ABSTRACT

Relative Toxicity of Organophosphate Flame Retardants in Embryonic Zebrafish

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Organophosphate flame retardants (OPFRs) delay ignition of commercial products manufactured in the textile and furniture industries. In the current study, embryonic zebrafish (*Danio rerio*) were exposed to varying concentrations of seven selected OPFRs at 6 hours post fertilization (hpf) and evaluated daily for morbidity and mortality. Glutathione S-transferase (GST) activity was assessed in zebrafish at 120 hpf following OPFR exposure in order to probe oxidative stress as a mode of action, and acetylcholinesterase (AChE) activity was evaluated as an indicator of neurotoxicity. Our study found that OPFRs elicited a variety of effects, with triphenyl phosphate, tributyl phosphate, tris-(2-butoxyethyl) phosphate and tris-(1,3-dichloro-2-propyl) phosphate inducing mortality in a concentration dependent manner. Furthermore, several of the selected OPFRs impacted spontaneous movement and AChE activity. Due to the rising production volumes of OPFRs, these results underscore concerns regarding human exposure and adverse health effects.

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RELATIVE TOXICITY OF ORGANOPHOSPHATE FLAME RETARDANTS IN EMBRYONIC ZEBRAFISH

A Thesis Submitted to the Faculty of

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By

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DEDICATION

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CHAPTER ONE

Introduction

History of OPFR Usage

Organophosphate flame retardants (OPFRs) are compounds commonly used to suppress the ignition of commercial products, including furniture, electronics and textiles.[1] Polybrominated diphenyl ethers (PBDEs) and brominated flame retardants (BFRs) were once commonly used flame retardants, but have gradually been phased out of usage due to growing evidence of their bioaccumulation, toxicity, and persistence in the environment.[1,2] As a result of the growing concerns regarding PBDEs, the EPA initiated laws with the aim of limiting PBDE production and exposure.[3] By 2004, PBDE production had been voluntarily terminated in the United States.[3] Furthermore, the European Union has banned PBDE sales as well as their production.[3]

Due to the phase out of PBDEs, manufacturers have searched for alternative flame retardants for use in flammable products. OPFRs have replaced PBDEs and BFRs. Consequently, OPFR usage has gradually increased so that larger volumes of OPFRs are now prevalent in the environment.[4] In fact, recent studies have detected OPFR concentrations at higher levels than those of PBDEs.

Routes of Exposure and Environmental Contamination

Organophosphate compounds are used as flame retardants, plasticizers and lubricants in a variety of products. In the early 1990s, worldwide OPFR production

accounted for approximately 20% of all flame retardants, and with the recent phasing out of PBDEs and BFRs, this value has likely increased.[5] In 1998, OPFR consumption in Western Europe was approximately 58,000 tons while it increased to approximately 83,000 tons in 2001.[1] Furthermore, regulations regarding the disposal of products containing these compounds are limited and, as a result, OPFR-containing products are often either incinerated or placed in landfills. These disposal methods can result in further air and soil contamination.

Several studies have confirmed the presence of OPFRs in dust samples.[1,2] This suggests that OPFRs are being leeched out of materials, thus contaminating the environment and potentially reducing their purpose as flame retardants in the original material. Many OPFRs are additives and not covalently bonded with the products that they are used in.[6] Because they are not chemically bonded to their products, OPFRs may easily be released into the environment. Studies have even found dust samples containing OPFRs at concentrations equal to that of PBDEs.[2] Additionally, a portion of dust samples in Boston, Massachusetts during 2002 to 2007 contained concentrations of TPP in excess of that of PBDEs.[2] TPP was detected at an average concentration of 7360 ng/g in indoor dust samples collected in suburban Massachusetts. Beyond their presence in dust samples, OPFRs have also been found in water and air samples, worldwide. A study of water samples from the Ruhr River in Germany revealed concentrations of 20-200 mg/L TCPP, 12-130 mg/L TCEP, 50 mg/L TDCPP, 10-200 ng/L TBEP and 20 mg/L TPP.[7] Another study found concentrations of 49 ng/m^3 TBEP and 2.2 ng/m^3 TBP in indoor air samples at German daycare centers.[8]

Initial research efforts focused on detecting and quantifying OPFR contamination in the environment. As the occurrence of OPFRs in the environment continues to rise, however, there is growing concern regarding human exposure and adverse health effects. Once a compound is leeched out of its original material, it can accumulate in indoor and outdoor environments, leading to human exposure. Furthermore, humans regularly come into contact with products that contain OPFRs, such as furniture, mattresses and paint. Consequently, dermal absorption of OPFRs, especially the lipophilic compounds which may easily be able to cross the dermis, is a cause for concern. The main routes of human exposure include inhalation, ingestion and direct skin contact.[9–11] A study in Washington D.C. estimated adult inhalation of TCPP to be 4540 ng/day, while another study in Belgium found adult ingestion of TCPP in Belgians to be 8000 ng/day.[10,11] Interestingly, human exposure of TPP and TDCPP was recently studied by collecting hand wipes of individuals living in North Carolina, USA. TPP and TDCPP were frequently detected, and the geometric mean of OPFR concentrations was significantly greater than that of PBDEs.[9]

Another study evaluating human breast milk samples from individuals in Japan, the Philippines and Vietnam demonstrated the presence of TPP at a median concentration of 4.9 ng/g lipid weight.[12] TBP, TCEP and TBEP were also detected, though at lesser concentrations. The total median concentration for the sum of all of the OPFRs was 10 ng/g lipid weight.[12] These findings are of particular concern due to the potential for exposure to children during a critical developmental window. Other studies have also demonstrated the presence of OPFRs in human hair and nail samples at concentrations higher than those of PBDEs.[13,14]

Due to the prevalence of OPFRs in the environment, there is also concern regarding the contamination of vegetable and animal food sources. However, there have only been a limited number of studies examining OPFR contamination in food sources. One of the few studies conducted thus far evaluated rice and other food samples, including vegetables, meats, dairy products and fruits, from major suppliers in China.[15] Rice and vegetables had the highest OPFR concentrations, with all of the rice samples containing at least some degree of OPFR contamination. The mean OPFR concentration found in the rice samples was 69.9 ng/g, with TCEP and TEHP present at the highest concentrations.

Overall, the concentrations of the OPFRs detected in the environment varies among studies, depending on the location of the study and the OPFR being analyzed. Nonetheless, the rapidly increasing production volume and demonstrated human exposure of OFPRs underscore the need for further research on the chemicals' potential toxicity to humans and wildlife.

Prior OPFR Toxicity Studies

Due to the rapidly rising production volume of OPFRs and their prevalence in the environment, there have been several recent endeavors to evaluate their toxicity. A number of OPFR studies suggest adverse neurological, endocrine and reproductive effects in a variety of organisms.[4,16,17] For instance, kestrals exposed to TCEP or TBEP show a significant disruption in circulating levels of endocrine hormones, triiodothyronine (T3) and thyroxine (T4).[4] Other studies have examined OPFR effects on glutathione *S*-transferase (GST) activity, a common indicator of oxidative stress. A recent

study in male mice orally exposed to TPP and TCEP demonstrated a significant decrease in GST activity in the liver as well as a decrease in body and testis weights in the treated group.[18] Although the doses utilized in the study (100 mg/kg and 300 mg/kg) are not environmentally relevant, the results still raise concern due to the potential effects of long term OPFR exposure at low concentrations and the rising production of OPFRs.

Neurological Function as Indicator of Toxicity

Exposure to organophosphate pesticides is known to inhibit acetylcholinesterase (AChE) activity.[19–22] AChE is an important enzyme that catalyzes the hydrolysis of acetylcholine in the neuromuscular junction. The hydrolysis of the neurotransmitter, acetylcholine, terminates neurotransmission. When AChE is inhibited, a buildup of acetylcholine at the post-synaptic terminal results. Consequently, acetylcholine receptors are overstimulated and the duration of impulse transmission is inappropriately extended. Symptoms of AChE inhibition include muscle weakness, difficulty breathing, sweating and muscle spasms.[23,24] The current study investigates AChE activity in order to determine if OPFRs produce similar inhibitory effects as organophosphate pesticides.

The structure of OPFRs is similar to that of organophosphate pesticides, several of which are potent neurotoxins.[19,25–27] Neurotoxic effects following exposure to organophosphate pesticides include cognitive impairment, developmental delays and behavioral changes. Organophosphate pesticides cause a build-up of acetylcholine at the post-synaptic terminal, thus overstimulating the post-synaptic neuron and interfering with signal transduction. Table 1 below illustrates the basic structure of an organophosphate ester (OPE) and the organophosphate insecticides, parathion, chlorpyrifos and diazinon.

The 7 OPFRs selected in this study (Table 2) share the basic OPE structure, but possess a variety of different R substituents. The R substituents vary from phenyl groups to alkyl chains, making the organophosphate class very large and diverse. Organophosphate pesticides also contain a similar organophosphate backbone, but they typically have a bulky R_1 group and smaller R_2 and R_3 groups. Nonetheless, due to the structural similarities between OPFRs and organophosphate pesticides, prior studies have examined whether OPFRs exhibit similar neurotoxic effects as organophosphate insecticides. One such study in zebrafish demonstrated that long-term exposure to TDCPP can lead to neurotoxicity, as evidenced by decreases in dopamine and serotonin levels and downregulation of genes in the nervous system.[28]

OPFR studies focusing on AChE activity have exhibited mixed results with outcomes depending on the OPFR being examined. A study employing Japanese medaka, an aquatic model organism, found AChE activity inhibition by TPP, stimulation by TBP, and no change following TCEP and TBEP exposure.[29] A similar study in embryonic zebrafish demonstrated only a slight increase in AChE activity when exposed to TBP and TCEP and no change when exposed to TBEP.[30] Nonetheless, no studies have clearly shown negative effects of OPFR exposures on gross neurological functions.

Table 1: General Structure of an Organophosphate Ester (OPE) and Several Organophosphate Insecticides

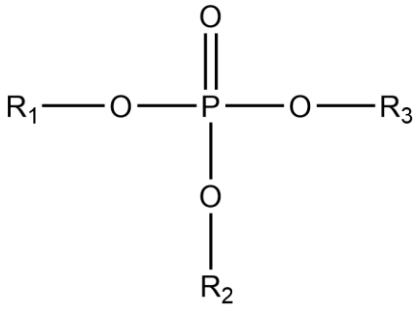
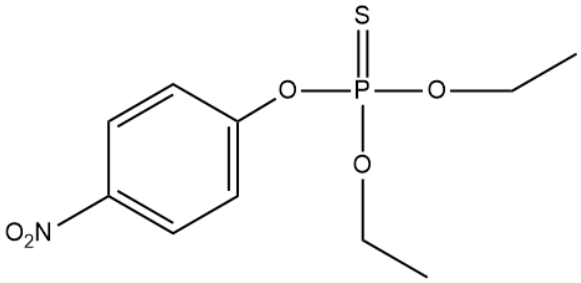
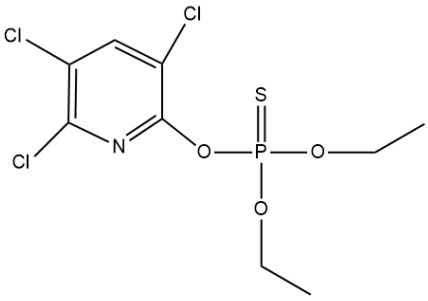
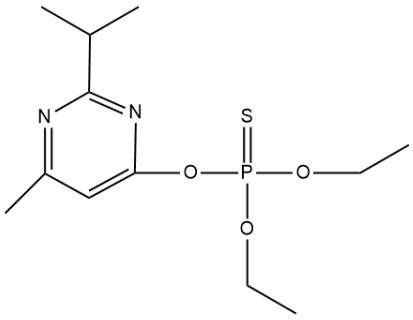
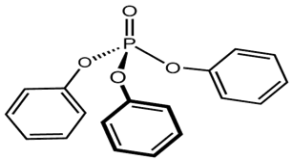
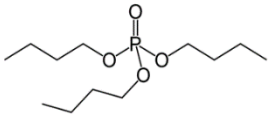
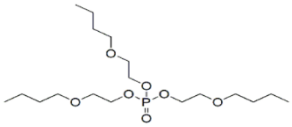
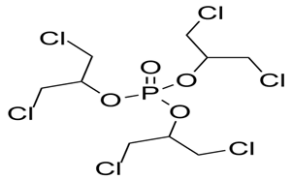
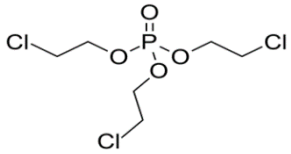
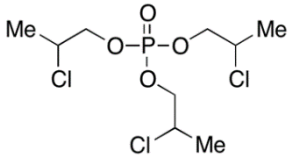
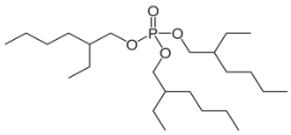
General Structure of an OPE	
Parathion	
Chlorpyrifos	
Diazinon	

Table 2: CAS Number, Structure, Log K_{ow} and vendor for the 7 selected OPFRs

CAS Number	Name (abbreviation)	Structure	Log K_{ow}	Vendor
115-86-6	Triphenyl phosphate (TPP)		4.60	Wako Pure Chemical Industries Ltd
126-73-8	Tributyl phosphate (TBP)		4.00	Wako Pure Chemical Industries Ltd
78-51-3	Tris-(2-butoxyethyl) phosphate (TBEP)		3.75	Wako Pure Chemical Industries Ltd
13674-87-8	Tris-(1,3-dichloro-2-propyl) phosphate (TDCPP)		3.65	AccuStandard
115-96-8	Tris-(2-chloroethyl) phosphate (TCEP)		1.78	AccuStandard
1067-98-7	Tris-(2-chloro-1-methylethyl) phosphate (TCPP)		2.59	Wako Pure Chemical Industries Ltd
78-42-2	Tris-(2-ethylhexyl) phosphate (TEHP)		4.20	Wako Pure Chemical Industries Ltd

*CAS numbers are from the review of Van der Veen et al[31]

Oxidative Stress as Indicator of Toxicity

Glutathione S-Transferases (GSTs) are a group of enzymes that function in the detoxification of xenobiotics as well as endogenously produced free radicals as a cellular response to oxidative stress.[32–34] The enzymes catalyze the nucleophilic attack of glutathione (GSH) on electrophilic substrates, or toxins. The electrophilic substrate is illustrated as RX in Figure 1 below. The resulting products of the reaction are generally less reactive with cellular macromolecules and more water soluble.[33] The GST reaction, as shown in Figure 1, generally reduces the substrate's toxicity, thus protecting the cell.

Because GSTs protect against oxidative stress, their expression is often upregulated following exposure to cellular stressors. The three main classes of GSTs in mammals are α, μ and π, and all of them have complex mechanisms of expression. The antioxidant-responsive element (ARE) is located in the promoter region of genes encoding detoxification enzymes, such as GSTs.[35] Thus, ARE plays a particularly important role in the gene expression of GSTs. In response to an increase in reactive oxidative species, the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), binds to and activates ARE.[35] Gene expression of GSTs is upregulated in this manner during oxidative stress. Alternatively, as a mechanism of action, toxins may suppress GST expression thereby exacerbating oxidative stress.

Prior studies of flame retardants suggest that chemical exposure induces oxidative stress, which may be a disease mechanism. Furthermore, oxidative stress is a common endpoint underlying, in part, numerous diseases, including diabetes, cancer and heart disease. As a result, it was hypothesized that OPFR toxicity may induce oxidative stress and affect GST activity. Thus far, there has been a scarcity of research examining

oxidative stress following OPFR exposure, making the current study one of the first to consider the role of GST activity in relation to OPFR toxicity.

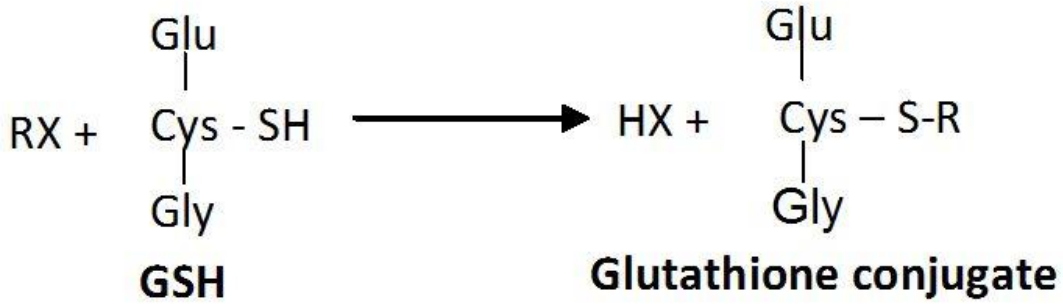


Figure 1: Reaction catalyzed by GSTs[36]

Embryonic Zebrafish Model of Human Toxicity

In the present study, zebrafish (*Danio rerio*) embryos served as the model organism to evaluate the relative toxicity of the selected OPFRs and to probe two potential mechanisms of action, oxidative stress and acetylcholinesterase interference. Zebrafish were utilized due to their short generation time, high fecundity, easy laboratory maintenance and significant physiological and genetic homology with humans.[37,38] Additionally, zebrafish embryos' rapid development and transparent body make them ideal to examine morphological changes over time.[39] The small size of the model organism allows for small quantities of chemicals to be used during exposure and an ease of evaluation of the biological response.[40] Furthermore, zebrafish have become an accepted model organism for developmental neurotoxicity testing due to the highly conserved neurobehavioral responses between zebrafish and humans.[41] For these reasons, the zebrafish model was used as a means of testing the structurally diverse OPFR compounds for toxicity as an indicator of human health hazards.

Hypothesis

It was hypothesized that the diverse group of OPFRs examined in this study would exhibit a high degree of variability in toxic effects, partially due to their distinct structural differences. The octanol-water partition coefficient, or K_{ow}, value is an important property that can predict the solubility or lipophilicity of a compound. It was predicted that the OPFRs with higher log K_{ow} values, or the more lipophilic compounds, would be more toxic because they were more likely to bioaccumulate in fatty tissues. Furthermore, due to the established inhibition of AChE activity following organophosphate pesticide exposure and the structural similarities between organophosphate pesticides and OPFRs, it was predicted that the acetylcholine pathway would be impacted by exposure to OPFRs. To test these hypotheses, embryonic zebrafish (6 hpf) were exposed to the 7 selected OPFRs and evaluated daily for mortality and malformations, including pericardial edema, yolk sac edema and curved body. At 120 hpf, AChE activity and GST activity were measured at each OPFR's maximum tolerated dose (MTD). Lastly, spontaneous movement was evaluated at 24 hpf to quantify any changes in locomotor behavior.

CHAPTER TWO

Methods and Procedures

Fish Husbandry and Care

Wildtype zebrafish (*Danio rerio*) originated from Oregon State University (Corvallis, OR, USA) and were raised in the Abel and Usenko Toxicology Laboratory at Baylor University. Zebrafish were fed twice daily with flake food, and all appropriate IACUC approved protocols were followed in caring for the zebrafish. Adult zebrafish were contained in plastics tanks filled with deionized water containing 0.26% Instant Ocean Aquarium Salt^{TM}. Each tank held 3-5 adult zebrafish and the system was maintained at a pH of 7.0 and conductivity between 300-400 µSem/cm. The system was sustained at 28°C and on a 14:10 hour light:dark cycle.

Chemicals and Materials

OPFRs were purchased from the following sources: tris-(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris-(2-chloroethyl) phosphate (TCEP) from AccuStandard and tributyl phosphate (TBP), triphenyl phosphate (TPP), tris-(2-ethylhexyl) phosphate (TEHP), tris-(2-butoxyethyl) phosphate (TBEP) and tris-(2-chloro-1-methylethyl) phosphate (TCPP) from Wako Pure Chemical Industries Ltd. All chemical standards were analytically verified with purity >99% except TEHP which had >97% purity. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at room temperature in the dark. DMSO, protease, tricaine methanesulfonate, 1-chloro-2,4-dinitrobenzene

(CDNB), L-glutathione reduced (GSH), dithiothreitol (DTT), dithiobisnitrobenzoic acid (DTNB), acetylthiocholine iodide (AcSCh) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. Ethylenediaminetetraacetic acid (EDTA) and Tris were purchased from Bio-Rad Laboratories. Ethanol was purchased from ACROS and sucrose was purchased from Fischer Scientific.

Exposure Methods

Selected zebrafish tanks were spawned twice weekly. Approximately 2 hours after spawning, embryos were collected, rinsed using fish water, and placed into plastic petri dishes. Embryos were dechorionated at 6 hpf using Pronase and thoroughly rinsed with fish water. They were carefully transferred into a 48-well microplate, with each well containing 5 embryos and 750 µL of the selected OPFR treatment solution.

Treatment solutions were prepared using DMSO as the solvent vehicle. The treatment solution concentrations were prepared by diluting stock OPFR solutions to yield concentrations of 2.5, 5, 10 and 20 ppm in the final treatment solution. The control group consisted of 0.5% DMSO for all experiments. Following exposure, the microplates were stored in an incubator and embryos were visually assessed daily for morbidity and mortality for up to 168 hpf.

Protein Isolation

Zebrafish protein extract was used to assess both AChE and GST activity levels at 120 hpf. Embryos were exposed to selected OPFRs in a similar manner to the dose response protocol. Treatment solutions were prepared in the same manner and the

transfer process was conducted at 6 hpf. Exposures were conducted at the maximum tolerated dose for each OPFR. Embryos were removed from the 48-well microplate and placed into a centrifuge tube at 120 hpf. Thirty embryos were pooled into each centrifuge tube for each replicate. 100 mM PBS solution containing 500 mM DTT was added to the centrifuge tube and the embryos were homogenized using a motorized pestle. The homogenate was centrifuged at 14,000 rpm and the supernatant was collected and stored at -80°C. Four replicates were used for each treatment group.

Spontaneous Movement

Spontaneous movement of zebrafish embryos is often used as an indicator of proper neurological development and locomotive behavior in embryonic zebrafish. Zebrafish embryos begin exhibiting spontaneous flexions as early as 17 hpf. Embryonic zebrafish were exposed to OPFRs using the dose response protocol as described above at concentrations of 2.5, 5, 10, and 20 ppm. Spontaneous movement was visually assessed at 24 hpf using a microscope. Number of tail contractions were counted and recorded for each well over a 30 second interval. The measurements were divided by the number of embryos in the well and multiplied by 2 in order to get the average flexions/minute for 1 embryo.

GST Assay

The method by Habig et al. was adapted to examine GST activity in embryonic zebrafish lysates.[42] 35 µL of each protein sample was mixed with 15 mM GSH in 100 mM sodium phosphate buffer. In the final step, 60 mM CDNB substrate was added,

resulting in a total of 1.5 mL. The appearance of the GST-CDNB conjugate was measured over 70 seconds at a wavelength of 340 nm using a Shimazu UV-1800 spectrophotometer. Protein concentration was measured using a NanoDrop Spectrophotometer and used to normalize GST activity.

AChE Assay

The Ellman protocol was used to measure AChE activity in embryonic zebrafish lysates.[43] 0.7 mM DTNB was dissolved in 100mM PBS to make a PBS/DTNB solution at a pH of 7.0-7.5. AcSCh was added to the solution to make a final concentration of 3 mM PBS/DTNB/AcSCh solution. For each sample, 90 µL of PBS/DTNB/AcSCh solution was pipetted into a 96-well microplate. 10 µL of protein sample was added in each well and the absorbance at 450 nm was measured at 2 minutes and 10 minutes using a DTX880 microplate reader. The control well contained deionized water. Protein concentration was quantified using a NanoDrop Spectrophotometer and used to normalize AChE activity.

Statistical Analysis

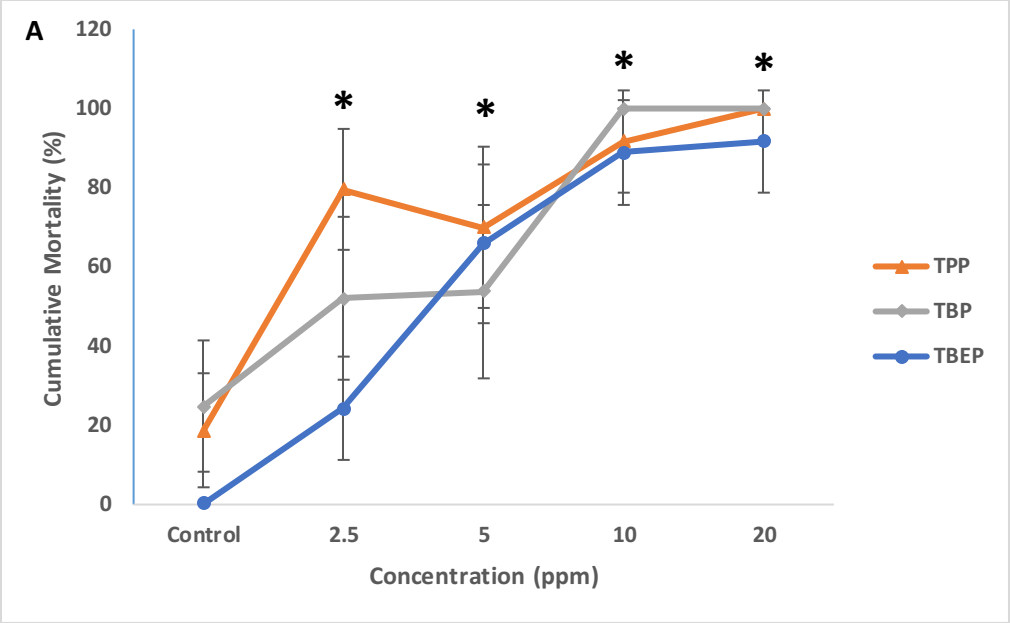
One-way analysis of variance (ANOVA) compared to the controls was used in SigmaPlot to determine the significance for mortality, malformations, and spontaneous movement (N=12). The significance for GST activity and AChE activity was also determined using one-way ANOVA (N=4). $P < 0.05$ was considered significant in this study. Microsoft Word was used to establish the regression line and the R^2 value for the log K_{ow} and LC50 relationship.

CHAPTER THREE

Results

Dose Response: Mortality

The viability of the zebrafish exposed to the seven OPFRs was observed daily until 168 hpf and incidence of mortality was recorded. As anticipated, great variety was noted in the response to the diverse OPFRs. Figures 2A & B show a summary of the mortality elicited by the compounds by 168 hpf. Of the seven OPFRs tested, TBP, TPP, TBEP and TDCPP induced mortality in a concentration dependent manner. Concentrations as low as 2.5 ppm induced significant mortality in developmental embryos. Of the chlorinated OPFRs that were tested, only TDCPP elicited adverse effects; the remaining OPFRs did not induce a significant increase in the incidence of mortality. Furthermore, TEHP was tested at concentrations up to 100 ppm with no significant mortality or developmental malformations observed (data not shown in Figure 2 due to different scaling of the graph).



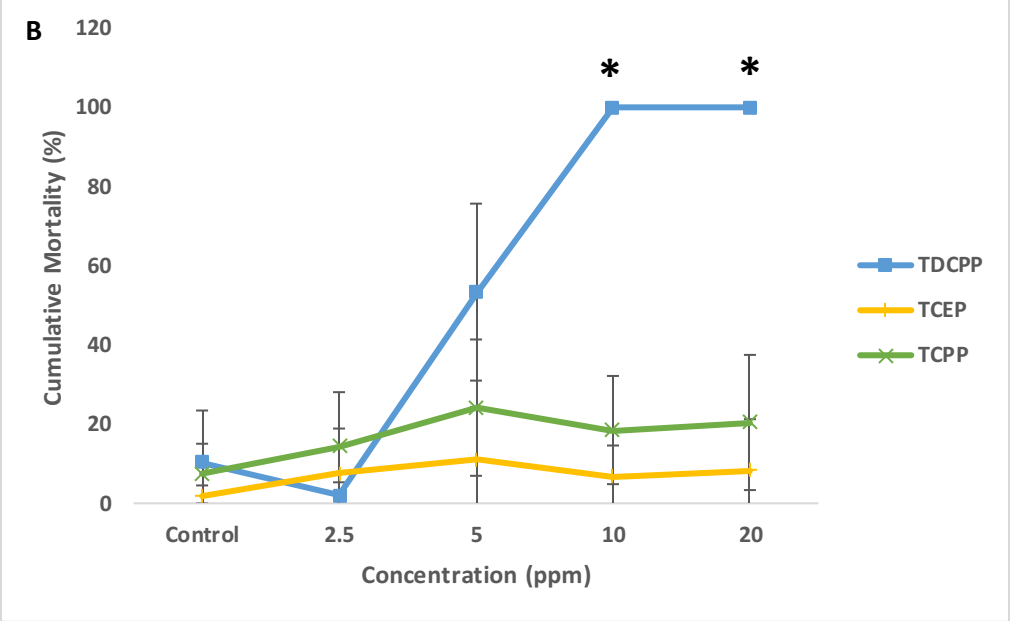


Figure 2: Embryos were exposed at 6 hpf to the indicated OPFRs at varying concentrations and evaluated daily for incidence of mortality until 168 hpf. Results shown are the cumulative % mortality at 168 hpf. (A) All 3 non-chlorinated OPFRs, TPP, TBP, and TBEP, induced mortality in a concentration-dependent manner up to 20 ppm. (B) Of the chlorinated OPFRs, only TDCPP resulted in a significant increase in mortality. N= 12, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

Log K_{ow} vs LC50

The median lethal concentration (LC50) values of the selected OPFRs are shown in Table 3 below. The LC50 value is defined as the dose that induces mortality in 50% of the population following exposure to a compound. Our study compared the LC50 values of each OPFR with their log K_{ow}, which is a measure of a compound's solubility. (As a result of the absence of mortality in embryos exposed to TCEP, TCPP and TEHP, the LC50 values for these compounds could not be determined.). A negative correlation was observed, as can be visualized in Figure 3 below. Essentially, the OPFRs with higher lipophilicity were observed to induce significant mortality at relatively lower OPFR concentrations. For instance, TPP, the most lipophilic compound, is predicted to induce mortality in 50% of the zebrafish embryos at a concentration as low as 1.5 ppm.

Table 3: LC50 values of the 7 selected OPFRs

OPFR Name	**LC50 (ppm)**
TPP	1.5
TBP	3.3
TBEP	4.3
TDCPP	5.3
TCEP	N/A
TCPP	N/A
TEHP	N/A

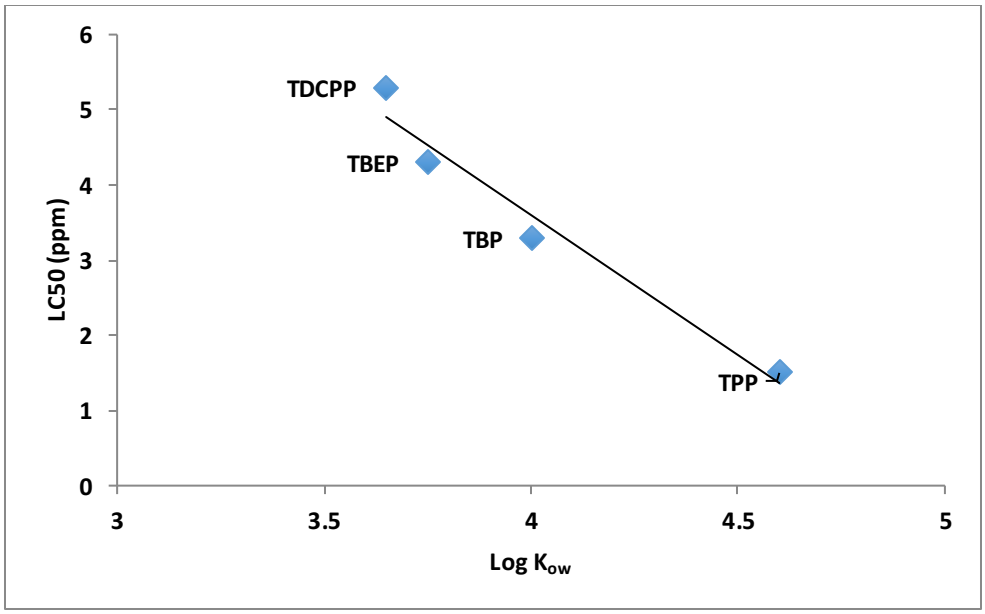


Figure 3: Log K_{ow} vs. LC50 (ppm) plot of the selected OPFRs. The compounds that did not have quantifiable LC50 value are not shown. $R^2 = 0.96$.

Dose Response: Developmental Malformations

Developmental malformations can be indicative of toxicity or frank teratogenesis. Thus, the manifestation of pericardial edema, yolk sac edema, and notochord malformations was recorded daily in zebrafish exposed to the seven OPFRs. Dechorionated embryos at 6 hpf were transferred into wells containing concentrations of 2.5, 5, 10 and 20 ppm of the selected OPFRs or 0.5% DMSO. Examples of the developmental malformations noted in this study can be visualized in Figure 4 below.

Figures 5A & B, Figures 6A & B, and Figures 7A & B, show a summary of the incidence of pericardial edema, yolk sac edema, and notochord malformations, respectively. Following exposure to TPP, pericardial edema and yolk sac edema were observed at concentrations of 10 and 20 ppm. Curved body malformations following TPP exposure were observed at 10 and 20 ppm as well as 5 ppm. Interestingly, TPP was the

only OPFR to elicit significant developmental malformations. Furthermore, after exposure to 20 ppm TPP, zebrafish embryos developed all 3 of the assessed malformations as early as 48 hpf (Figure 8). Exposure to TBP also induced developmental malformations, but their incidence was not statistically significant. No significant changes in malformations were observed following exposure to any of the remaining OPFRs. TEHP was tested at concentrations up to 100 ppm with no significant developmental malformations (data not shown in Figures 5, 6 and 7 due to different scaling of the graphs).

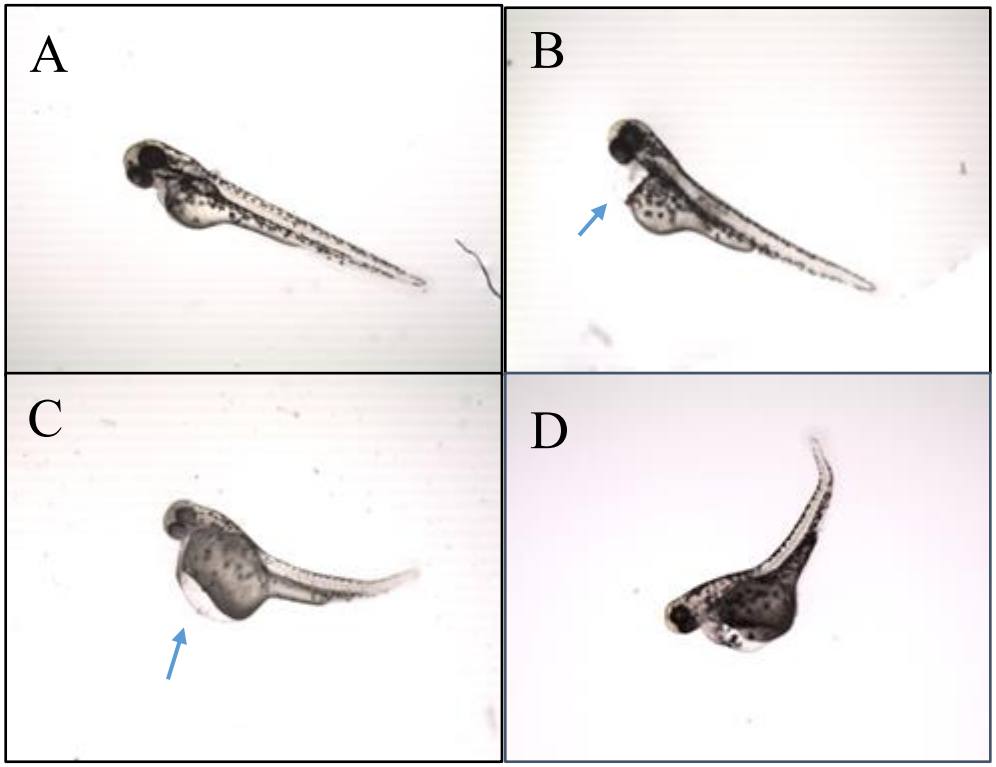


Figure 4: (A) Example of a normal control embryo at 72 hpf. (B) Specimen exposed to TPP at 72 hpf exhibiting pericardial edema, as indicated by the arrow. (C) Specimen exposed to TPP at 72 hpf exhibiting yolk sac edema, as indicated by the arrow. (D) Specimen exposed to TPP at 96 hpf exhibiting curved body malformation.

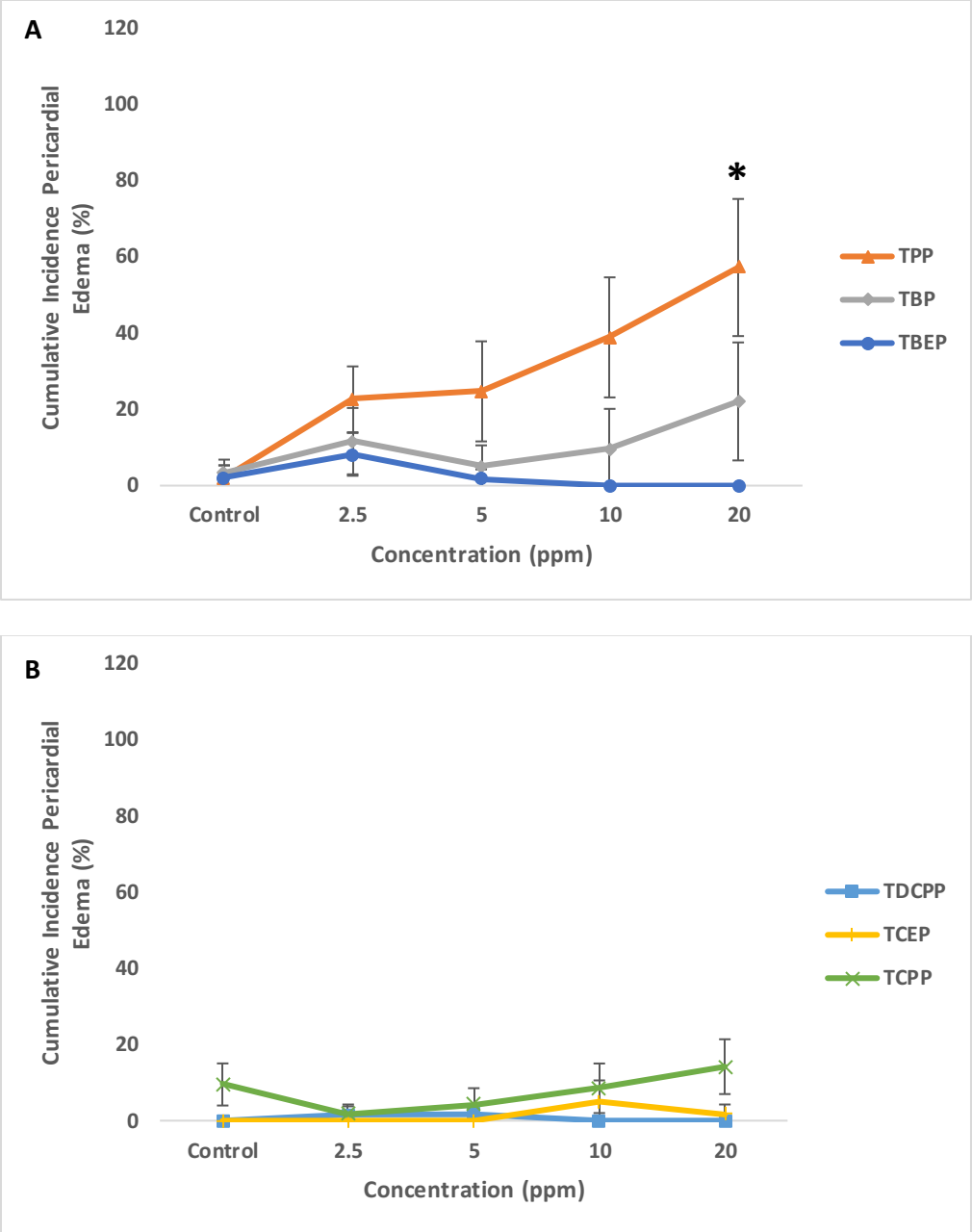


Figure 5: Embryos were exposed at 6 hpf, and evaluated daily for pericardial edema until 168 hpf. Results shown are the cumulative incidence (%) of pericardial edema at 168 hpf. (A) Incidence of pericardial edema malformations in embryos exposed to non-chlorinated OPFRs. (B) Incidence of pericardial edema malformations in embryos exposed to chlorinated OPFRs. N= 12, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

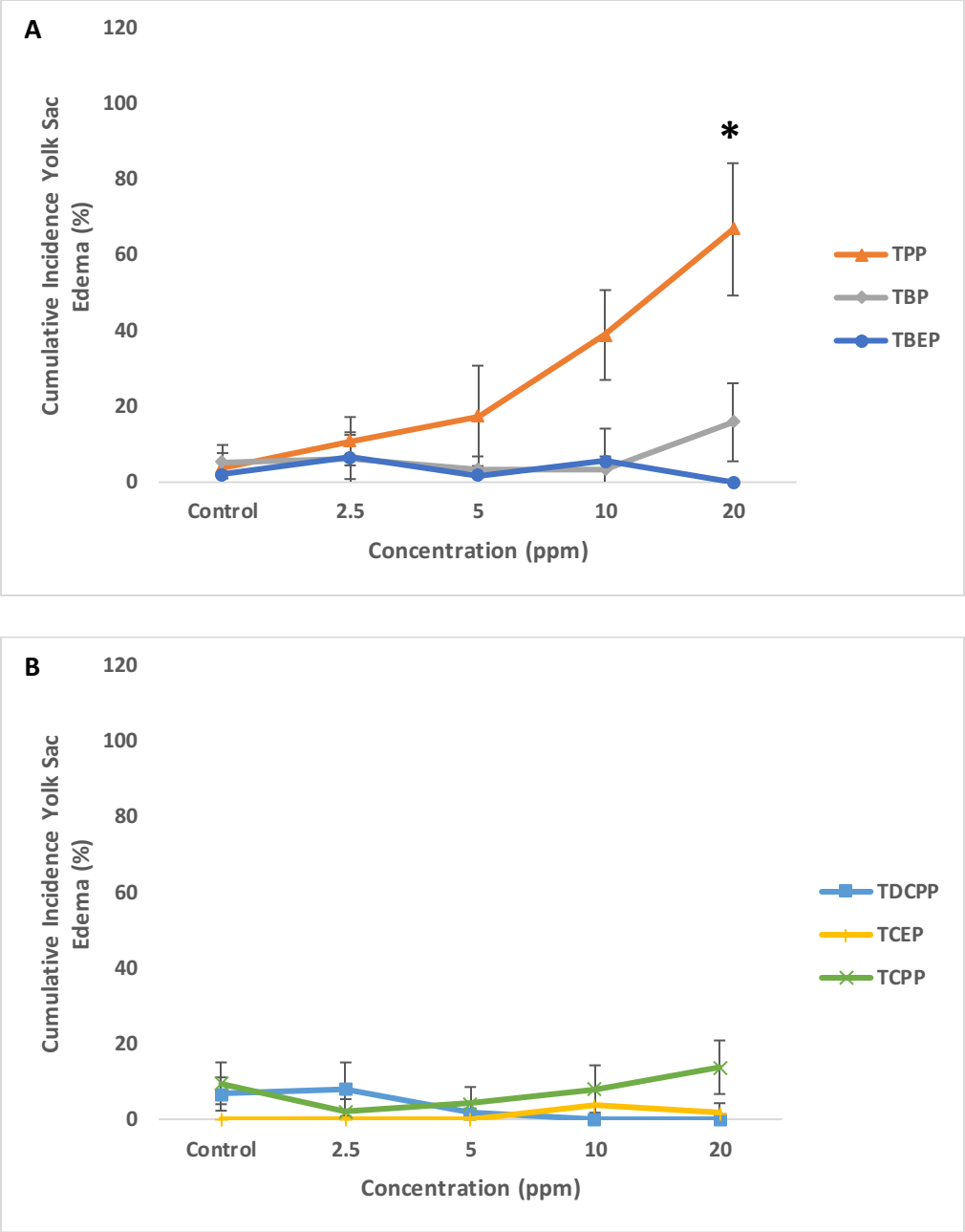


Figure 6: Embryos were exposed at 6 hpf, and evaluated daily for malformations until 168 hpf. Results shown are the cumulative incidence (%) yolk sac edema at 168 hpf. (A) Incidence of yolk sac edema malformations in embryos exposed to non-chlorinated OPFRs. (B) Incidence of yolk sac edema malformations in embryos exposed to chlorinated OPFRs. N= 12, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

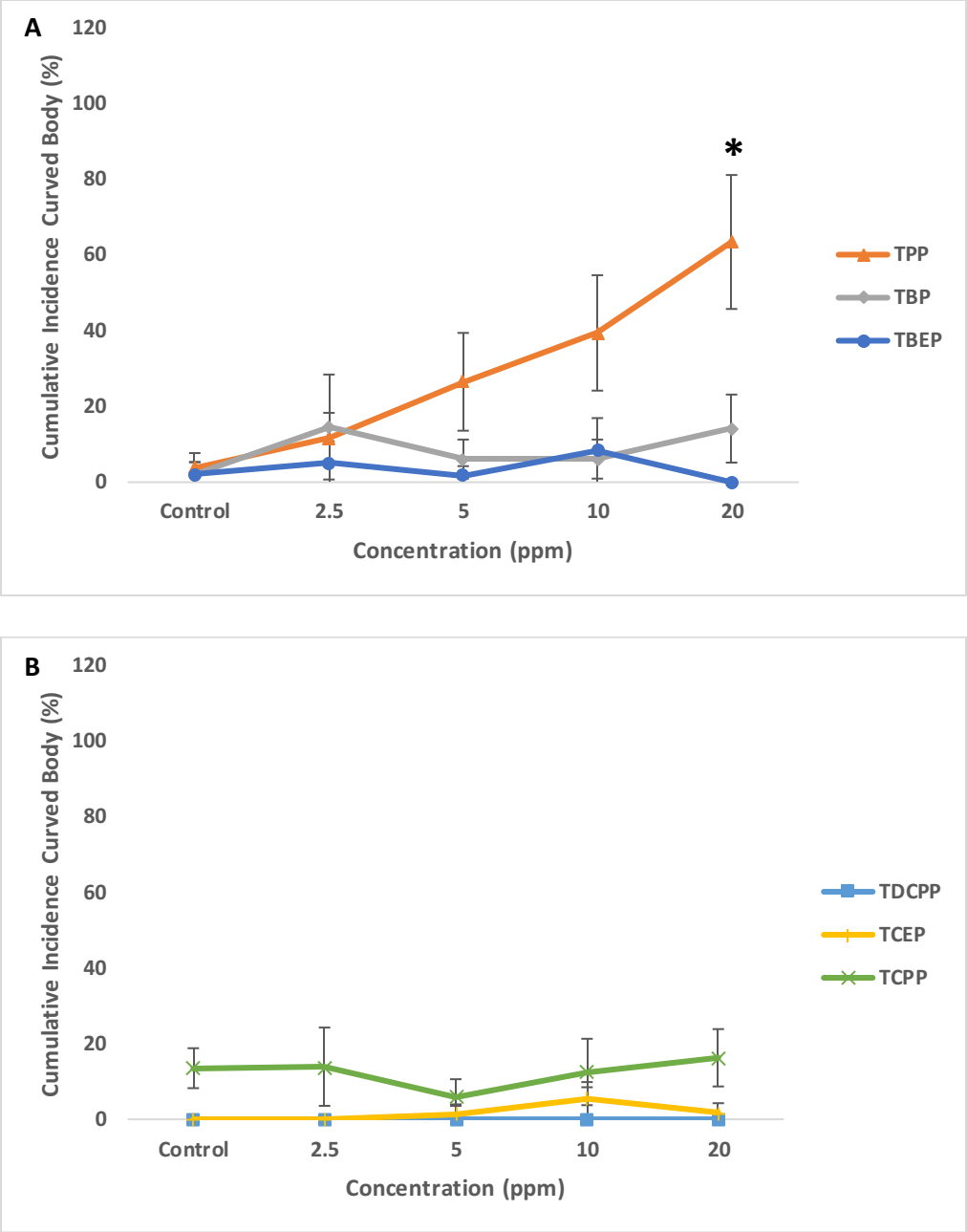


Figure 7: Embryos were exposed at 6 hpf, and evaluated daily for malformations until 168 hpf. Results shown are the cumulative incidence (%) curved body malformations at 168 hpf. (A) Incidence of curved body malformations in embryos exposed to non-chlorinated OPFRs. (B) Incidence of curved body malformations in embryos exposed to chlorinated OPFRs. N= 12, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

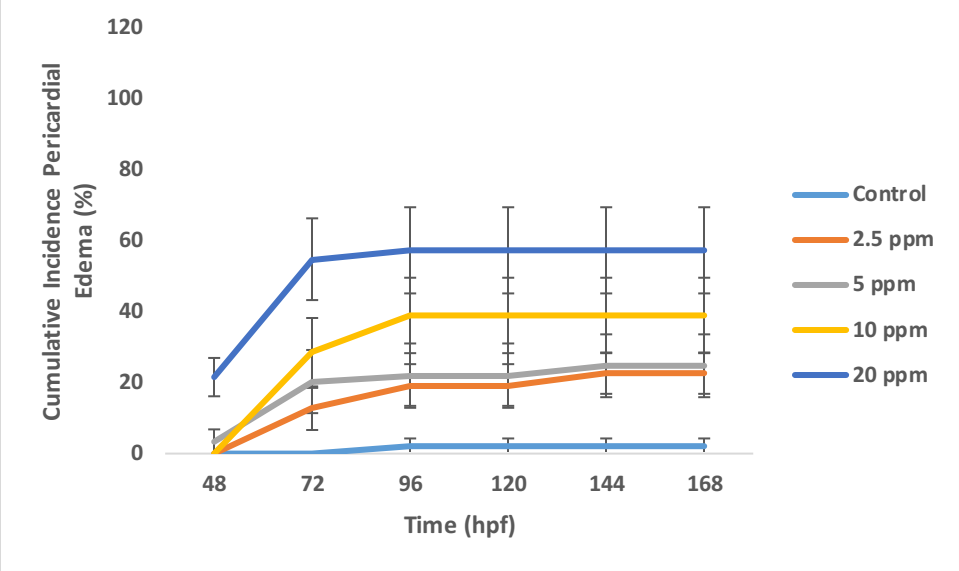


Figure 8: Cumulative incidence (%) pericardial edema in embryos exposed to varying concentrations of TPP at 48, 72, 96, 120, 144, and 168 hpf. N=12. Error bars represent (+/-) standard error.

Spontaneous Movement

Spontaneous movement rate was calculated in embryos exposed to 7 OPFRs by counting the number of tail flexes per larva per 30 seconds at 24 hpf. Embryos were exposed to the selected OPFRs at 6 hpf at concentrations of 2.5, 5, 10, and 20 ppm. Larvae exposed to non-chlorinated OPFRs, TPP, TBEP, and TBP, experienced significant hypoactivity at 20 ppm. Furthermore, significant hyperactivity was observed in larvae exposed to TPP at 2.5 ppm. Some of the effects seen can be attributed to frank toxicity; however, other effects may be attributed to direct disruption of the nervous system. Significant alterations in flexion rate were observed with exposure to only one of the chlorinated OPFRs, TDCPP (Figure 9B). TEHP (not shown in Figure 9 due to different scaling of the graph) expressed no significant change in spontaneous movement and remained consistent with the control (N=12).

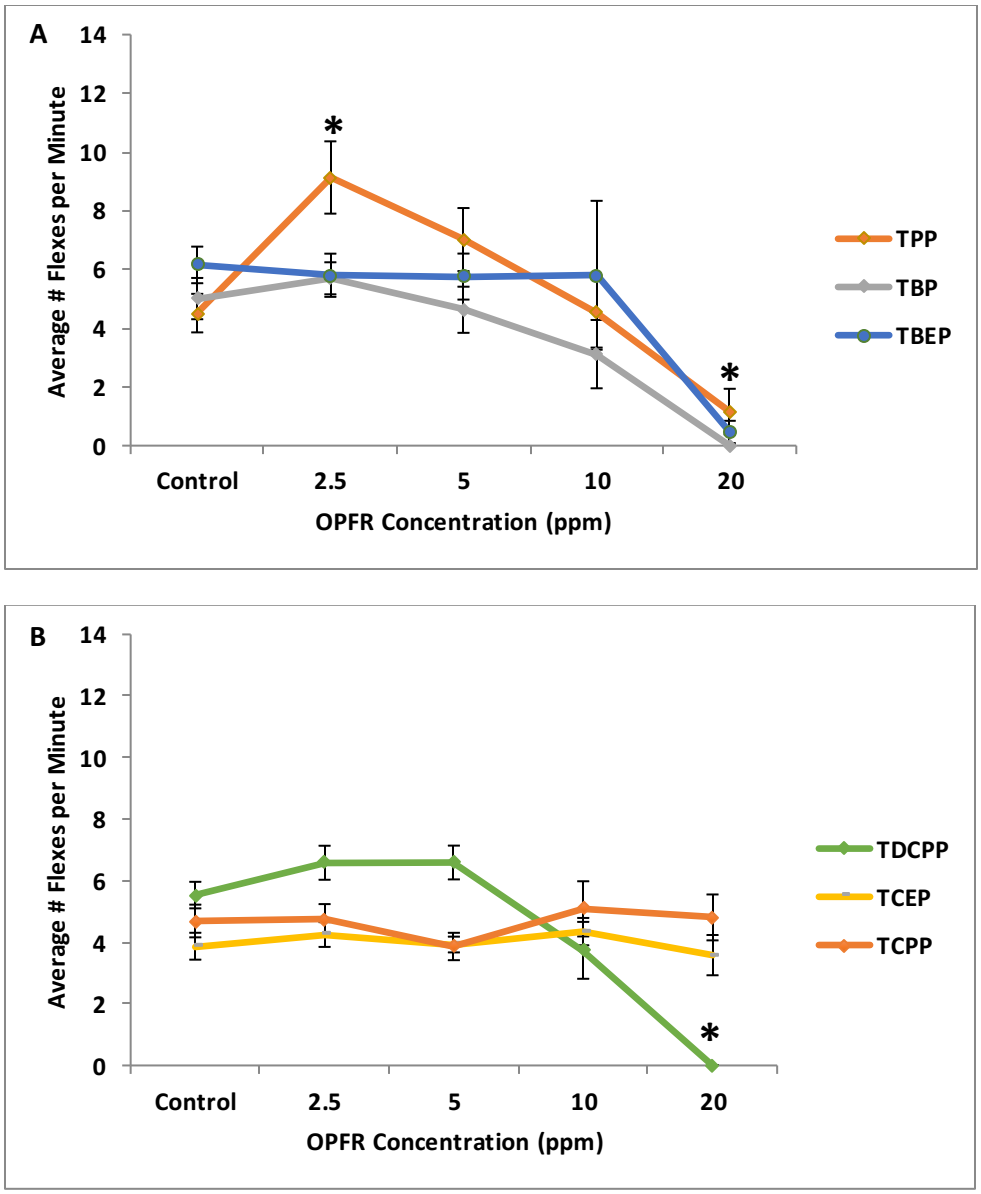


Figure 9: Average rate of spontaneous movement at 24 hpf in embryos exposed to OPFR concentrations of 2.5, 5, 10 and 20 ppm. (A) Spontaneous movement following exposure to the non-chlorinated OFPRs, TPP, TBP and TBEP. (B) Spontaneous movement following exposure to the chlorinated OFPRs, TDCPP, TCEP and TCPP. N= 12, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

Acetylcholinesterase Activity

Acetylcholinesterase activity was measured as an indicator of neurological toxicity following OPFR exposure. Acetylcholinesterase is the enzyme responsible for

hydrolyzing acetylcholine (ACh) in the neuromuscular junction. An accumulation of ACh in the synapse leads to overstimulation of the post-synaptic neuron and one of the possible consequences is muscle overexcitation. As a result, spontaneous movement may actually be disrupted due to AChE inhibition.

AChE activity was evaluated at 120 hpf in zebrafish embryos exposed to OPFRs at the MTD. Of the non-chlorinated OPFRs, 2.5 ppm TPP and 2.5 ppm TBEP inhibited AChE activity as compared to the control by 51% and 27.2%, respectively. Exposure to 2.5 ppm TPP also resulted in hyperactivity in regards to spontaneous movement. Since exposure to TPP inhibits AChE activity and there is also an observed increase in spontaneous movement following TPP exposure, the two biological responses may likely be related. Interestingly, a concentration of 20 ppm TPP results in hypoactivity, suggesting that extreme overstimulation of ACh receptors can lead to loss of muscle movement or overall organism failure, rather than overexcitation.

Of the chlorinated OPFRs, a concentration of 5 ppm TCPP and 5 ppm TCEP inhibited AChE activity as compared to the control by 24.7% and 34.9%, respectively. There were no significant alterations in AChE activity following exposure to the remaining OPFRs, 2.5 ppm TDCPP, 2.5 ppm TBP and 100 ppm TEHP. There was no correlation between OPFR chlorination and change in AChE activity.

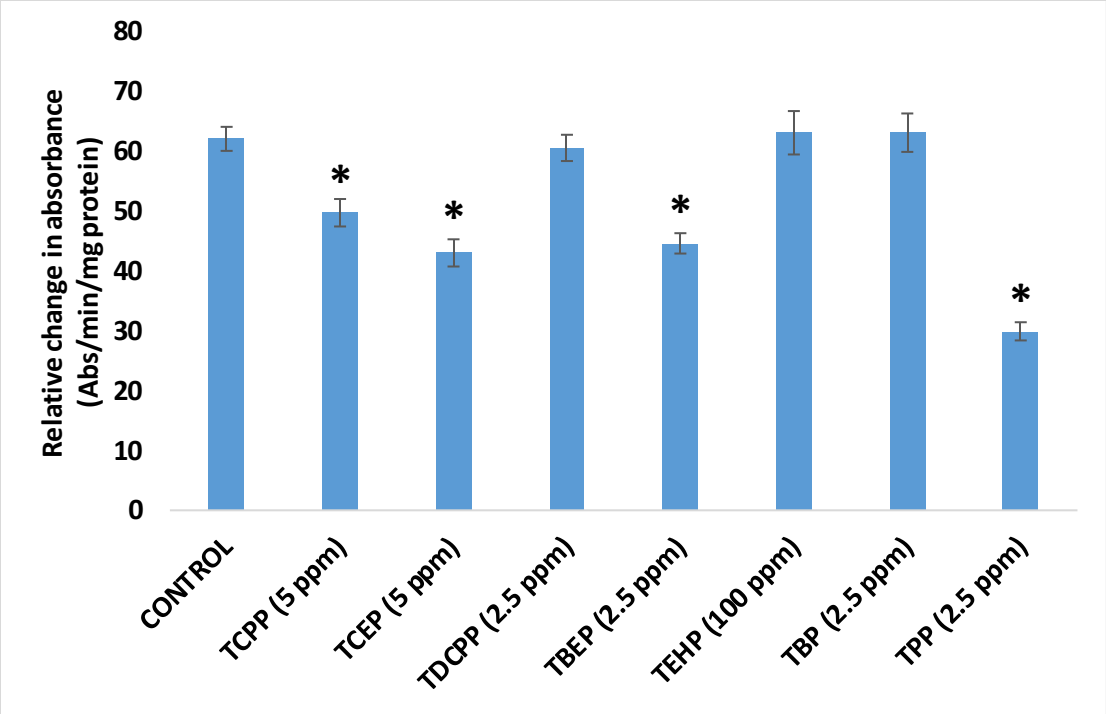


Figure 10. Embryos exposed to the 7 OPFRs, TCPP (5 ppm), TCEP (5 ppm), TBEP (2.5 ppm), TPP (2.5 ppm), TDCPP (2.5 ppm), TEHP (100 ppm) and TBP (2.5 ppm) and AChE activity was measured at 120 hpf. N=4, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard error.

GST Activity

Glutathione *S*-Transferase (GST) is an enzyme that functions in the detoxification of free radicals as a cell's response to oxidative stress.[32] As a mechanism of action, OPFRs may repress expression of GST, thereby exacerbating oxidative stress. To determine if GST activity was disrupted following exposure to the selected OPFRs, the specific activity toward the substrate, CDNB, was measured for 70 seconds. The OPFR concentrations used were those at which the highest observable morbidity was observed without significant overall mortality. At 120 hpf, none of the 7 selected OPFRs elicited a significant change in GST activity as compared to the control. Importantly, the most toxic

OPFR did not impact GST activity, suggesting that its mechanism of action is not related to oxidative stress or the cellular response to it.

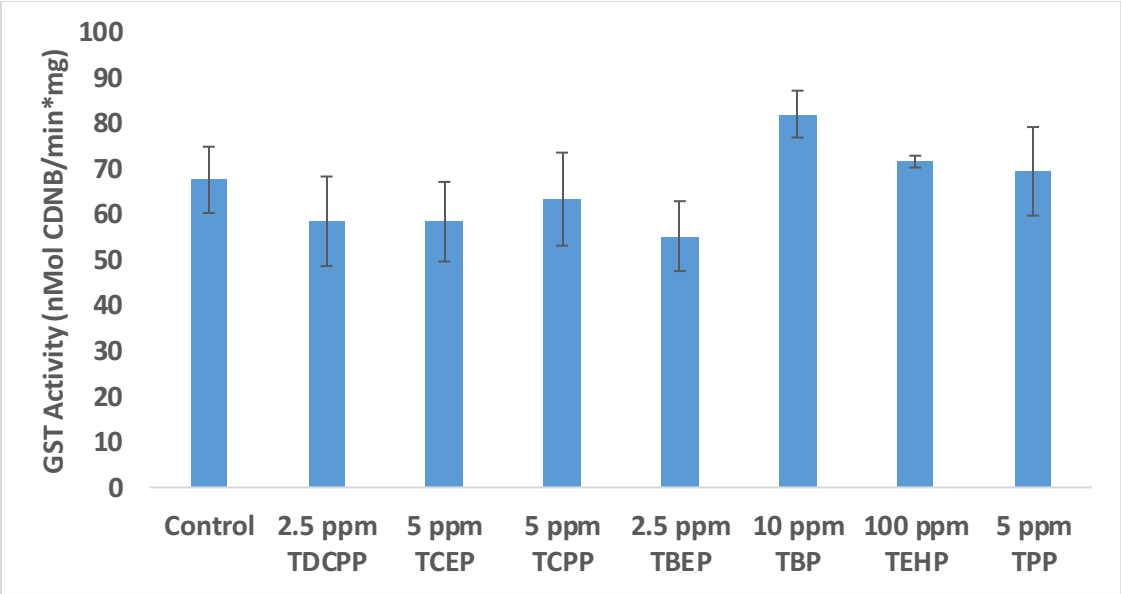


Figure 11: Embryos were exposed to the 7 selected OPFRS, TDCPP (2.5 ppm), TCEP (5 ppm), TCPP (5 ppm), TBEP (2.5 ppm), TBP (10 ppm), TEHP (100 ppm), and TPP (5 ppm) and GST activity was measured at 120 hpf. N=4, (*) indicates statistical significance as compared to the control, $p < 0.05$. Error bars represent (+/-) standard deviation bars.

CHAPTER FOUR

Discussion

In this study, embryonic zebrafish were utilized as the model organism to investigate the relative toxicity of seven OPFRs, TPP, TBP, TBEP, TDCPP, TCEP, TCPP and TEHP. Mortality, malformations and spontaneous movement were evaluated as indicators of toxicity. In addition, GST activity was assessed to probe oxidative stress as a mechanism of action and AChE activity was assessed as an indicator of neurotoxicity. Our study also compared the toxicity of the non-chlorinated OPFRs, TPP, TBP, TBEP, and TEHP and the chlorinated OPFRs, TDCPP, TCEP and TCPP. Our findings revealed that chlorination cannot consistently predict OPFR toxicity. Several chlorinated and non-chlorinated OPFRs induced significant mortality and malformations. In regards to the OPFR mechanism of action, the results do not support oxidative stress as a mechanism of action. No change in GST activity was observed following exposure to any of the 7 selected OPFRs. However, several OPFRs did inhibit AChE activity at 120 hpf. In particular, TPP was found to induce mortality, significant alterations in spontaneous movement as well as a 51% decrease in AChE activity as compared to the control. Due to the drastic inhibition of AChE activity, even at a concentration as low as 2.5 ppm, the mechanism of action for TPP likely involves nervous system disruption.

In the present study, the malformations observed following OPFR exposure were pericardial edema, yolk sac edema and notochord deformities. These malformations are indicative of chemical toxicity in the embryonic zebrafish. Specifically, the presence of

pericardial edema during embryonic development is a common endpoint for cardiac toxicity.[44] Defective cardiac systems are often observed in embryonic zebrafish exposed to toxic compounds and thus, pericardial edema can be used to assess the developmental toxicity of a compound.[45] Nonetheless, due to the variety of mechanisms that can lead to the formation of pericardial edema, this endpoint cannot deduce a toxin's mechanism of action.

Like pericardial edema, a variety of mechanisms can induce the development of yolk sac edema. The embryonic zebrafish yolk has a high lipid composition and is a vital source of nutrients and energy for the developing organism. The formation of yolk sac edema disrupts blood flow between the yolk and the embryonic body, and thus, can impair embryonic development. The formation of yolk sac edema is a useful tool for assessing toxicity, but limited in its ability to identify a toxin's mechanism of action.

Apart from assessing mortality and malformations, our study also evaluated GST activity and AChE activity in order to probe the mechanism of action of the selected OPFRs. We found that none of the selected OPFRs elicited changes in GST activity. However, significant decreases in acetylcholinesterase activity were found in TPP, TBEP, TCEP, and TCPP. In particular, TPP, an OPFR known for its high production volume and presence in the environment, was found to drastically inhibit AChE activity. TPP has been detected in indoor and outdoor air samples at urban sites including Chicago, Cleveland and Houston.[46–49] Due to the high concentrations of TPP found in the environment, it is vital to further investigate its toxic effects. Prior studies evaluating TPP have remarked on the compound's potential neurotoxic effects.[7,29,50,51] The current study confirms the neurotoxicity of TPP in embryonic zebrafish as evidenced by the significant

inhibition of AChE activity. Furthermore, TPP also induced mortality and malformations in a concentration dependent manner, inhibited spontaneous movement behavior, and significantly decreased AChE activity. Because TPP inhibits AChE activity so drastically, its mechanism of action for mortality and malformations likely involves nervous system disruption. Due to the homology and conserved body systems between early-stage zebrafish and humans, the neurotoxic effects following TPP exposure on zebrafish embryos cause concerns for human health.

The other non-chlorinated OPFRs, TBP and TBEP, also showed notable decreases in zebrafish spontaneous behavior and increases in mortality in a concentration dependent manner. TBP and TBEP share a common structure of unbranched alkyl chains and both compounds induced similar effects on zebrafish embryos upon exposure. On the other hand, TEHP, another non-chlorinated OPFR, has a branched structure and it is one of the few OPFRs not regulated by the Environmental Protection Agency (EPA).[52] Several studies have demonstrated the limited toxic effects of TEHP and as a result, the EPA officially stated that TEHP has a low level of acute oral toxicity, is non-carcinogenic and non-mutagenic.[52] As expected, our results revealed that TEHP induced no adverse effects in all of the experimental assessments. Concentrations up to 100 ppm of TEHP did not induce malformations or mortality and resulted in no change in spontaneous movement, GST activity or AChE activity. The lack of toxicity that TEHP exhibits makes it a desirable flame retardant as compared to other OPFRs.

Since the phasing-out of PBDEs, TDCPP has been of particular importance due to the compound's prevalence in human seminal plasma and breast milk.[53–55] A previous study demonstrated that TDCPP increased malformations, including spinal curvature and

growth inhibition, in zebrafish embryos.[53] As anticipated, TDCPP elicited an increase in mortality upon exposure to concentrations of 10 and 20 ppm. Conversely, the other chlorinated OPFRs, TCPP and TCEP did not induce mortality. They did, however, prompt significant inhibition of AChE activity at 120 hpf (Figure 10).

As the usage of alternative flame retardants, such as PBDEs, becomes increasingly restricted worldwide, environmental contamination with OPFRs continues to escalate. For instance, samples from the Great Lakes basin contain OPFR concentrations up to 2-3 orders of magnitude greater than the concentrations of BFRs.[46] As environmental concentrations rise globally, it is essential to better understand the mechanism by which OPFRs affect human health. Our findings suggest short-term neurotoxic effects of OPFRs in developing zebrafish, but the relevance of our findings is undetermined in the early development of humans. Furthermore, additional research is needed to determine the consequences of the long-term neurotoxic effects of OPFRs. Due to the severe inhibition of AChE activity and mortality induced by TPP, it is particularly important to focus on TPP and its adverse effects on human health.

In conclusion, the seven selected OPFRs of varying physical-chemical properties had a wide variety of toxic effects and there was no correlation between chlorination and toxicity. Our findings suggest that TPP is the most toxic OPFR and its mechanism of action likely involves disruption of the nervous system. TBEP, TBP and TDCPP induced mortality in a concentration dependent manner, but additional research needs to be conducted to determine the compounds' mechanism of action. Our findings, as well as the findings of previous studies, revealed a lack of toxicity following exposure to TEHP.

As a result, manufacturers should consider using TEHP as the flame retardant of choice in commercial products.

Another focus of future research should target OPFR metabolites. Several studies have detected the presence of OFPR metabolites in human urine samples.[56,57] Over time, OPFRs biodegrade to form metabolites, and thus, it is important to understand the metabolites' toxic effects as well. Metabolites can occasionally be more toxic than their parent compounds, so it is important to evaluate the toxicity of each OPFR's metabolites.

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