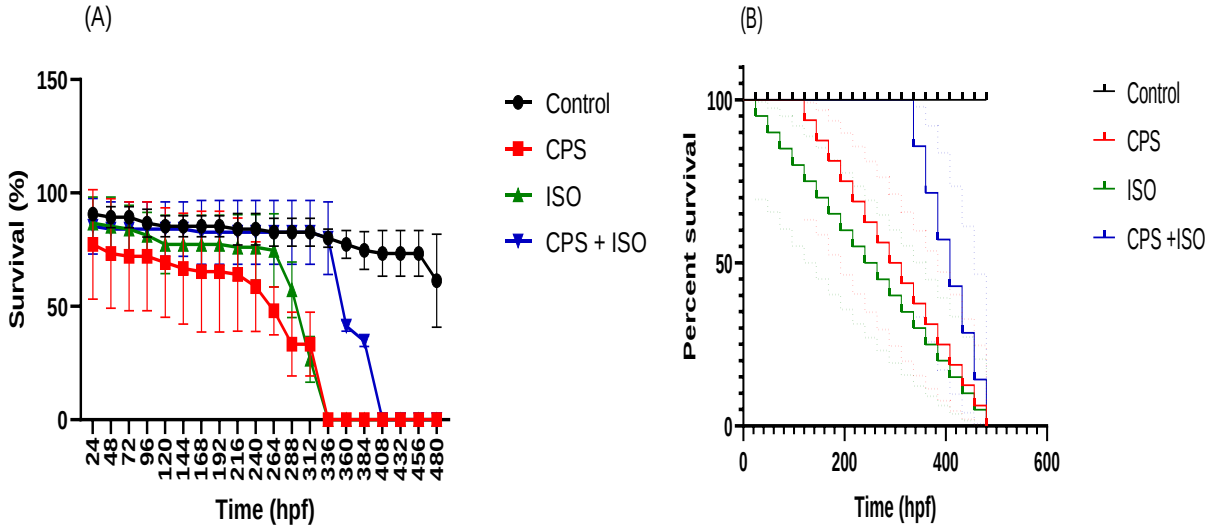
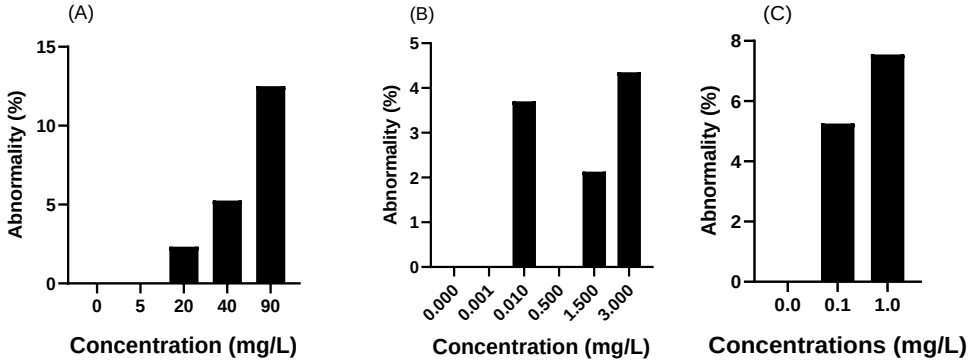



Figure 14: Survival (mean ± SD) of *D. rerio* exposed to either CPS, ISO, or their mixture for 21 days (A) percentage survival, (B) probability of survival proportions. (n= 4 treatments, 20 organisms per treatment, 3 replicates/treatment).

The least effective concentrations was 20 mg CPS/L (Figure 15A) and 0.01 mg ISO/L (Figure 15B) in this study showed that ISO has greater toxic potency. In all cases, numbers of abnormalities were directly proportional to concentration (Figure 15). Various abnormalities were observed (Figure 15D); some common examples were cardiac and pericardia edema, craniofacial abnormality, cranial hemorrhage, spinal malformation and curved tail. Chemicals that cause pericardial edema have been linked to toxic effects on the cardiovascular system (Lu et al., 2022). In-silico studies (Table A.2 and A.3) also estimated CPS and ISO as developmental toxicants. Heart rate (Figure 16) of *D. rerio* without physical damage showed no significant difference from the control, demonstrating that effects of these chemicals might not be easily monitored with heart rate. The heart is the first organ to develop in an embryo, is fully formed, and attains physiological function after 72 hpf (Lu et al., 2022); thus, the effect of moderately toxic chemicals might not be readily observable since these chemicals are mobile. Alternatively, ISO affects the length of *D. rerio* > 1.5 mg ISO/L (Figure 17), showing that a greater concentration of ISO would inhibit growth. CPS and the mixture do not affect the length of *D. rerio* larvae.



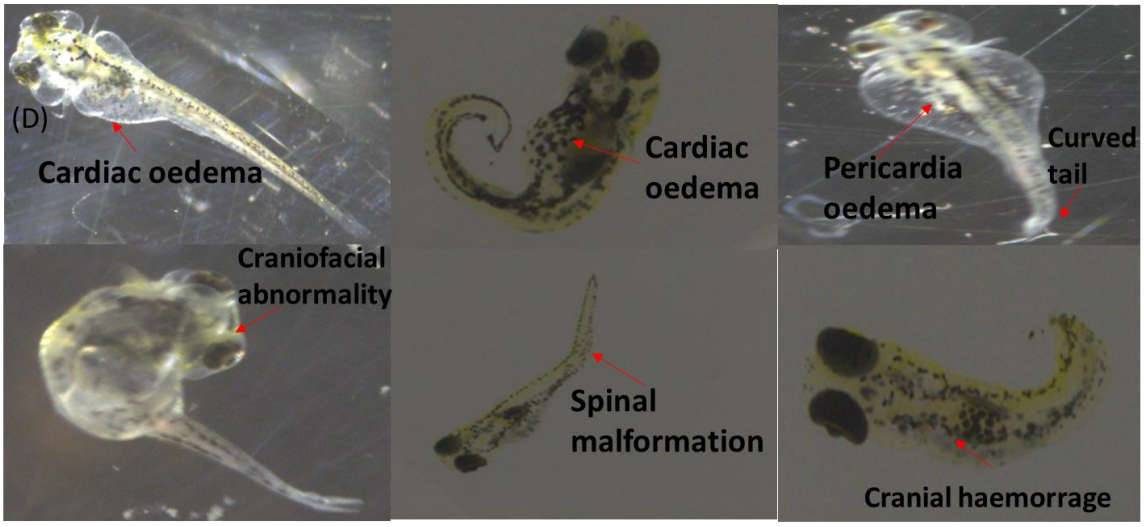



Figure 15: Morphological alteration of *D. rerio* larvae exposed to various concentrations of (A) CPS, (B) ISO (C) CPS and ISO (D) Examples of selected abnormalities.

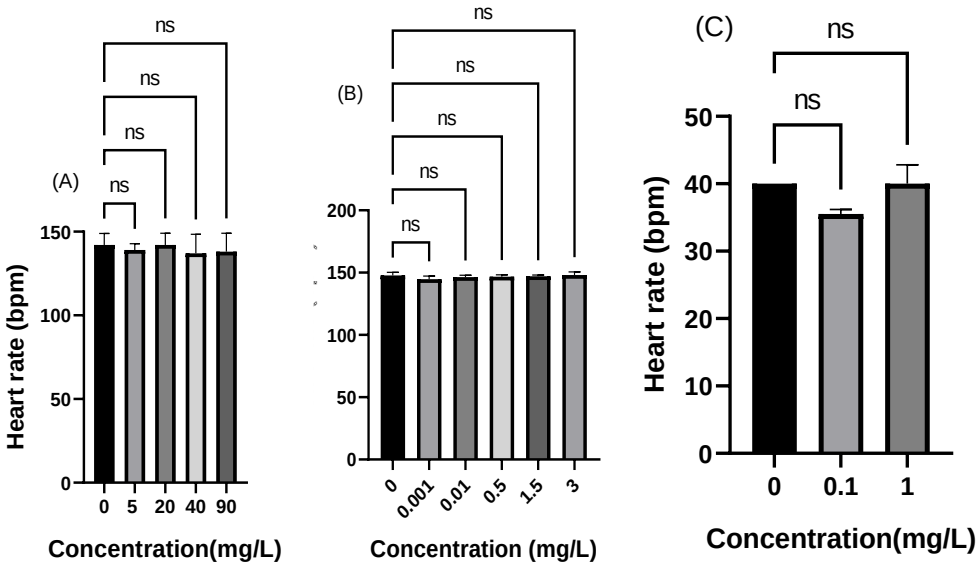



Figure 16: Heartbeat (% (mean ± SD) rate of *D. rerio* after exposure to (A) CPS (B) ISO (C) CPS + ISO for 96 hours, 0 =solvent control.

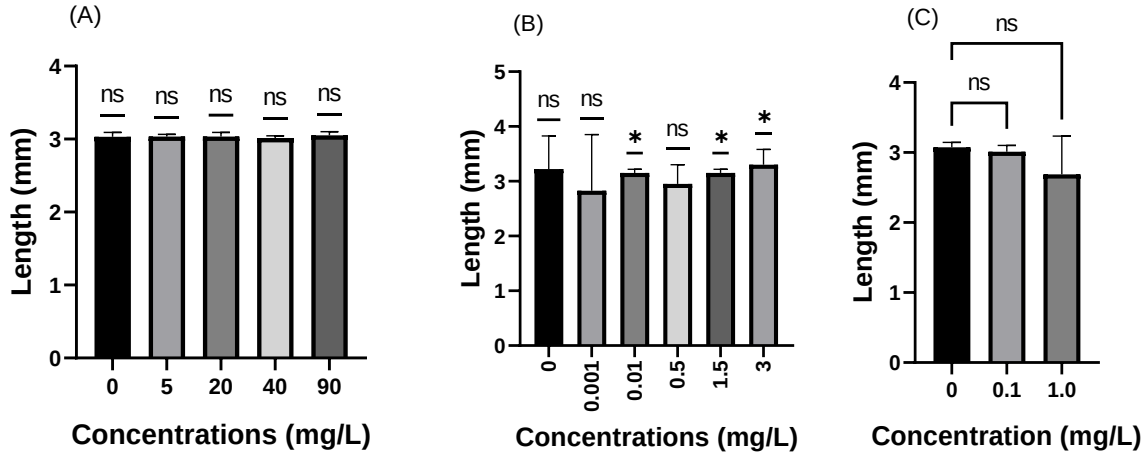



Figure 17: Length (mean ± SD) of *D. rerio* (without visible deformation) exposed to (A) CPS (B) ISO (C) CPS +ISO. *P<0.05 (one sample t test).

### 4.5.3 Antioxidant enzymes/ 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 electron transport chain from monooxygenase activity (Wu et al., 2011). SOD and GST are ideal indicators of oxidative stress. 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 (Figure 18), which leads to a decreased SOD activity in *D. rerio* embryos or due to the saturation of SOD during converting $O^{2-}$ to hydrogen peroxide. Similarly, a reduction in SOD indicates production of excess ROS and intense reutilization of SOD. GSTs are a group of enzymes that are important in detoxifying many different xenobiotics in organisms, and it contributes to cellular protection against oxidative damage. The enzymes protect cells against toxicants by conjugating the thiol group of glutathione

to electrophilic xenobiotics, thereby defending the cells against the compounds' mutagenic, carcinogenic and toxic effects (Kavitha and Rao, 2009). CPS and ISO did not show any significant difference compared with the control group for the activity of GST because *D. rerio* were able to detoxify them without the need to enhance GST activity (Figure 19). Therefore, the difference between activities of GST in the control group and mixture group indicates that the mechanism of action of detoxification in the mixture group was different from that of the single chemicals group. Under normal physiological conditions, there is a balance between production of ROS and activities of a family of antioxidant enzymes. So, oxidative damage occurs when ROS generation exceeds these enzymes' protective capacity (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). Another type of safener, "benaxacor," has been implicated to cause oxidative stress in *D. rerio*, which resulted in pericardial edema and decreased embryo hatching rates (Zhang et al., 2021). Hence, the lack of GST enhancement by CPS and ISO at ≤ 1mg/L could potentially explain why there is no significant difference in the hatching rate.

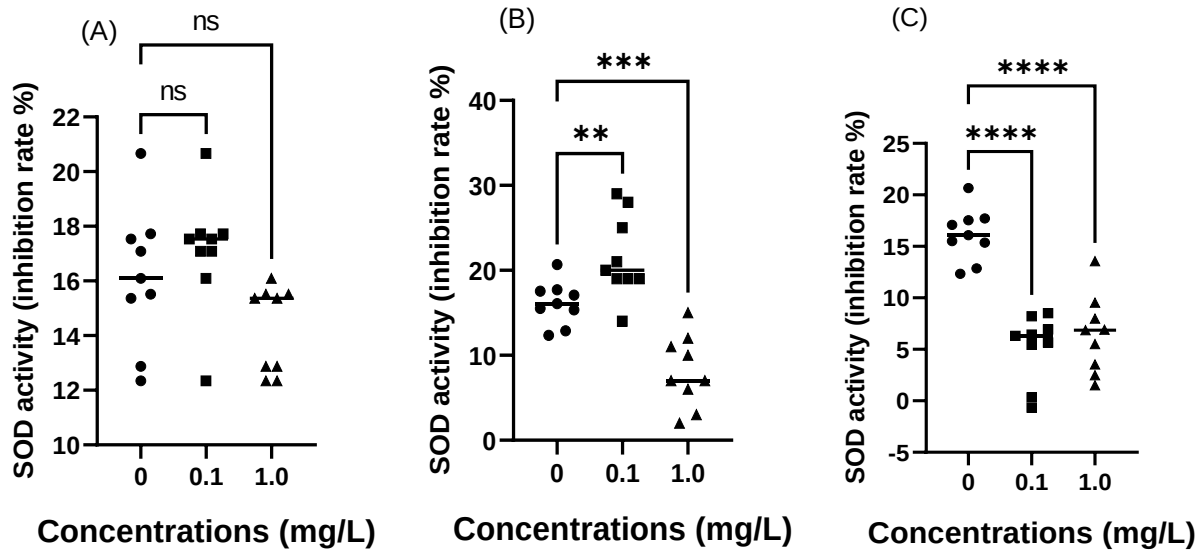



Figure 18: SOD activities of *D. rerio* exposed to (A) CPS (B) ISO (C) CPS +ISO for 21 days. ****P<0.0001, ***P=0.0006, **P=0.008, 0 = solvent control

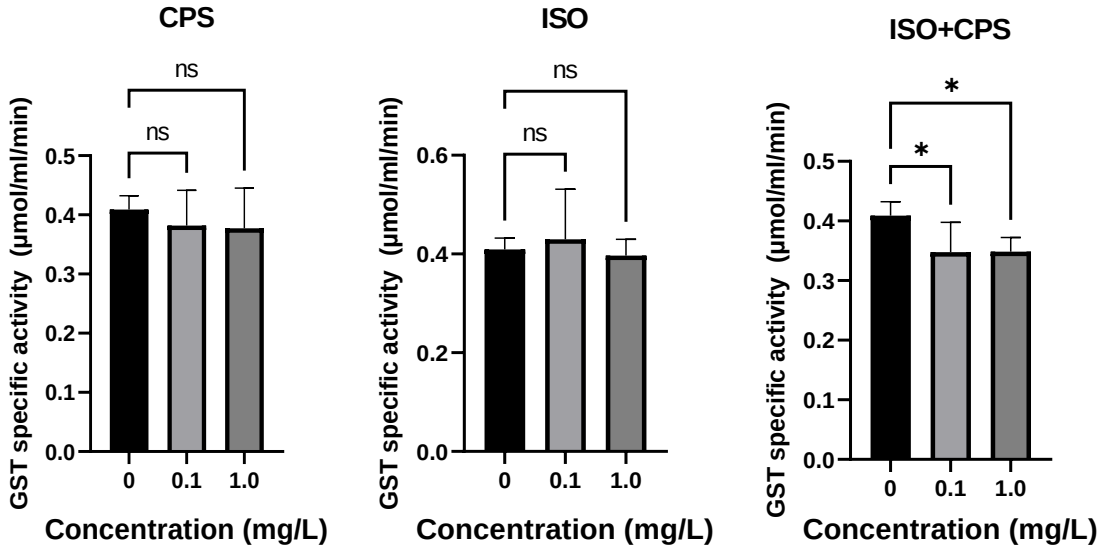



Figure 19: GST activities of *D. rerio* exposed to (A) CPS (B) ISO (C) CPS +ISO for 21 days. *P<0.05, 0 = solvent control.

### 4.5.4 Molecular docking

Receptor of the hatching enzyme (ZHE1 (3lqb)) in *D. rerio* was assessed for molecular docking to CPS (Figure 20) and ISO (Figure 21) because hatching is critical for the development, survival, and reproduction of *D. rerio*. Any disruption to these hormones can have detrimental effects on their health and well-being. ISO (-7.2) and CPS (-7.1) have binding energies similar to that of ZHE1 (Table 13), which would be because they have similar molecular masses and, therefore, similar or close steric hindrance. Their binding energies explain why they behaved similarly on hatching rate (Figure 11). Both ISO and CPS are sulfonated and arenes compounds and have similar structures that have been implicated in causing competitive binding with the herbicide at the active site to increase the tolerance of treated plants (Wang et al., 2021). Protein inhibition was solely based on significant interactions between CPS or ISO and the combination of several amino acid residues found at ZHE1's active site. The similarity in structures might be responsible for them having similar binding energies. However, while ISO binds to only one histidine acid site (His 109), CPS binds to additional sites, arginine (Arg182) and Tyr155. These additional residues mean that CPS has the capacity to inhibit other functions apart from His 109. However, the function of such sites determines if it is essential or not. Arg 182 is an essential residue because it plays an important role in cell division, wound healing, removing ammonia from the body, immune function, and releasing hormones (Wu, 2013). It also helps regulate blood pressure, so binding to and preventing it from functioning is not ideal. One of the mechanisms of action of safeners is to induce detoxifying enzymes, which may be why it binds to more proteins than ISO.

Adsorption, distribution, metabolism, Excretion and Toxicity (ADMET) screening was also done for the compounds by probing the physicochemical properties, pharmacokinetics properties,

drug-likeness, and toxicity of the compounds (Table 14). The lipophilicity of CPS (1.88) and ISO (3.03) showed that their ability to pass through and bind to the cells varies. To pass through the small intestinal lumen, a chemical must exhibit a high level of lipophilicity. Neither ISO nor CPS violated Lipinski and Veber's rules, with both having zero scores. Similarly, the bioavailability score was 0.55 for both chemicals, meaning they are both available for cell uptake. In addition, the gastrointestinal absorption capacity was determined to be large. However, neither ISO nor CPS crosses the blood-brain barrier (BBB) permeability test, and neither were predicted to be substrates for P-glycoproteins (Pgp). ProTox II has has categorized CPS and ISO as class 5 and 4 thereby indicating moderate toxicity. ISO inhibits the mixed function mono-oxygenase enzymes, CYP1A2, CYP2C19, and CYP2C9. While CPS inhibits CYP2D6 and CYP3A4. These enzymes are required for drug metabolism in the first phase. Interestingly, CPS is mutagenicity negative, while ISO was predicted to be positive. Indicating that ISO has a higher probability of causing mutagenic effect, compared to CPS.

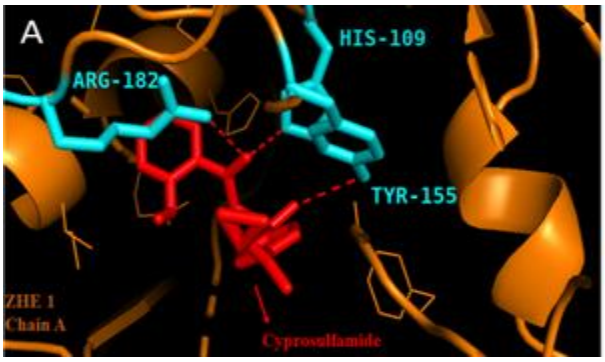



Figure 20: Molecular interaction between CPS and ZHE1 (3lqb) receptor

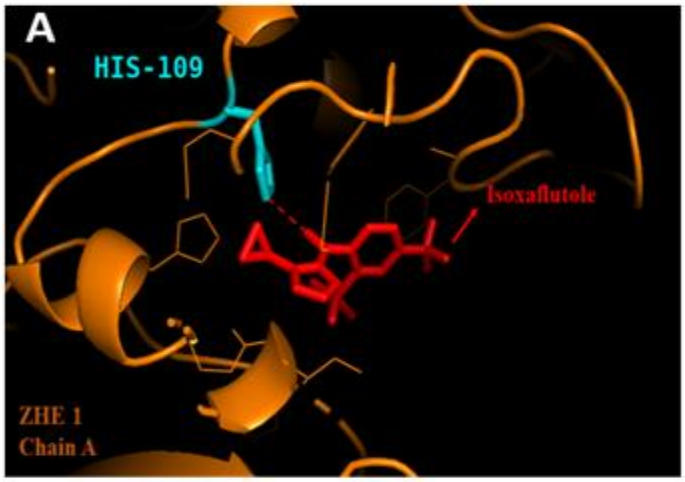



Figure 21: Molecular interaction between ISO and ZHE1 (3lqb) receptor

Table 13*:* Binding properties of selected CPS and MEF with ZHE1

| Chemical | Protein | Protein function | Chain | Binding energy | Residues |
|---|---|---|---|---|---|
| CPS | ZHE1 | Hatching | Whole | -7.1 | His109, Arg182, Tyr155 |
| ISO | ZHE1 | Hatching | Whole | -7.2 | His109 |

Table 14*:* ADME property prediction

| Compounds | GI absorption | BBB permeant | P-gp substrate | CYP1A2 inhibitor | CYP2C19 inhibitor | CYP2C9 inhibitor | CYP2D6 inhibitor | CYP3A4 inhibitor |
|---|---|---|---|---|---|---|---|---|
| CPS | High | No | - | - | - | + | + | + |
| ISO | High | No | - | + | + | + | - | - |

+active -inactive; P-gp – P glycoprotein

## 4.6 Conclusions

CPS is a safener commonly used to prevent harmful effects of herbicides, such as ISO and thiencarbazone-methyl on the crop plants on which they are used. It protects crops by elevating its detoxifying enzymes, such as GST and SOD. In combination with herbicides, this chemical has been found in surface water because of its relative mobility and the possibility of leaching or runoff. CPS and ISO at concentrations ≤ 3 mg/L had no significant effect on the hatching rate of *D. rerio* embryos. Their mixture also did not cause significant inhibition of hatching rate. Nevertheless, at ≥ 20 mg CPS/L and 1.5 mg ISO/L, the chemicals caused some lethal toxicity. The $LC_{50}$ was 136 mg CPS/L and 7 mg ISO/L for CPS and ISO, which indicates that ISO has greater toxic potency than CPS. The mixture of CPS and ISO reduced mortality by 8%; thus, CPS also mitigated the toxic effects of ISO in *D. rerio* embryos. ISO also affects the length of *D. rerio* at < 3 mg ISO/L; this effect was not significant in the mixture. CPS did not cause significant changes in SOD or GST, but ISO altered SOD compared to control, but not GST. The mixture significantly changed SOD and GST, which suggested that the mechanism of action of the mixture is different from those of single chemicals. Results of molecular docking showed that CPS and ISO have

similar binding energy to the hatching enzyme. The docking score of CPS and ISO were similar, but CPS has two more residues than ISO. This work has shown that the mechanism of action of the mixture of ISO and CPS differs from that of each of them and that of each singly.

# 5 Chapter 5: Toxicity of Individual and Combined Effect of Mefenpyr di-Ethyl Safener and Its Co-Herbicide, Fenoxaprop-P-Ethyl, to *D. rerio*

## 5.1 Preface

Building upon the results of Chapter 5, this chapter leveraged the compelling evidence of the link between individual and mixture effect of CPS and ISO with exposure concentrations coupled with their binding affinity to substrates. Similar to chapter 5, this chapter aimed to test the hypothesis that early embryonic life stages *D. rerio* are not affected differently when exposed to MEF individually or with a binary mixture of MEF and FEN. Acute and chronic studies using approximately 2hpf *D. rerio* embryos, coupled with biochemical tests using plate reader were used to determine difference in effects of MEF, FEN and their mixture on ELS *D. rerio*, and checking for effects on survival, hatchability and growth. In this chapter, *D. rerio* embryos were exposed to varied concentrations of MEF, FEN and MEF + FEN from day 0 to 4 (acute) and day 0 to 21 acute studies. Similar to chapter 4, this paper also attempted to link safener capacity to protect from herbicide to its effects on the observed endpoints.

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Abstract

Mefenpyr diethyl (MEF), a supposed inert chemical, which is a safener used in herbicide formulations with active ingredients such as Fenoxaprop-P-ethyl (FEN), has been found in surface water. Since hazards of this safener, individually and in combination with its herbicide, toward non-target aquatic organisms, were not known, therefore, acute and chronic studies were conducted on various endpoints in embryos of *D. 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 was more toxic 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 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.

Keywords: Chronic exposure, Acute toxicity, Mixture toxicity, Mortality, Reproduction, Molecular docking.

## 5.2 Introduction

Safeners are compounds used in combinations 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 Tukar, 2008). Relatively great concentrations of FEN have been implicated in causing injuries to plants and 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 & 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 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 *D. rerio* (*D. 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 (*D. 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. *D. rerio* embryos are well-studied model organisms that have been used for testing toxicities of contaminants (Chen et al., 2011).

Effects of various chemicals on *D. rerio* have been well documented, but no studies on the toxicity of MEF or its mixture with FEN have been reported for *D. rerio*. *D. rerio* 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 *D. rerio* are becoming more popular (Chen et al., 2011). Morphology of the spinal column and vertebral structure of *D. rerio* is similar to that of humans (Boswell & Ciruna, 2017). Therefore, using this organism will provide better understanding of the toxicity of this chemical singly and in combination with FEN.

Because of its ability to show the possibilities of chemicals binding with enzymes or proteins within the body of animals or plants, molecular docking of chemicals is useful in determining the most likely molecular initiating events. 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 toxicity to the protein targets compared to FEN will determine the feasibility of various molecular events. These chemicals were docked with the hatching enzyme ZHE1 because hatchability was a major endpoint in this study and the ability of MEF and FEN to bind this target can give a clue and help predict the effects that will be observed in the experimental part of this study. Safeners are hypothesized to bind to this receptor because several studies have shown that safener could delay and inhibit hatching altogether (Liu et al., 2022).

This study assessed the toxicological effects of MEF and FEN, singly and in a mixture, on *D. rerio* 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 different toxic effects different from the effect observable with single chemicals. In this study, *D. rerio* were exposed to varying concentrations of MEF and FEN for 96 hours, 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.

## 5.3 Materials and methods

### 5.3.1 Chemicals and reagents

The safener, MEF and herbicide, FEN, were purchased from Sigma-Aldrich (Canada). Stock solutions were prepared with 0.01% acetone v/v. 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).

### 5.3.2 *D. rerio* maintenance and egg collection

*D. rerio* were bred at a ratio of 2:1 (female: male), with approximately 20 females and ten males per breeding tank (Li et al., 2018). 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 *D. rerio* 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 - ½ 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.

5.3.3 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 stone 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 and several drops of methylene blue were added, and additional feces were removed with a pipet. *D. rerio* 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.

5.3.4 Embryotoxicity test

Embryo toxicity tests were performed following the Organization for Economic Co-operation and Development (OECD, 1992) 236 guidelines with some modifications to assess the effects on some endpoints, including mortality and hatching. Solvent concentration was kept at 0.01% Acetone (v/v) throughout the experiment and the solvent control contains acetone and water alone. A semi-static approach with up to 98% water renewal per day was adopted to retain stable concentrations of the test compounds in the exposure plates and petri dishes. Briefly, embryos, at 2 hpf, were exposed to six concentrations of either MEF or FEN (solvent control(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 (solvent control (), 0.1 and 1 mg/L) until 96 hpf. The mixtures concentrations were selected considering the ratio of safeners to the active ingredient in the herbicide formulation

(up to 20% to 80% herbicide to safener) (EPA 2015) and the environmental relevant concentration. 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 of 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 *D. rerio* 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. The temperature during the test was 26 ± 1 °C. The daily photoperiod was 16 h of light and 8 h of darkness.

### 5.3.5 Assessment of deformations

Detection of deformations in the zebrafish embryos were done by a visual assessment and phenotype scoring of morphological features. Each embryo and larvae were viewed under the microscope and every form of deformations were recorded. An overall incidence of deformities was thereafter calculated by dividing the number of individuals classified as deformed by the total number of individuals in that replicate. The results were then converted to the percentage of the population for each concentration.

### 5.3.6 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.

### 5.3.7 Determination of oxidative stress biomarkers

Sixty embryos were randomly selected and placed into Petri dishes for each concentration (90 mm diameter) with 50 mL of exposure solution per 20 eggs/larvae. More than 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 minutes 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.

### 5.3.8 Molecular docking analyses

The hatching enzyme (ZHE1 (3lqb)) in *D. rerio* was selected as target as a molecular initiating event because hatchability is one of the endpoints chosen for the experimental study. 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 (20X20X20) was created for ZHE1 protein 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 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.

### 1.1 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.

### 5.4 Statistical analysis

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.

### 5.5 Results and Discussion

#### 5.5.1 Hatching rate

No significant difference was observed in the hatching rate between the control and concentration ≤ 1.5 mg MEF/L, while a significant difference was evident at concentrations ≥ 3 mg MEF/L (Figure 22A). Therefore, the concentration of MEF at which no observable effect was

observed was determined to be 1.5 mg/L. The analysis was done using one way ANOVA. was observed on rate of hatching *D. rerio* embryos was 1.5 mg/L. There was no significant effect on rate of hatching of *D. rerio* embryos exposed to concentrations ≤ 5 mg FEN/L (Figure 22B).

Mixtures of MEF and FEN did not result in any significant changes in the hatching rate compared to the control (Figure 22C). There was also no significant difference in *D. rerio* 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 (Figure 23). 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 *D. rerio* embryo hatching, in which lesser concentrations of 0.01 mg/L delayed hatching, while greater concentrations, such as 0.1 mg/L, resulted in hatching inhibition (Liu et al., 2021). Chemicals such as PFOS have also been shown to cause delayed hatching in *D. rerio* (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 ELS of *D. rerio* 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 with log Kow equal to 3.9, may be more soluble than FEN (log Kow= 4.1) (PubChem), and could penetrate inside the pore faster than FEN if the Log Kow will make a difference. 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.

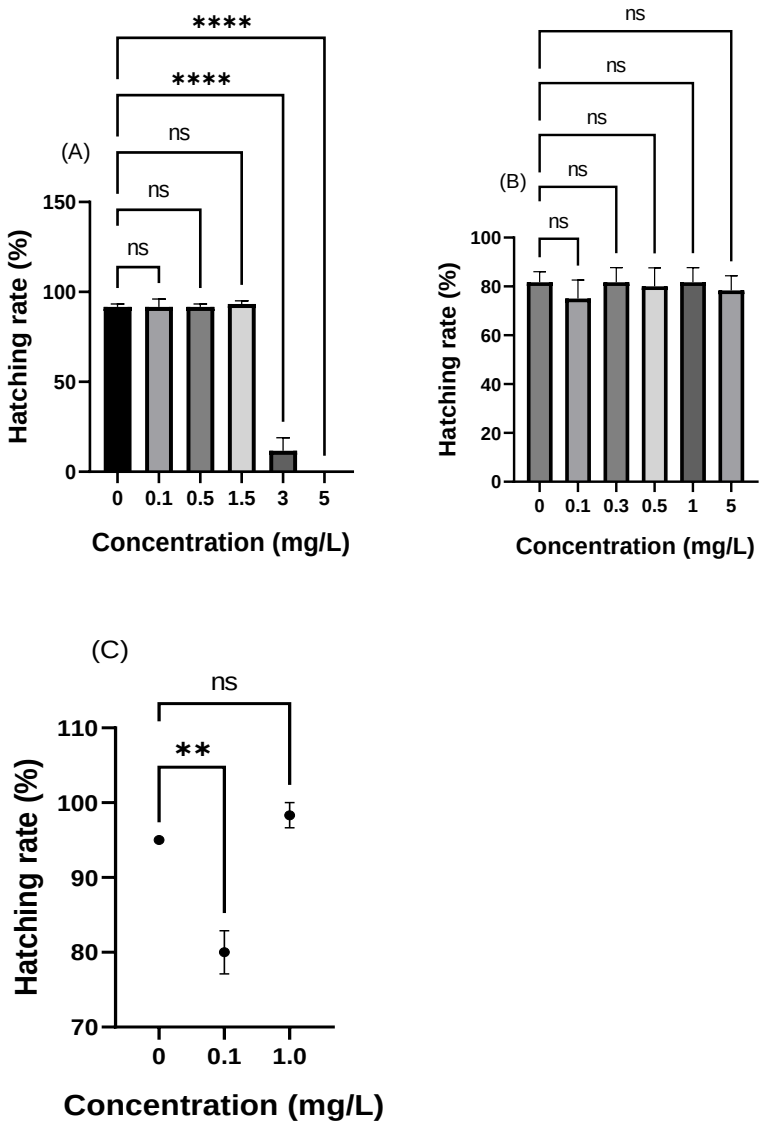


Figure 22: Average number of hatched *D. rerio* larvae after exposure to a varied concentration of (A) MEF, (B) FEN, (C) MEF + FEN for 96 hours *P<0.01, **P<0.005, ****P<0.0001.

### 5.5.2 Survival rate and morphological changes

Exposure to MEF or FEN caused lethality of *D. rerio* embryos at concentrations ≥ 0.1 mg/L in the 96-hour study. Exposure to greater concentrations, > 3 mg MEF/L resulted in 100% mortality during the yolk sac stage (Figure 24A), while there was 100% survival of embryos exposed to concentrations > 3 mg FEN/L (Figure 24B). 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 *D. rerio* embryos (Figure 24C). Since MEF was more potent than FEN, this result can be interpreted as FEN antagonizing the effects of MEF.

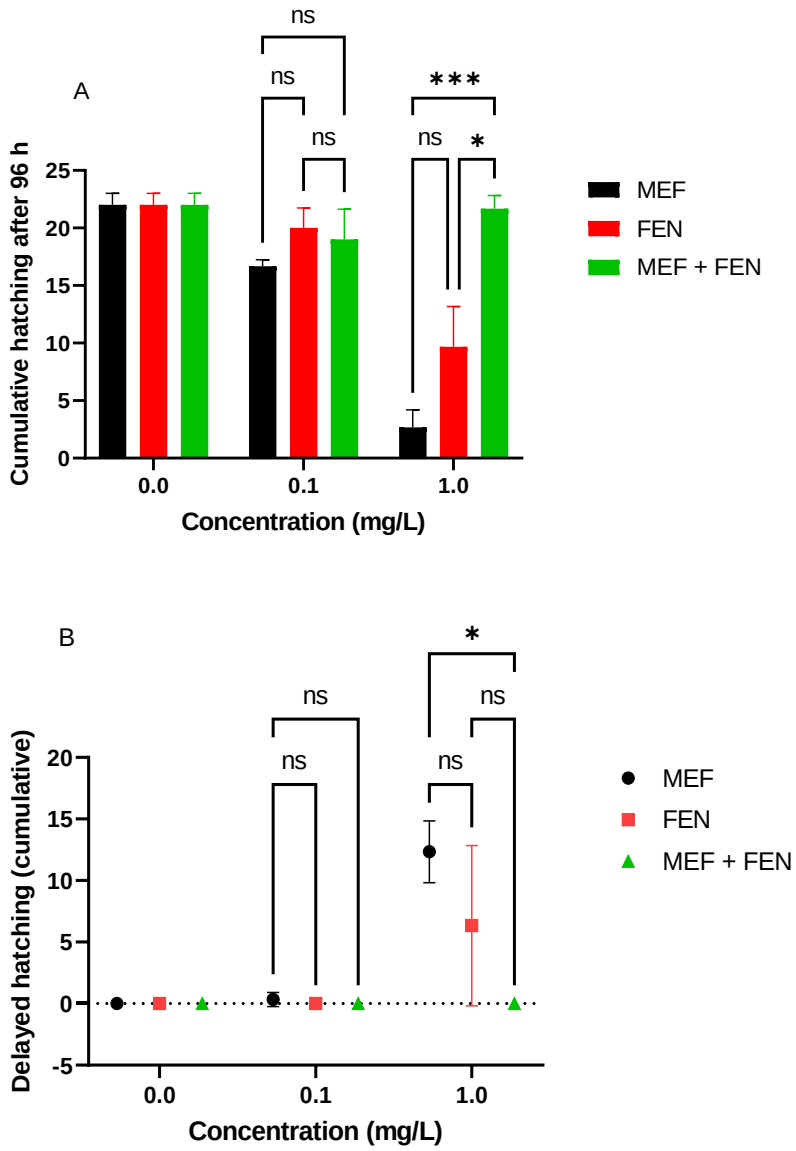


Figure 23: Average number of hatched *D. rerio* larvae (A) during the normal hour after 96 h and (B) delayed period after 120 h for MEF, FEN and their mixture at different concentrations. *P<0.05, ***P<0.001.

The $LC_{50}$ of MEF to Rainbow trout was reported to be 4.2 mg/L after 96hpf (PubChem), which was greater than the value of 2.93 mg/L observed for *D. rerio* after 96 hpf in this study (Table 15). For FEN, the $LC_{50}$ for rainbow trout was 0.46 mg/L (PubChem), while the observed

$LC_{50}$ for *D. rerio* 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 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).

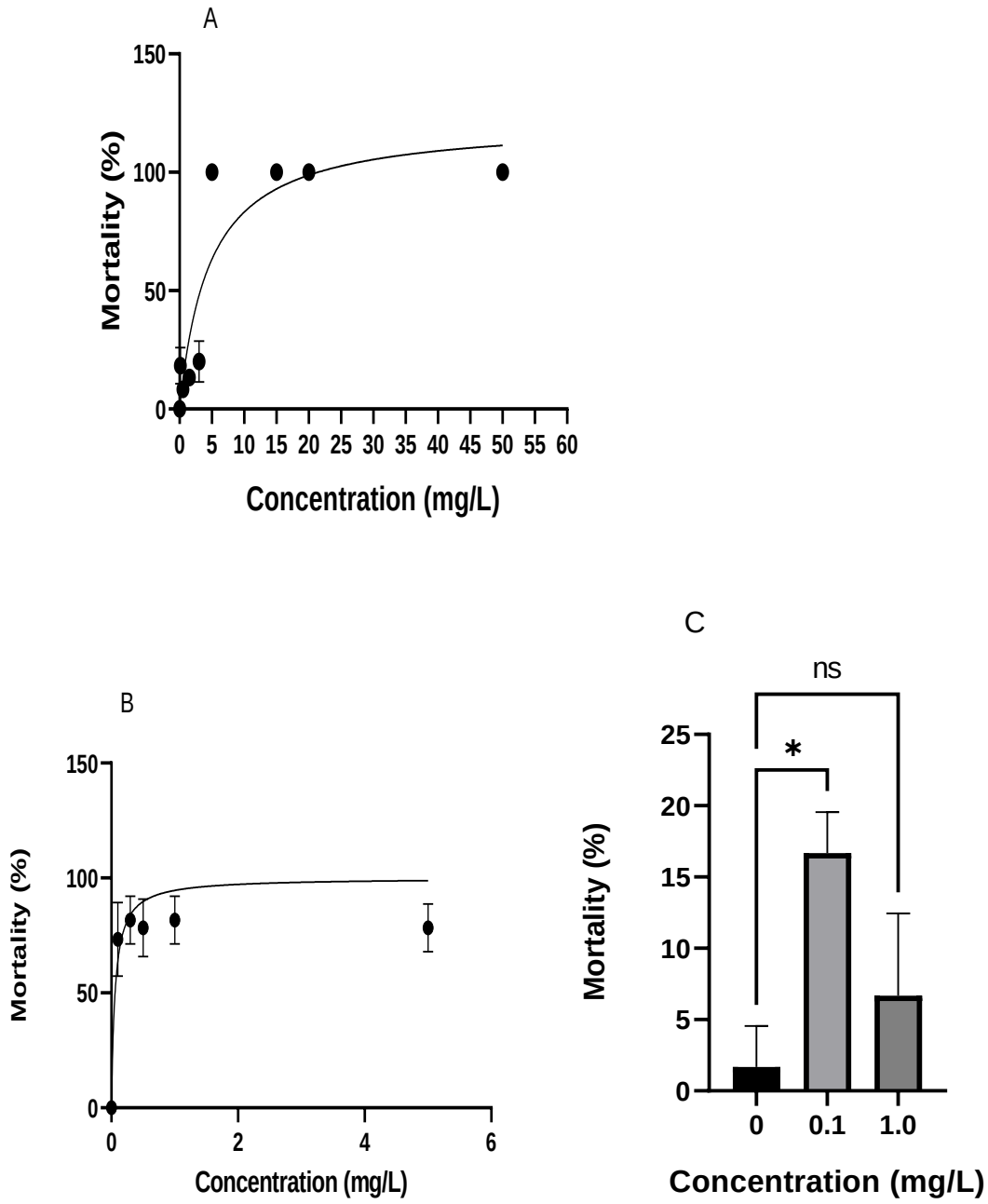


Figure 24: Mortality (mean ± SD) rate in *D. rerio* larvae after exposure to (A) MEF, (B) FEN and (C) MEF+FEN for 96 hours. (n =3 treatments, 20 organisms/treatment; 3 replicates/treatment) *P<0.05.

Table 15: Lethal concentration ($LC_{50}$) of MEF and FEN that could kill half the exposed population in a 24-hour period for 4 days and their confidence interval (CI)range.

| | MEF | | FEN | |
|---|---|---|---|---|
| | $LC_{50}$ | CI | $LC_{50}$ | 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 |

Toxicity curves for MEF and FEN (Figures 25 and A.4) had $LC_{50}$ that were smaller at greater durations, which confirmed that toxicity to *D. rerio* 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 in when exposed to MEF, FEN individually or as a mixture, respectively. This observation implies that MEF is more toxic than FEN and since it took longer for the mortality to be 100%, the toxic potency of MEF was less when embryos were exposed to a combination of FEN and MEF. This observation suggests 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.

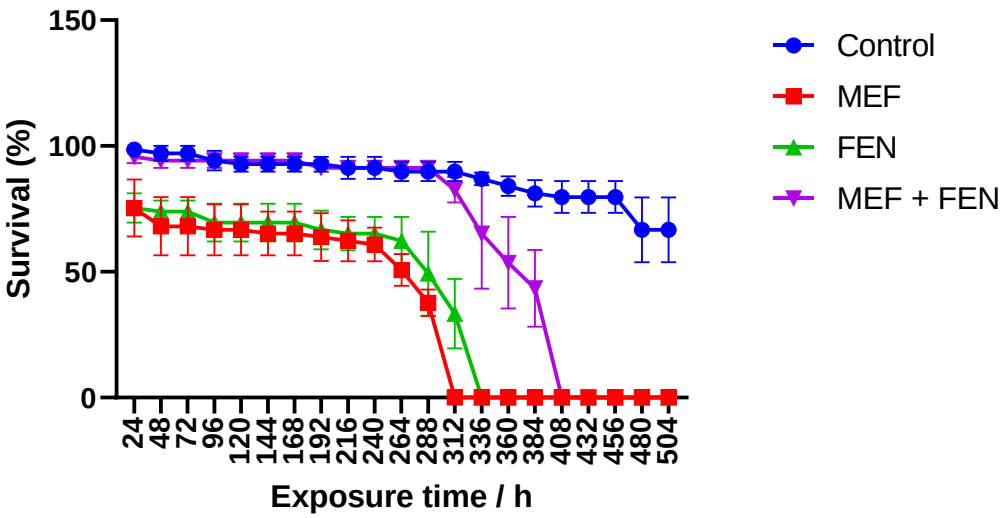


Figure 25: Survival of *D. rerio* exposed to MEF, FEN, and a mixture of MEFl and FEN for 21 days.

Several deformities, including pericardial edema and spinal malformation, were observed in *D. rerio* larvae (Figure A.5-A.7). The common sublethal effects observed in embryos exposed to MEF or FEN are 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 will be toxic to *D. rerio* 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 the heart rate (Fig. A.8) and length (Fig. A.9) of organisms exposed to MEF or FEN were not significantly different from 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 *D. rerio* in the different treatment groups, even when there were different degrees of damage in the internal organs (Yang et al., 2016).

### 5.5.3 Antioxidant enzymes/ 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 antioxidant enzyme whose expression could predict tolerance to the chemicals and inhibition could predict oxidative stress. MEF, FEN and mixture of MEF and FEN had significant effect on SOD activities by reducing it in *D. rerio* at all concentrations compared to the control group (Figure 26). 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 *D. rerio* embryos or due to the saturation of SOD during the process of converting $O^{2-}$ to hydrogen peroxide. A reduction in SOD suggests the production of excess ROS and intense SOD reutilization. MEF had a significant effect on *D. rerio* by altering the GST activities across concentrations when compared to the control groups (Figure 27). 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 *D. rerio* by causing a reduction in the activity of GST at both concentrations compared to control group (Figure 27). GST contributes to cellular protection against oxidative damage and detoxification of many xenobiotics. It converts

xenobiotics to non-toxic metabolites by conjugation with glutathione (Kavitha and Rao, 2009). Therefore, an increase or decrease in GST might indicate tolerance and oxidative stress respectively. 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 *D. rerio*, which induced oxidative stress, pericardial edema and decreased embryo hatching rates (Zhang et al., 2021).

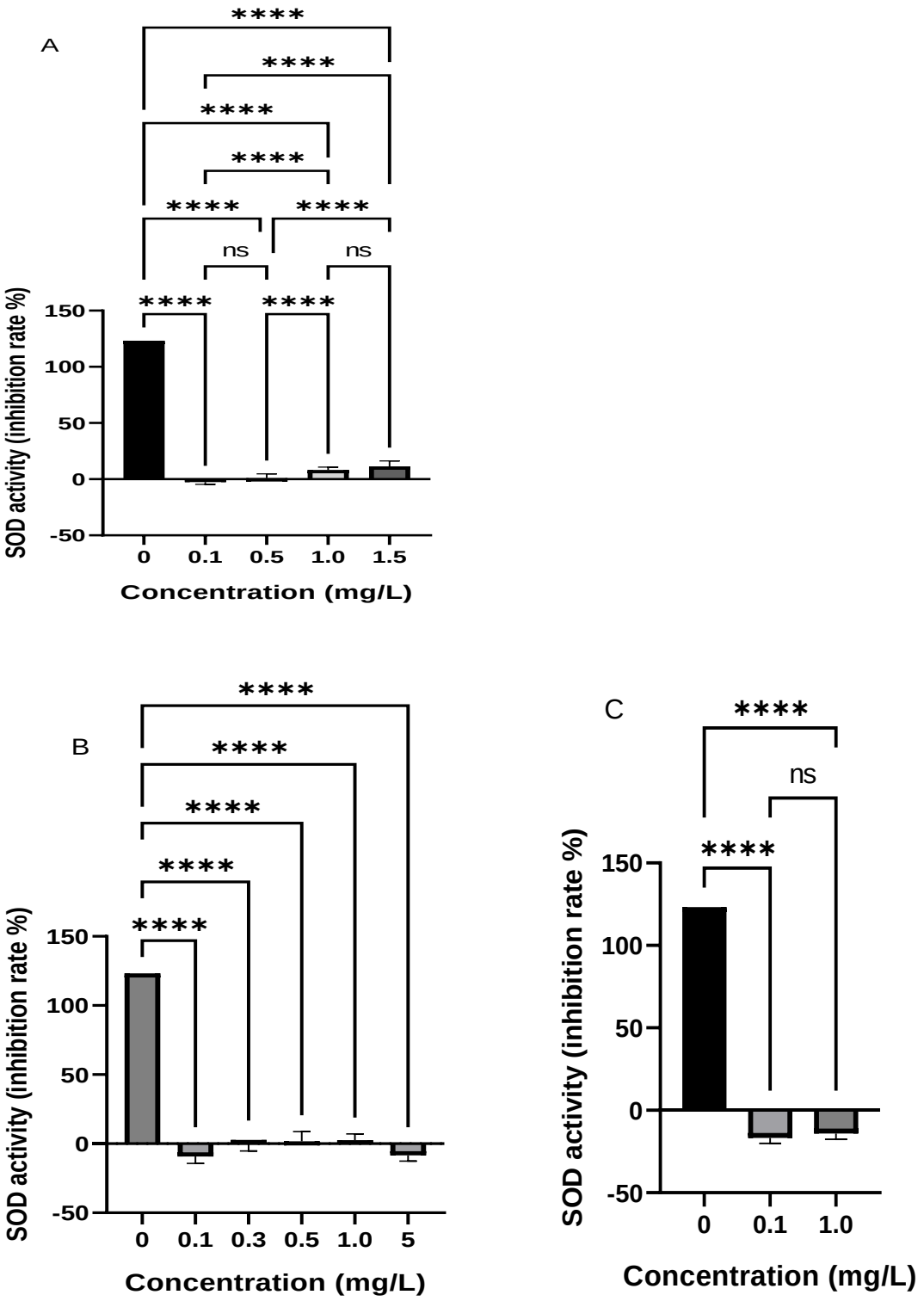


Figure 26: SOD activities of *D. rerio* exposed to (A) MEF (B) FEN (C) MEF + FEN. ****P<0.0001, 0= solvent control.

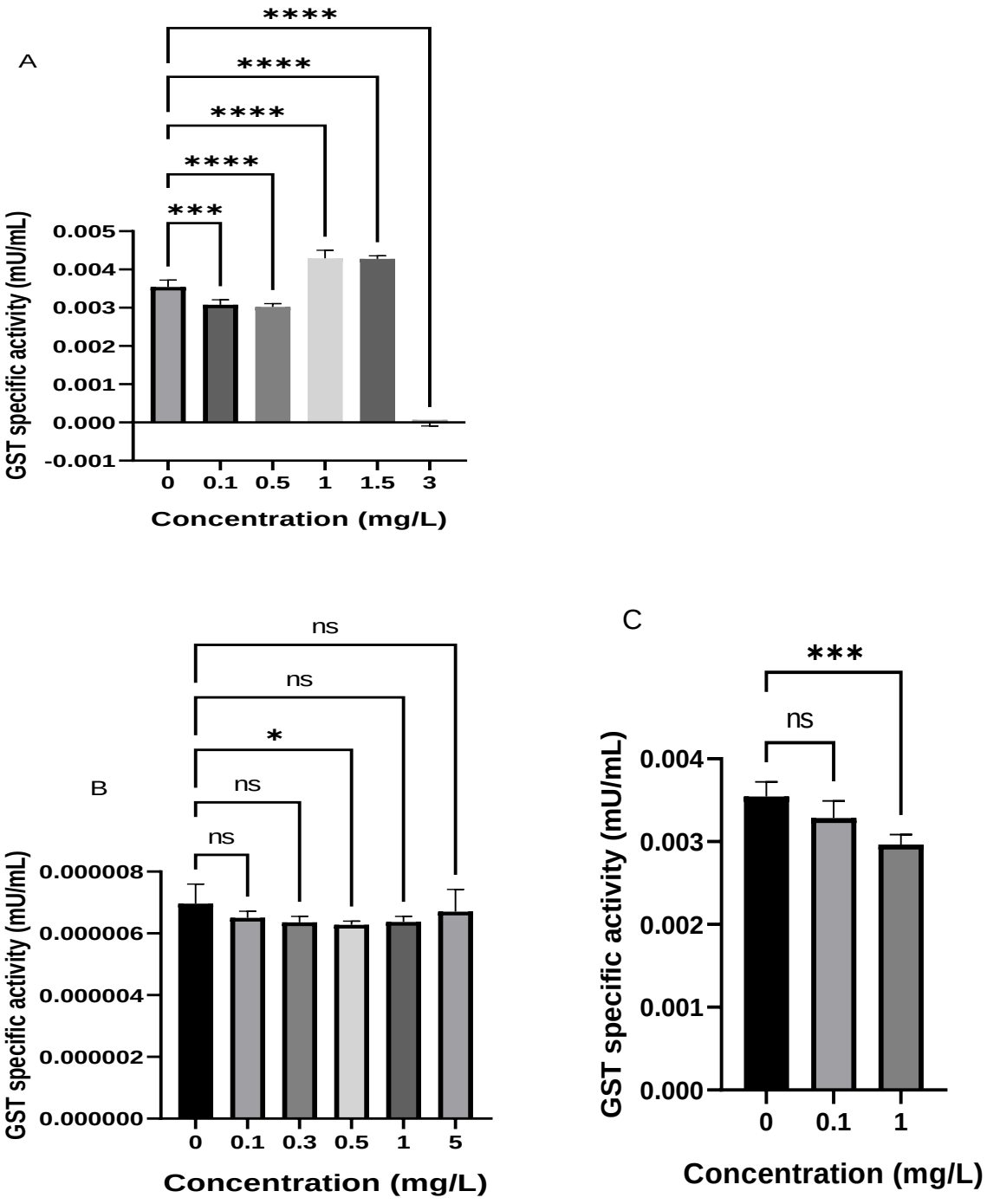


Figure 27: GST activities of *D. rerio* exposed to (A) MEF (B) FEN (C) MEF + FEN. ****P<0.0001, *P<0.05, ***P=0.0001, 0 = solvent control.

### 5.5.4 Molecular docking

Hatching enzymes is important to determine reproductive success, and any chemicals that bind strongly with their substrates have potential to affect reproduction. Therefore, the hatching enzyme (ZHE1 (3lqb) (Okada et al., 2010) in *D. rerio*, which has been well characterized was docked with MEF (Figure 28) and FEN (Figure 29). 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 (Fig 28, 29 and Table 16). 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 (dG) of protein-ligand complexes more accurately. It is one of the techniques that help to improve the virtual screening of toxicity results. 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 for the 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., 2012). Histidine has been shown to have the ability to undergo various types of molecular interactions such as cation –π interactions, π-π stacking interactions, hydrogen-π interactions, coordinate bond interaction 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 16). This confirmed the ligation of histidine. These amino acids

are essential because they cannot be sufficiently synthesized during certain physiological periods of growth or recovery from stress (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). They showed that the chemical binds to three amino acids, His 99, His 109 and Arg182, through hydrogen bonding and thus inhibits hatching of *D. rerio* 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 (Figure 23). 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).

However, as much as binding energy is important to determine the binding affinity, which translates to toxicity since binding to a site will prevent its functionality, the binding location also matters. Thus, MEF binding with both Thr 332 and Asn 330 will inhibit the activities of the site, while FEN will only inhibit the activity 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 *D. rerio* reproduction since they both bind to hatching enzyme active site, 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 17). 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).

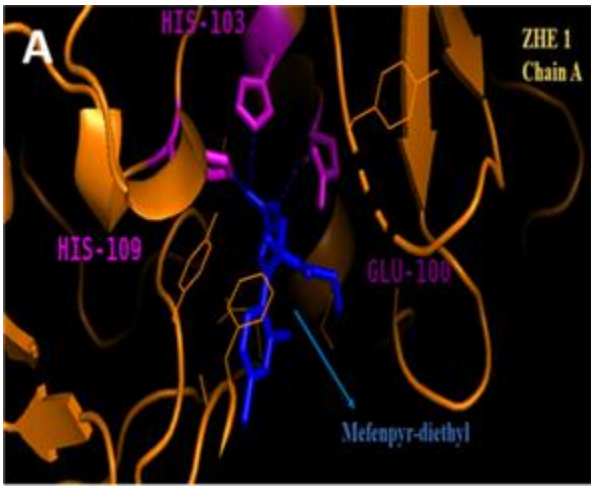


Figure 28: Molecular interaction between MEF and ZHE1.

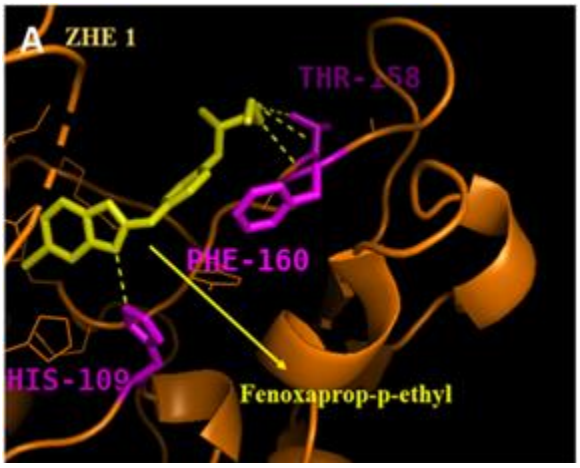


Figure 29: Molecular interaction between FEN and ZHE1.

Table 16*:* Binding properties of ZHE1 with MEF and FEN

| Chemical | Protein | Protein function | Chain | Binding energy | Residues |
|---|---|---|---|---|---|
| Mefenpyr-diethyl | ZHE1 (3lqb) | Hatching enzyme | Whole | -6.2 | His 109, His103, Glu 100 |
| Fenoxaprop-p-ethyl | ZHE1 (3lqb) | Hatching enzyme | Whole | -6.9 | Phe 160, Thr 158, His 109 |

Table 17*:* ADME property prediction

| Compounds | GI absorption | BBB permeant | Pgp substrate | CYP1A2 inhibitor | CYP2C19 inhibitor | CYP2C9 inhibitor | CYP2D6 inhibitor | CYP3A4 inhibitor |
|---|---|---|---|---|---|---|---|---|
| Mefenpyr-diethyl | High | Yes | - | + | + | + | | + |
| Fenoxapro-p-ethyl | High | Yes | - | + | + | + | - | - |

+active -inactive

## 5.6 Conclusion

MEF and FEN are chemicals commonly formulated and applied for weed control. While FEN is the active ingredient, MEF mitigates the negative effect of FEN on crops probably by chemical inactivation, considering that MEF is more potent than FEN. FEN was found to have slightly less toxic potency than MEF in the ELS of *D. rerio* 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 ≥ 3mg/L, while no significant differences were observed for such concentration in 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 level of *D. rerio* larvae to MEF is moderate to high, and its toxicity level is higher 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 many malformations in embryos and larvae, singly and as a mixture. Some examples of the malformations are pericardial edema, spinal curvature, tail malformation and yolk sac edema. SOD and GST expression and inhibition showed tolerance to the chemicals and predicted that that oxidative stress occurred in the organisms respectively, due to the chemicals, and molecular docking showed that the chemicals bind with both the hatching enzyme and other proteins. FEN has a higher binding affinity to the hatching enzyme, 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.

# 6 Chapter 6: General Conclusion

## 6.1 Introduction

The use of safeners in herbicides' formulation is crucial for improving crop yield and quality. However, it cannot be ignored that they have negative effects on crops. To reduce or eliminate these negative effects, safeners are added to herbicides, which change their mechanisms of action. It is important to note that safeners have become an important component of herbicides and have been widely used either during pre- or post-emergence of crop plants. This study has shown the role of safeners on various endpoints in *D. magna* and ELS of D. *rerio*. This study was important because toxicity of safeners to non-target organisms was not well documented, and where available, they are associated with earlier safeners such as dichloroacetamide. Safeners such as CPS and MEF have become popular, and their toxicity study would be beneficial for both regulatory and environmental purposes. While in vivo studies remain the best in evaluating chemical toxicities, in vitro and computational methods are encouraged as they hold promise as alternatives to live animal tests even though they have limitations with regards to quantitatively predicting adverse outcomes and their corresponding toxicity thresholds as these models do not represent the entirety of an organism. Recent developments suggest that ELSof fish are sensitive surrogates of adult fish (Wheeler et al., 2014) while some of the embryonic stage is not being considered as live animals in many jurisdictions (Canadian Council on Animal Care, 2005; European Union, 2010; UK, 1993). Thus, this dissertation aimed to evaluate and determine toxicity threshold for common widely accepted toxicity testing models, *D. magna* and *D. rerio* embryos, exposed to SESs in support of environmental risk assessment (ERA). Specifically, less than 24 hours *D. magna* and approximately 2hpf *D. rerio* embryos were exposed to graded concentrations of two SESs, CPS and MEF and its co-herbicides, ISO and FEN. Whole body tissues that were

continuously exposed to either the safener or with the herbicides for up to 21 days, were collected for biometric measurements, survival analysis and biochemical activity assays including GST and SOD. Heart rate and hatchability were assessed at 4dpf. Biochemical activity assays were conducted to link observations with downstream effects and apical outcomes. Data from the studies were analysed and effects on the animal models were compared across safeners and co-herbicides. Toxicity software and other computational studies were also used to predict toxicity of these chemicals.

## 6.2 Physicochemical properties of SESs influencing bioavailability

This study has shown that safeners' mobility and chemical properties vary. For instance, CPS is mobile and hydrophilic, while MEF is relatively hydrophobic because it has higher log Kow than CPS. MEF's log Kow is like that of FEN. The common route by which safeners could enter the non-target organisms includes surface runoff, leaching, sorption, and volatilization (chapter 1). There are also possibilities that hydrolysis, photolysis, and microbial degradation could cause the transformation of the parent's compounds. While most safeners may not persist in the soil system, there is evidence of the availability of safeners in some rivers. The presence of safeners such as CPS in the river could be because of the low sorption capacity and thus prone to be transported to the nearby surface water. In chapter two, the sorption and toxicity of CPS were examined because CPS has gained significant attention in recent years due to its unique properties and potential for use as a safer alternative to traditional safeners. Soil properties affect the sorption of the CPS to the soil, which invariably determine their possibility to leach to nearby river. Soil pH is the major property of the soil, which determine the sorption coefficient. It is known for its high efficacy, low toxicity, and ability to provide broad-spectrum protection against different herbicides, making it a promising candidate for crop protection in modern agriculture.

## 6.3 Toxicity from acute and chronic studies

In chapter 2 we showed that CPS has no lethal toxicity to *D. magna* at ≤120 mg/L for acute study, while MEF induced some degree of mortality at ≤ 6 mg/L (chapter 3). The difference in lethality for both CPS and MEF is related to wide variability in their chemical and physical properties. Thus, the effect of safeners on *D. magna* is chemical-specific and cannot be generalized. In-silico prediction confirmed that the toxicity of MEF is higher than that of CPS. The twenty-one-day chronic study also showed that CPS is low to moderately toxic to *D. magna* while MEF is moderate to high. However, both chemicals are classified under the category of developmental toxicants due to their potential to cause deformities in organisms. The higher the concentrations, the higher the probability of the chemicals causing a significant effect on the number of neonates and brood size. Thus, there was a negative correlation between longevity, number of broods per adult and days to first brood for both chemicals.

CPS has no significant effect on hatching rate of *D. rerio* embryos at < 3 mg/L, while MEF has a significant effect at the same concentrations. The $LC_{50}$ of CPS to *D. rerio* was 120 mg/L, while that of MEF was 3 mg/L. Thus, the toxicity of MEF was 40 times more than CPS in *D. rerio*. Nevertheless, some common malformations include pericardial edema, yolk sac edema, spinal curvature, and tail malformation. The ability of these chemicals to cause malformations means that their use needs to be regulated. As expected, both herbicides, ISO and FEN induced some degree of toxicity on *D. rerio* embryo which was highlighted in chapter 4 and 5. Nevertheless, FEN is more toxic than ISO. This observation could be associated to their respective chemistry such as logKow and solubility.

### 6.3.1 Mixture of herbicides and safeners

Since compounds are commonly found in mixture in an aquatic environment, then mixture of compounds is necessary to have idea of a real-life situation. Mixtures of compounds can cause changes in biological systems which might reduce or increase the toxicity individual compounds (Hernado et al., 2003). Different type of interaction could occur when mixtures of compounds interact with biological system, with the common interactions being antagonistic, additive, or synergistic. In chapter 4 and 5, a mixture of safeners with herbicides has shown that antagonistic interaction occurred, which is beneficial to the organism as the percentage of toxicity is reduced in the mixture. This result is consistent with a study which states that safeners may act as 'bioregulators' whereby they regulate the amount of a given herbicide that reaches its target site in an active form or as 'antagonists' of herbicidal effects at a similar site of action (Hatzios, 1991) The mixtures extended the longevity of *D. rerio*. The mechanism of action of safeners when applied singly differed from when applied jointly with their corresponding herbicides. Thus, the mixtures of safeners and herbicides are not only beneficial to crops but also helpful in reducing the potential toxicity to aquatic organisms. For a more potent safener like MEF compared to it less potent co-herbicide, FEN, the mechanism of action could be by chemical inactivation rather than antagonistic, considering the reduction in toxicity on the model organisms, as a result of the binary mixture. Antagonistic interaction occurred when inhibition of enzyme by one of the chemicals modify the organism's response towards the second compound. Similarly, both chemicals might be competing with the other at the target site in the organism. Metabolic processes related to acetyl-CoA metabolism have been implicated as likely target sites for a competitive antagonism between safeners and chloroacetanilide or carbamothioate herbicides (Hatzios, 1991).

## 6.4 Biochemical activity study

GST and SOD were considered in this study because they are both important in understanding the effect of toxicant in various biological system. GST is a soluble protein with low molecular weight in various cells and tissues (Song et al., 2017). They are a family of detoxification enzymes that catalyse the conjugation of glutathione with electrophilic compounds (such as safeners and herbicides), thus preventing toxicity. They generally activate defence against oxidative damage and peroxidative products of DNA and lipids that might results from the introductions of chemicals. Hence, they are playing important role in detoxification mechanism. In Chapter 4, it was shown that CPS and ISO did not significantly affect GST, which indicate that at the concentration tested, they did not cause significant change in GST activities compared to control. However, their mixture caused significant difference by decreasing GST activities compared to the herbicide alone, suggesting that the mechanism of action of the mixture is different from single chemicals. The observed difference could also be because the presence of two chemicals is causing competition for binding sites, which leads to altered GST activities significantly.

In chapter 5, low concentration of MEF caused significant reduction in GST, while higher concentration caused significant increase in GST activity compared to control. This observation showed that as the toxicity increase the biological system reacted by inducing more GST to detoxify the toxic effect of the chemical. However, FEN showed no significant difference at concentration below 0.5 mg/L FEN or above the concentration. The only concentration where a significant change occurred was 0.5 mg/L FEN. Therefore, the difference between GST in the control and treatment groups indicates oxidative stress caused by the chemical. The lower concentration did not induce GST activity in mixture, which indicate that mixture have different mechanism of action compared to MEF. However, higher concentration of mixture inhibits GST

activity, which showed that one of the chemicals might have altered the chemistry of the cells thus suppressing GST activity.

SOD play an important role in the antioxidant system, intervening in the first transformation by dismuting the most reactive, dangerous free radicals into ions that are less reactive. Thus, SOD assay gave a true picture of the antioxidant activity. SOD activity has potential to change as the organism adjust to the chemical condition, thus the time of exposure and the chemical concentration play important roles (Song et al., 2017). While no significant changes in SOD activity in CPS, there were significant changes in SOD activities in ISO and mixture of CYP and ISO. This observation suggested that ISO is more toxic than CYP. The lower concentration of ISO (0.1 mg/L) cause a significant increase in SOD activity compared to control, but a significant reduction was recorded at higher concentration (1.0mg/L). This observation is similar to early studies with pesticides (0.72 µg/L chorpyrifos, 0.33 mg/L trifluarlin, 0.18 mg/L chlorothaloni) which resulted a significant increase in SOD activities compared to control, while an increase in the concentration of the pesticides (5.72 g/L chorpyrifos, 1.33 mg/L trifluarlin, 0.72 mg/L chlorothaloni) resulted in a significant decrease compared to control (Song et al., 2012). A rapid generation of reactive oxygen species could occur at high concentration of an electrophilic compounds; thus, this high amount of ROS could cause the damage cell structure and function due to increased stress, which is capable of impeding the production of SOD, and thus reduced or impede SOD activity. This is because excessive ROS can affect biological redox system, destroy the oxidative balance, change the activity of related enzymes, and lead to oxidative damage (Tang et al., 2019).

MEF and FEN caused significant effect of SOD singly and in mixture. This observation is slightly different from what was obtained in chapter 4, with ISO and CPS, because excessive

generation of ROS by MEF and FEN could damage the redox system in *D. rerio*, inhibit the activity of antioxidant enzymes, reduce their content, and cause oxidative damage (Tang et al., 2019). Thus, MEF and FEN have higher potential to generate ROS compared to ISO. Also, inability of CPS to cause significant changes in SOD might be related to its low potential of generating ROS.

## 6.5 Molecular docking

A detailed and comprehensive discussion of molecular docking analyses using the SESs is found in chapters 5 and 6 of this dissertation. In general, the usefulness of molecular docking in showing binding affinities of ligands to receptors to predict effects is consistent with what is observed in this study. Molecular docking has been helpful in predicting binding orientations of one molecule (e.g., safener or herbicide) with another (e.g., hatching receptor), when both interact with each other to form a stable complex. This information is always helpful to predict energy profiling (such as binding free energy), strength and stability of complexes. Binding parameters such as bond angle, scoring function, type of bonding and residues are important to understand the ability of the ligands to bind with biological receptor. Molecular docking was earlier used to showed that ligands could bind to *D. rerio* L-type Calcium channel (LTCC), which is a known pharmaceutically important target for arrhythmia (Sampurna et al., 2019). A recent study also conducted molecular docking analyses and observed that there were compatible binding sites between adriamycin and Wnt signaling pathway-related genes in *D. rerio* (Duan et al., 2023). In a similar way, chapter 4 and 5, showed that CPS, MEF, ISO, and FEN all bind to the hatching enzyme (ZHE1) receptor. This infers that the binding sites in *D. rerio* were compatible with that of the hatching protein. The amino acid residues at the binding sites provide important information on the effect of the binding, while the binding distance and nature of bonding at the sites provide

good information about the binding strength. Hence, MEF has a more adverse effect on hatching because it binds to two histidines, compared to FEN, which binds to one histidine. MEF and FEN can cross the blood-brain barrier, showing that they can cause toxic effects in humans and other vertebrates if accumulated. However, ISO and CPS are not blood-brain barrier permeants.

## 6.6 Future Work

Understanding toxicity threshold for safeners and their herbicides in animal models to assess chemical and environmental risk is a new and growing concept particularly for regulatory processes. Effects of these safeners and their herbicides on crops have been extensively evaluated in literature but there are very limited studies of the chemicals on non target animal species which could be exposed to them through run-off, leaching and other means. In this project, we evaluated and determine toxicity and binding capability of SESs and their co-herbicides using acute and chronic bioassays to derive toxicity thresholds. We used *D. magna* and *D. rerio* embryos that are not considered live animals under current legislations. This approach helps in timely understanding of effects of chemicals in early exposure but there is a need for the use of high throughput approach which is more robust in estimating toxicity thresholds and chemical hazard assessment. Regardless of the usefulness of the methods used in this study, it is still a new area where common methods are used, and much work is needed to gain confidence in the use of toxicity threshold using ELS derived data from *D. rerio* and acute and chronic toxicity tests for *D. magna*. The following recommendations are based on the experience and conduct of this dissertation:

1. Continuous testing and optimizing higher-throughput methods in ELS exposures using petri-dishes and/or micro-well plates.

2. Assess current statistical strategies in safeners environmental relevant concentration estimation and identify the best strategies to estimate thresholds that are protective of apical endpoints.
3. Identify strategies to link statistical computational estimates to biological context, this may require the use of the adverse outcome pathway (AOP).
4. Consult similar studies using other toxicological model species as it goes up the food chain to determine toxicity thresholds for regulatory purposes.
5. Using advance molecular sequencing including proteomics and metabolomics to better understand proteins and metabolites prese and expresses in model organisms.
6. Identify the optimal number of replications with considerations on reasonable experimental set-up and cost as well as the minimum number of dose/concentration/treatment groups that will give rise to substantial results for all biochemical and molecular bioassays.
7. Standardize and develop guidance documents for safeners evaluation from ELS fish and daphnids to allow for direct cross-comparison of datasets and standardization of dataset generation, processing, and interpretation for regulatory acceptability.

Furthermore, further studies are necessary to validate the methods used and establish a link between expressed genes and apical effects using sequencing to gain further confidence to help in its regulation. It is also imperative to assess the impact of these safeners on soils since safeners are used largely on soil and soil organisms are directly exposed to these chemicals. Sequencing and the use of omics techniques will help in understanding these chemicals better. Advancement in omics technologies will make it possible to investigate entire sets of molecular families without prior knowledge of a compound's modes of action (MOA). This approach can also offer a glimpse into molecular disturbances before any long-term effects are noticed. (Garcia-Reyero and Perkins,

2011; Zhang et al., 2018). Conclusively, once omics techniques especially proteomics and metabolomics are implemented to better understand safeners toxicity and prediction software instrumentation improves, it may be of interest to develop a pathway from the initiating event to downstream biological processes to adverse outcomes.

## 6.7 Conclusion

Conclusively, this study demonstrated that chemicals like safeners singly or in a binary mixture can evolve and behave unpredictably from what they used to be. These changes can cause various effects in targeted and especially non-target animal species. In addition, with the vast numbers of safeners being formulated, and which needed to be tested, the derivation of toxicity threshold should advance further than simply being termed ‘inert’. This will allow for chemical testing prioritization which can mature such that one can derive reference doses and margin of exposure values that are anchored to a biological context that is useful for evaluating human and environmental risks.

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# Appendices

## Appendix A

### 7.1 Methods for literature review

Properties of safeners, such as henry law's constant, bioconcentration factors (BCF), aqueous solubility, and octanol-water partition coefficient were estimated with programs within EPI Suite WEB 4.10 (USEPA, 2014). The input of chemical compounds was based on the CAS number and Simplified Molecular Information and Line Entry System (SMILES) notation. These data were obtained from either ChemSpider® or from the Pesticide Properties Database (PPDB): University of Hertfordshire. The structures were drawn using Chemdraw® Ultra 12.0 (Table 1). Some data were also sourced from PubChem. For the experimental method presented in this report, see supporting information.

### 7.2 Methods for Laboratory studies

For the determination of experimental Log $K_{OW,}$ a known volume (15 mL) of the test substance (benoxacor, MEF, CPS, furilazole and dichlormid) solution was added to an equal amount of octanol which had been saturated with water in a 50 mL polypropylene tube. The mixture was shaken for approximately 24 h at room temperature. Thereafter the mixture was centrifuge at 2400 rpm for 10 min and the two layers separated. Each of the phases were put into LC vials and spiked with atrazine-d5 at 50 ppb as a surrogate standard for LC-MS analysis. Then Log $K_{OW}$ was calculated from the concentration determined by a Vanquish UHPLC and Q-ExactiveTM HF Quadrupole-OrbitrapTM mass spectrometer (Thermo-Fisher). LC separation was achieved with a Kinetex 1.7µm C18 LC column (100 x 2.1 mm) (Phenomenex, Torrance, CA) using an isocratic elution of 45% $H_2O$: 55% methanol (each containing 0.1% formic acid) (Fisher Scientific) at a flow rate of 0.2 mL/min and column temperature of 40$^{o}$C. Samples were ionized by positive mode

heated electrospray ionization (HESI) with the following source parameters: sheath gas flow = 3; aux gas flow = 1; sweep gas flow = 0; aux gas heater = 350$^{o}$C; spray voltage = 4.0 kV; S-lens RF = 80; capillary temperature = 320$^{o}$C; Aux gas heater temperature = 300$^{o}$C. A targeted-SIM and PRM (collision energy was 10, 20, 35, 15, 45 and 35 for CPS, furilazole, benoxacor, MEF, dichlormid and atrazine, respectively) method at 60,000 resolution, AGC target = 1x106, max injection time = 30 ms, and a scan range from 100-1000 m/z was used to monitor [M+H]+ precursor and product ions of benoxacor (m/z 260.024 →149.083); MEF (m/z 373.071→327.029); furilazole (m/z300.016 → 241.975); CPS (m/z 375.100→ 254.081, 135.044)); dichlormid (m/z208.029→ 139.966) and atrazine (m/z 216.101→174.054) (surrogate standard). Precursor and product ions were used for quantification and confirmation, respectively.

7.3 Chemical quantification and analysis of MEF, CPS, ISO and FEN for the toxicity test

Actual concentrations of CPS, MEF, ISO and FEN were measured in water samples. 50 mL (n = 2) of water samples from each treatment group were collected before exposure and 24 h after the renewal of exposure solution on days 1 and 4. Water samples were filtered using 0.22 μm filters (Fisher Scientific, USA) and the safeners' and herbicides' concentrations were quantified by Vanquish UHPLC and Q-ExactiveTM HF Quadrupole-OrbitrapTM mass spectrometer (Thermo-Fisher).

7.4 Water quality parameters and physicochemical properties of exposure solutions

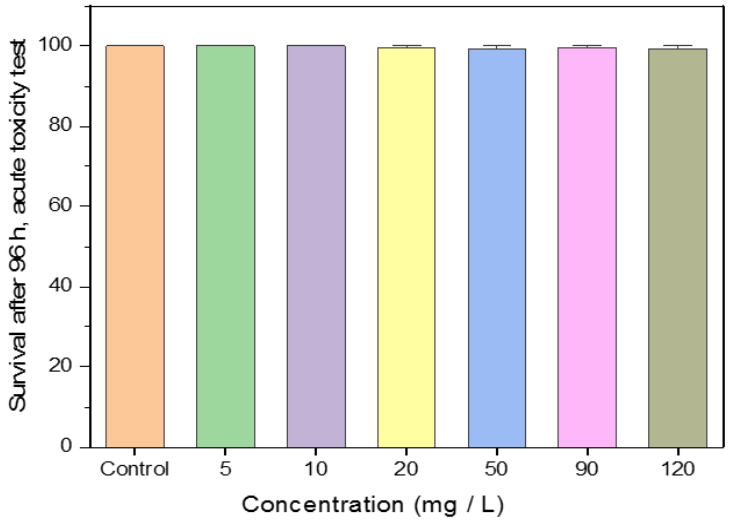
Water quality parameters and physicochemical characteristics including pH, Dissolved oxygen (DO), conductivity, ammonia, temperature, nitrites and nitrates did not differ in the entire study, probably because water change was done almost everyday in most of the studies. This showed that effect observed in this study were induced by the chemical stress. Another study showed that dissolved organic carbon and pH had significant effect on copper toxicity on D. magna, but water

hardness ($CaCO_3$ at 25 – 500 mg/L) had no effect (Schamphelaere et al., 2002). Yet another study noted water hardness ($CaCO_3$ at 10, 20 40 mg/L) had significant effect on copper toxicity to D. magna (Ryan et al., 2010). While it is possible to have no significant effect attributed to water quality in laboratory experiment, this might not be possible in a real-life experiment where other factors such as light might triggered change in the water quality parameter.

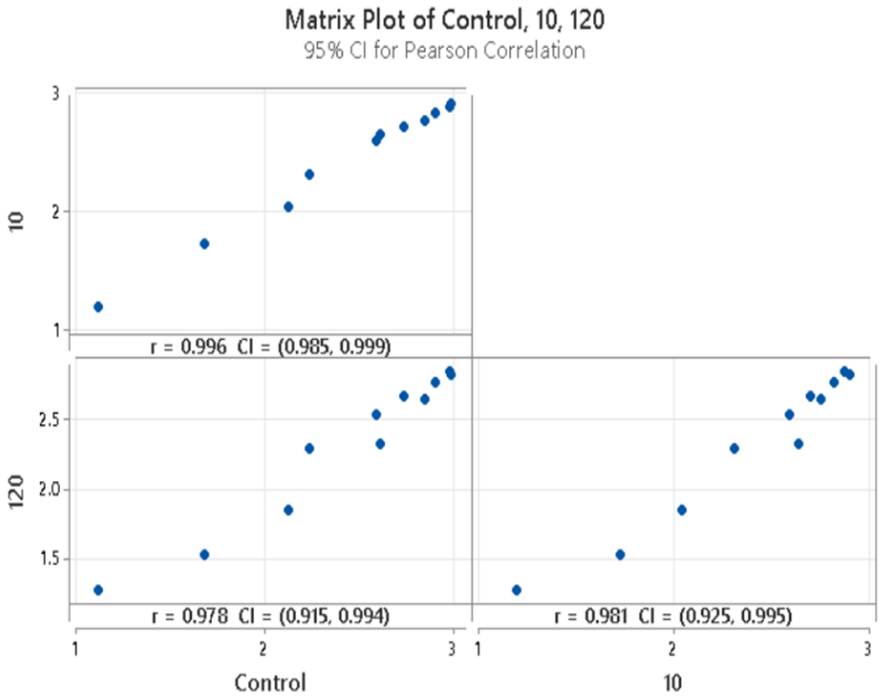
## Appendix B

7.5 Table *A.* 1. Freundlich model parameter of CPS on two selected soils

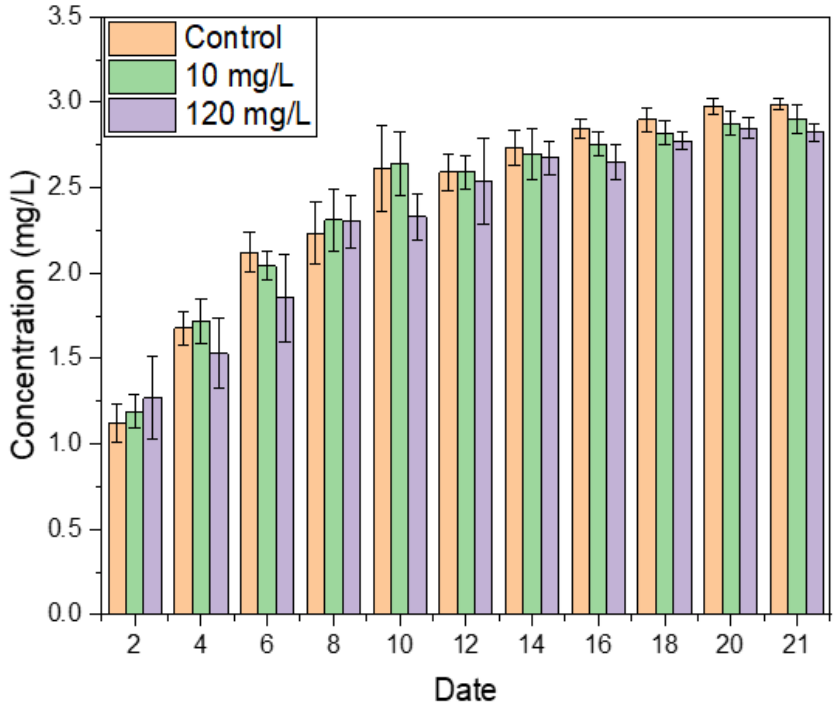
| Soil | $K_F$ | 1/n | $R^2$ |
|---|---|---|---|
| SK3 | 1.3867 | 0.9620 | 0.9217 |
| SK6 | 6.5119 | 1.2156 | 0.9639 |




7.6 Figure A. 1. Survival rate (mean ± SD) of D. magna after 96 h of exposure to varied concentrations of CPS. (n = 7 treatments, 10 organisms per treatment)

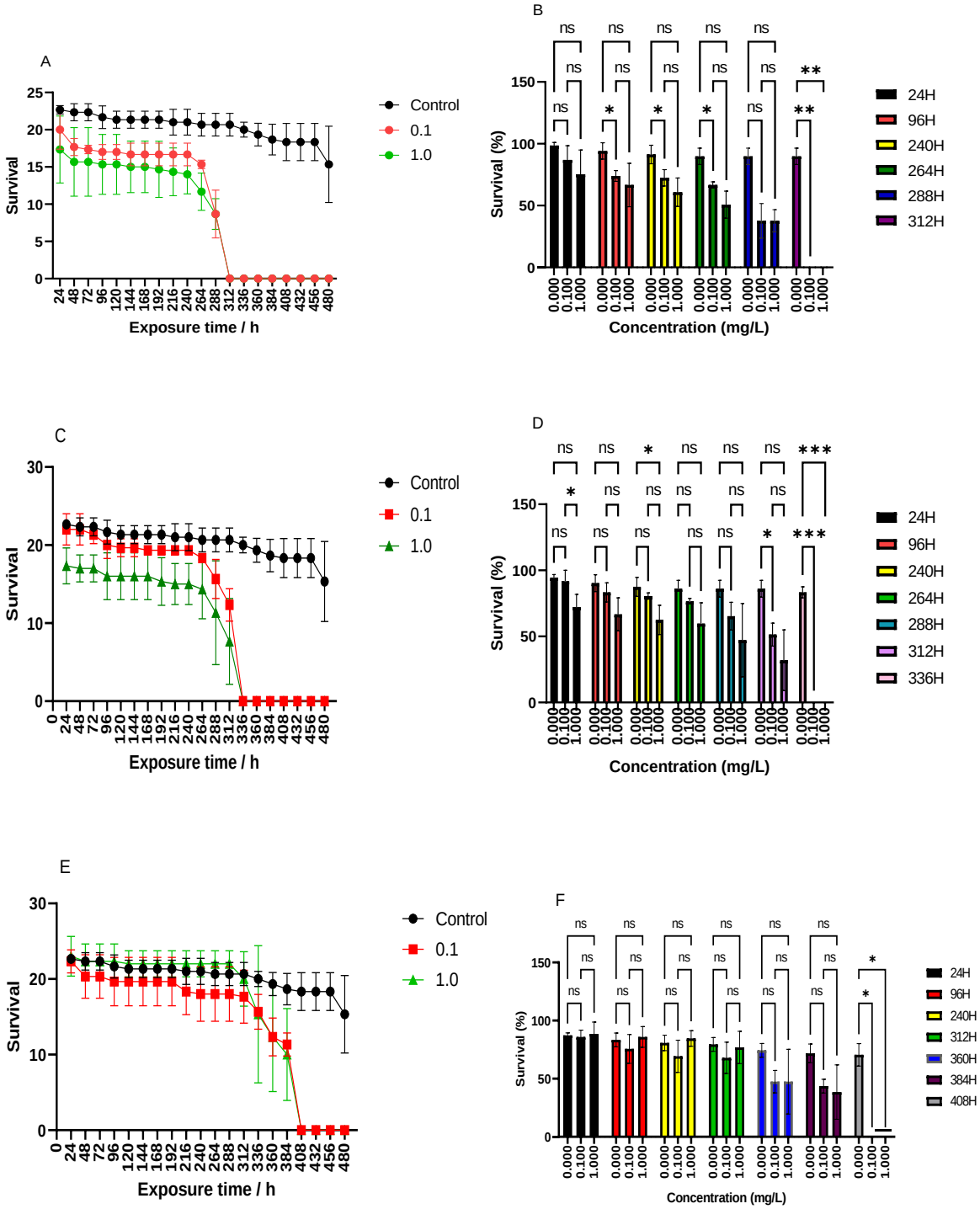



7.7 Figure A. 2. Correlation between the effect of different concentrations of CPS on the growth of *D. magna.*

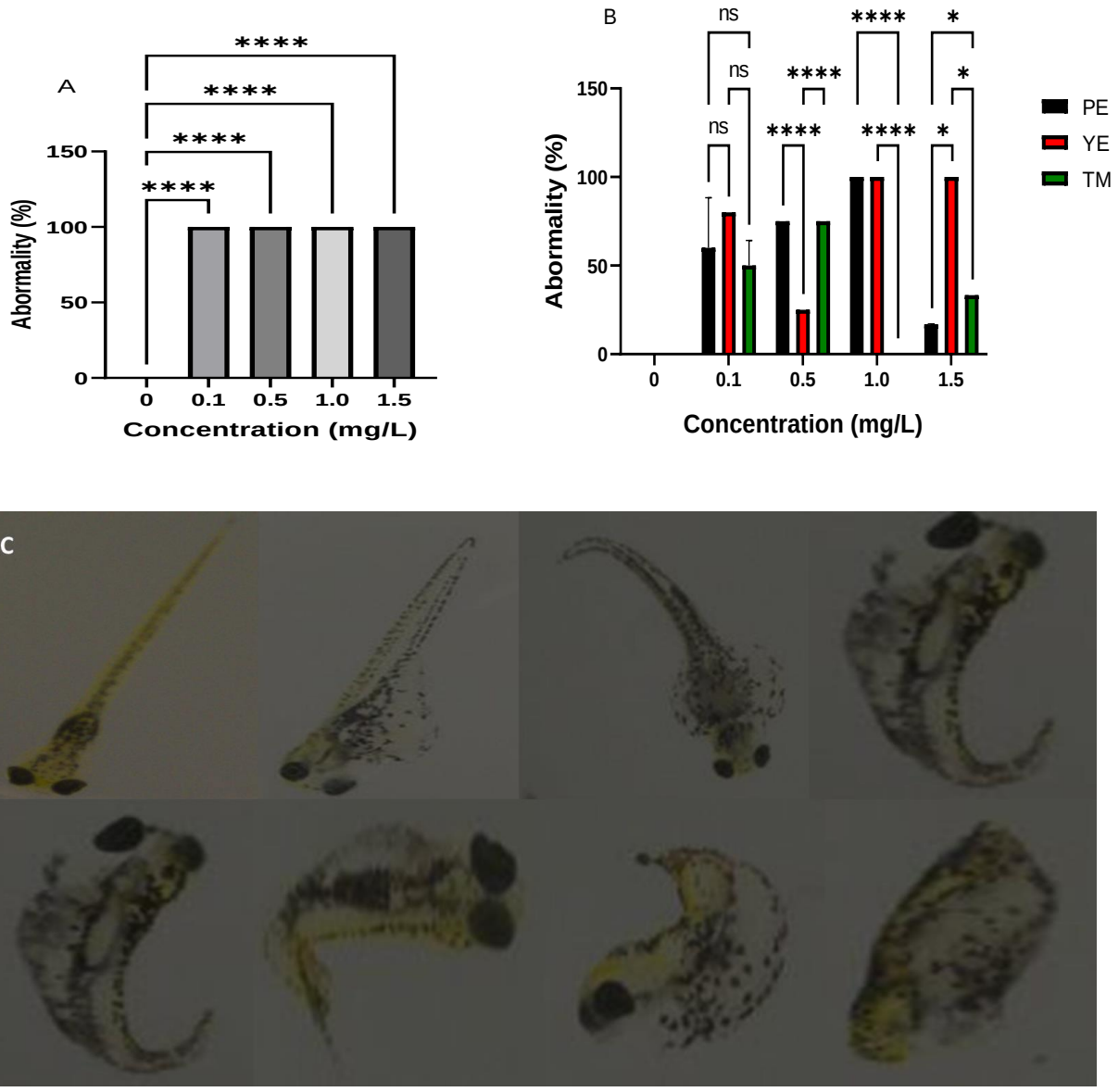



7.8 Figure A.3. The effect of different concentrations of CPS on growth of D. magna for 21days.

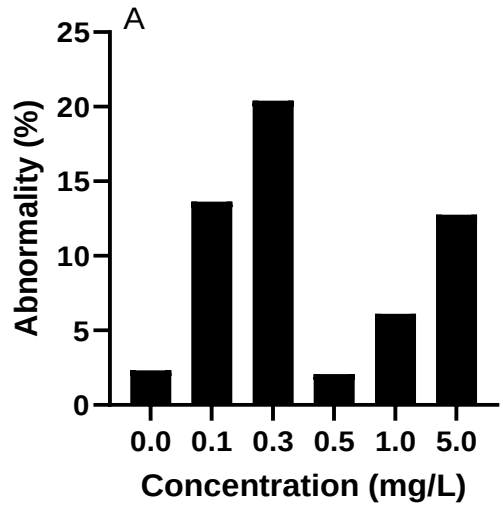
Appendix C

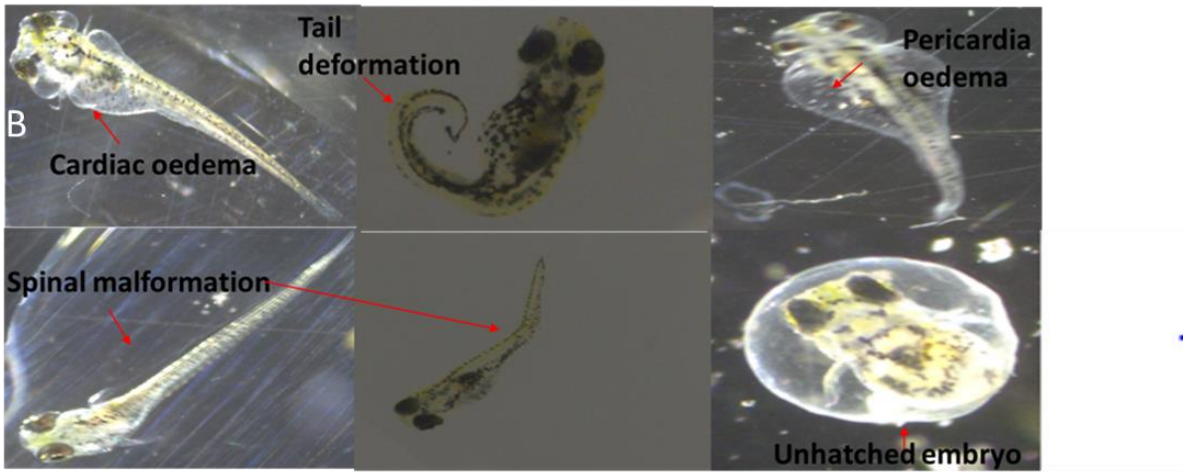



7.9 Figure A. 4. Survival of D. rerio exposed to (A and B) MEF (C and D), FEN (E and F) mixtures of MEF and FEN *P<0.05, ***P < 0.0001, 0=Solvent control.

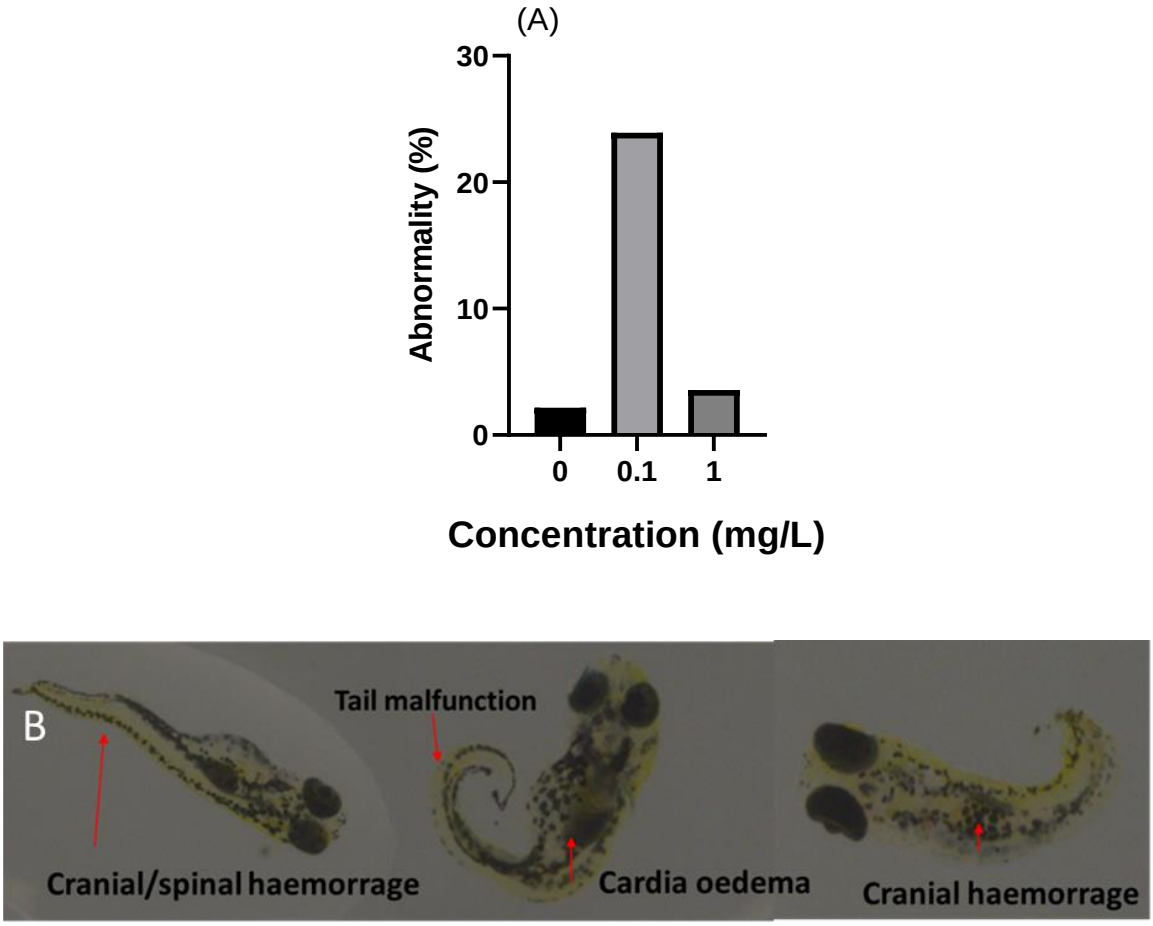



7. 10 Figure A. 5. Morphological alteration in *D. rerio* exposed to varying concentrations of MEF after 96 hpf (A) percentage of deformation (B) types of deformation (C) pictorial representation of the deformation. ****P<0.0001, *P<0.05, 0= solvent control

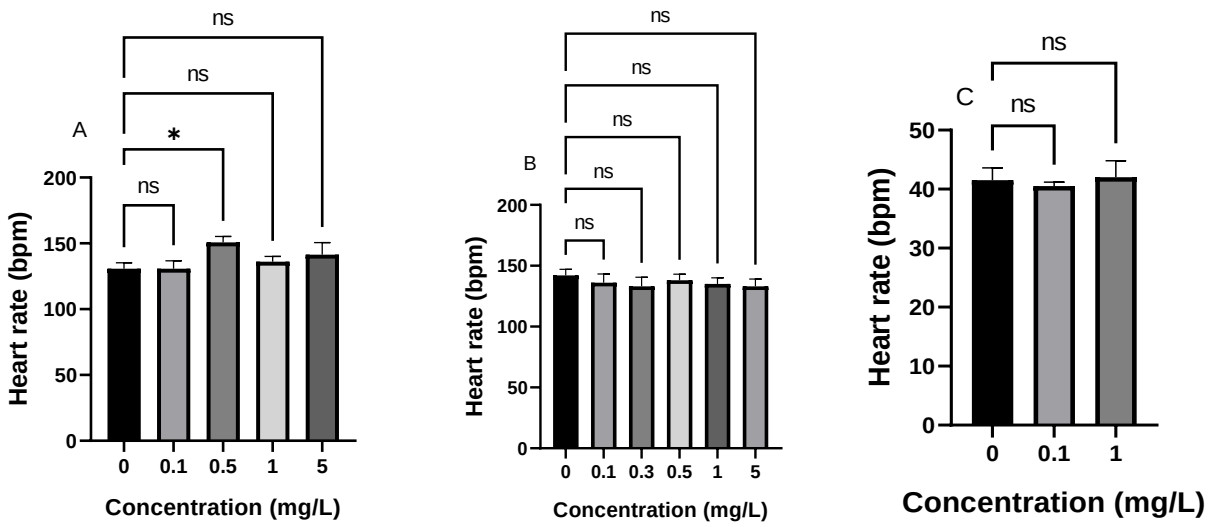


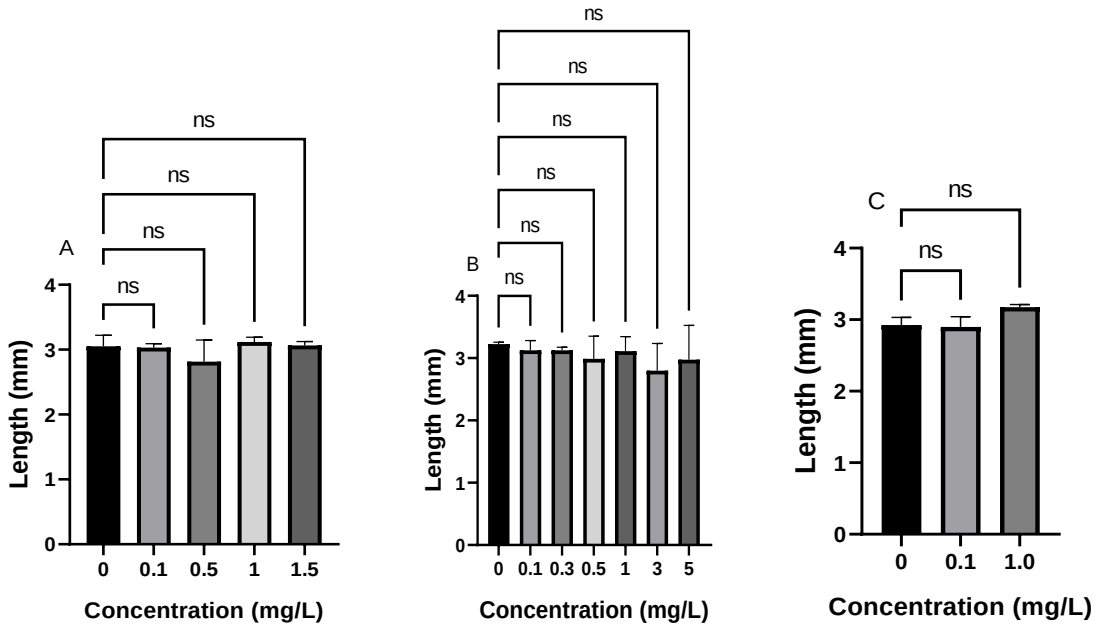

7.11 Figure A. 6. Morphological alteration in *D. rerio* exposed to varying concentrations of FEN after 96 hpf, 0 = solvent control (A) percentage of deformity (B) pictorial representation of deformities.




7.12 Figure A. 7. Morphological alteration in *D. rerio* exposed to mixture of FEN and MEF after 96 hpf, 0 = solvent control (A) percentage of deformity (B) pictorial representation of deformities.




7.13 Figure A. 8. Heartbeat rate of *D. rerio* exposed to (A) MEF (B) FEN (C) MEF + FEN. The analysis was done using one way ANOVA, 0= solvent control.




7.14 Figure A. 9. Length of *D. rerio* (without visible deformation) exposed to (A) MEF (B) FEN (C) MEF + FEN. The analysis was done using one way ANOVA, 0 = solvent control.

Appendix D

## 7.15 D.1. Toxicity Estimation Software Tool (T.E.S.T)

T.E.S.T is a free software for estimating the effect of various organic pollutants on different endpoint using quantitative structure activity relationship (QSAR). The input such as CAS, SMILES, name, InChi, InChiKey or DTXSID, for MEF was obtained from PUBCHEM and enter a search column. Then the software calculates the endpoint base on the method of choice. Here, consensus, hierarchical clustering, single model and nearest neighbour were chosen.

## 7.16 Table A. 2: In silico prediction of CPS toxicity

| End points | | Consensus | Hierarchical clustering | Single model | Nearest neighbour | Results |
|---|---|---|---|---|---|---|
| | Exp | Prediction | Prediction | Prediction | Prediction | Prediction |
| $LC_{50}$ Fathead minnow (mg/L) | N/A | N/A | N/A | N/A | 0.00665 | |
| Bioconcentration factor | N/A | 6.56 | 3.99 | 1.09 | 64.61 | |
| Development toxicity | N/A | 1.05 | 1.05 | 1.05 | N/A | Development toxicant |
| Mutagenicity | N/A | -0.03 | -0.06 | N/A | 0.00 | Mutagenicity negative |

7.17 Table A. 2: In silico prediction of ISO toxicity

| End points | | Consensus | Hierarchical clustering | Single model | Nearest neighbour | Results |
|---|---|---|---|---|---|---|
| | Exp | Prediction | Prediction | Prediction | Prediction | Prediction |
| $LC_{50}$ Fathead minnow (mg/L) | N/A | 0.0172 | 0.0355 | 0.0355 | 0.00406 | |
| Bioconcentration factor | N/A | 207.31 | 44.99 | 40.08 | 853.85 | |
| Development toxicity | N/A | 0.92 | 0.92 | 0.88 | N/A | Development toxicant |
| Mutagenicity | N/A | $0.62^{+}$ | $0.24^{-}$ | N/A | $1.00^{+}$ | Mutagenicity positive |

7.18 Mechanism of action

Safeners work to increase crop tolerance to herbicides by utilizing multiple mechanisms that work in synergy. The primary process involves using safeners to speed up the metabolic rate of the herbicide within the crop. This leads to a decrease in the harmful concentration at the area where the herbicide takes effect. (Zhao et al., 2023). Mechanisms of action of safeners involve inducing genes and proteins responsible for herbicide metabolism and crop detoxification, thereby lessening their effects on crops (Rosinger and Schulte, 2019). This mechanism of action is like the effects seen in the model animals used for the studies in this dissertation where the mixture of MEF and

FEN and CPS and ISO caused a reduction in the toxicity observed compared to the single herbicides. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione S-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. This is similar to the observation in this study where there is an elevation in the GST activities in the cells of D. rerio exposed to SESs. In plants, safeners enhance the vacuolar transport of glutathione or glucose conjugates of selected herbicides (Hatzios and Burgos, 2004). Dichlormid, another type of safener, was found to reduce lipid peroxidation induced by herbicides, suggesting safeners' role as antioxidants (Bernasinska et al., 2013). The mechanism of action of safeners in plant might therefore be similar to that of aquatic organisms.