AN ABSTRACT OF THE THESIS OF

Kimberly A. Britsch for the degree of Honors Baccalaureate of Science in Biology presented on June 4, 2014. Title: Evaluating the Toxicity of Organophosphate Flame Retardants in Zebrafish

Abstract approved:

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Robert Tanguay

Flame retardants are in many home products including couches, chairs, and electronics. The most commonly used flame retardants contained polybrominated diphenyl ethers (PBDEs) until 2004, when the majority of these chemicals were phased out. PBDEs were found to be persistent, bioaccumulative, and toxic to both humans and the environment. Organophosphate flame retardants have become an option for alternative flame retardants, but have not been tested extensively. There is concern that many of these organophosphate flame retardants may be carcinogenic, neurotoxic, or reproductive toxicants.

Using zebrafish as a model, three organophosphate flame retardants (TDCPP, TCPP, and TCEP) were tested for their developmental and neurotoxic effects. The results of this study indicated that there are both physical and behavioral effects of all three of these chemicals. With further testing, it may be found that these negative effects are linked to various neurological disorders in humans such as ADD and ADHD. Other neurological disorders, such as autism or depression, may also be affected, but additional studies would need to be performed in order to determine this.

Key Words: Polybrominated diphenyl ethers, Organophosphate flame retardants, zebrafish, developmental and neurotoxicity

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Evaluating the Toxicity of Organophosphate Flame Retardants in Zebrafish

by

Kimberly A. Britsch

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University Honors College. My signature below authorizes release of my project to any reader upon request.

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Kimberly A. Britsch, Author

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INTRODUCTION

Flame retardants are chemicals added to numerous home products including couches, chairs, and electronics in an effort to inhibit, suppress, or delay the production of flames, and the spread of fire (EPA, 2013). Polybrominated diphenyl ethers (PBDEs) are a major class of flame retardants that have commonly been used. However, the PBDEs have been subjected to ongoing phase-outs and bans due to concerns about their persistence, bioaccumulation, and toxicity potential to both humans and the environment (EPA, 2014).

California Technical Bulletin 117 (TB 117) is a law that was made in California in 1975. California TB117 required the testing of foam to ensure open flame resistance for at least twelve seconds. Flame retardants were used to adhere to this regulation. Although this began in California, companies began to place these flame retardants in all of their products rather than specifying different products just for California (Redford, 2013).

There continue to be efforts to assess appropriate uses of flame retardants and identify replacements for the PBDEs. Organophosphate flame retardants (OPFRs) have emerged as major alternatives. There is little known about the potential toxicity of OPFRs, but some evidence suggests that there may be similar toxicity outcomes as measured with PBDEs, including notable impacts on neurodevelopment. However, there

continues to be only a limited understanding of the potential for this class of compounds to impair development and elicit other adverse effects.

BACKGROUND

Overview of Flame Retardants

There are four major classes of flame retardants, based on their chemical composition: inorganic, halogenated, organophosphorous, and nitrogen based. Flame retardants can either be additive or reactive. A reactive flame retardant is one that is chemically bound to the raw materials used in the product. An additive flame retardant is one that is added to a product without bonding or reacting with the product. Most flame retardants are additive. Additive flame retardants work by either emitting a substance to displace the oxygen and other radicals necessary for flames to propagate, forming a protective coating on the surface of the flammable substrate, or by utilizing a combination of both (EPA, 2013).

How Flame Retardants Work

To reduce the likelihood of ignition, some flame retardants work by increasing the net heat capacity of the products to which they are added. Heat capacity is the amount of energy required to raise the temperature. Once a flame has already started, however, flame retardants can also reduce the tendency of the fire to spread. To do this, the flame retardant reacts with the product to form a noncombustible gaseous layer along the boundary line of the flame (EPA, 2013).

Halogenated flame retardants work in the gas phase by releasing either chlorine or bromine which then binds to radicals produced in the fire. PBDEs, which contain

bromine, act in this manner. Other kinds of flame retardants, such as nitrogen based flame retardants, reduce the flame by constructing a char that swells in reaction to heat. This char acts to insulate and therefore protect the product (EPA, 2013).

The OPFRs can use both of these mechanisms. Phosphorous containing flame retardants act in the solid phase of burning materials. When it is heated, the phosphorus reacts to produce a polymeric form of phosphoric acid which causes the material to char, forming a glassy layer which acts to inhibit the process of breaking down and releasing flammable gases. These gases are what fuel the fire, thus the char plays an important role in acting as a barrier which hinders the passage of the combustible gases towards the flame. Certain chemicals, such as TDCPP, TCPP, and TCEP, also contain chlorine. The effectiveness of these chemicals lies in their ability to release active chlorine atoms that can bind to oxygen and other high energy radicals produced during fires, thereby slowing their propagation (EFRA, 2013).

Polybrominated Diphenyl Ethers

PBDEs can have from 1-10 bromine atoms positioned on diphenyl ethyl, and the nomenclature for these compounds depends on the position and number of bromines. Three PBDE commercial mixtures have been produced: PentaBDE, OctaBDE, DecaBDE. PentaBDE and OctaBDE were phased-out from production in the US in 2004. Starting at the end of 2013, the DecaDBE commercial mixture was part of a voluntary phase out, with existing stock provisions.

It is important to understand the background of PBDEs because alternative flame retardants that are now being used in place of PBDEs may have similar toxicities. As

previously discussed, PBDEs have bromine atoms attached which are released and combined with free radicals in the event of a fire. Chlorine is a halogen like bromine, and because TDCPP, TCPP, and TCEP all contain chlorine atoms, they react in a similar way to PBDEs.

It was suggested in a 2006 study that PBDEs have endocrine disrupting effects. In female rats, the PBDEs down-regulated progesterone receptors, and up-regulated estrogen receptors (Ceccatelli, et al. 2006). Reproductive effects have been reported, and prenatal exposure to some PBDEs have shown a reduction in sperm count in male rats, as well as a change in ovarian cells in females (Zhou, et al. 2001). There are also studies using fish, and cell based assays that have come to similar conclusions (Meerts et al. 2001; Hamers et al. 2006; Muirhead et al. 2006).

Thyroid levels were also noted as being affected, and this is especially important due to the fact that thyroid hormones play a role in brain development. In a study using fathead minnows, fish were exposed to a low dose (~3 ng/g bw-day) of decabromodiphenyl ether, a heavily used PBDE, for 28 days. Compared to the control fish, these fish "experienced a 53% and 46% decline in circulating total thyroxine (TT4) and 3,5,3'-triiodothyronine (TT3), respectively" (Noyes, et al. 2013). Similar results were repeated in studies using mammals, cell based assays, and in vivo studies (Hallgren et al. 2001; Tseng et al. 2008; Hiroyuki et al. 2009; Zhou et al, 2001).

PBDEs have also been shown to induce neurobehavioral alterations. In one study, female rats were exposed to a PBDE at gestational day five. The exposure continued until the pups were 21 days old (post natal day 21). The exposed pups showed significant neurological delays compared to the vehicle controls as they did not exhibit the same

responses to the endpoints as the vehicle controls. One endpoint measured for neurobehavioral development was the cliff drop reflex. The elicited response in the vehicle control caused the pup to turn and crawl away from the cliff drop when placed on the edge of a cliff with the forepaws and face over the edge. The second endpoint was the negative geotaxis reflex. This is when the pup is placed on a 45° angle slope with his head pointing down the incline. The expected response, as seen in the vehicle control, was for the pup to turn and crawl up the slope (Cheng, 2009).

Another study exposed mice to BDE-99 at either postnatal day (PND) 3, 10 or 19. They then tested the mice using spontaneous motor behavior tests which observed the locomotor activity of the mice over three 20 minute periods. The mice exposed on PND 3 and 10 elicited a hypoactive response during the first 20 minute period compared to the control group. During the last 20 minute period, the mice exposed on PND 10 also elicited a hyperactive response in comparison to the control group. There were no changes in the response of the mice exposed on PND 19 when compared to the control. The conclusion of this study was that the behavioral disturbances following neonatal exposure to BDE-99 were induced during a specific critical period of neonatal brain development. The mice that were most susceptible to the effects of BDE-99 were those exposed on PND 10 (Eriksson, et al. 2002).

Viberg and coauthors carried out a similar study exposing neonatal mice to BDE-99 (ranging in concentration from 0 to 16mg/kg) during a period of rapid brain growth. The purpose of the study was to determine if this would lead to disruption of the adult brain function. They found that there were significant dose-related changes in

spontaneous motor behavior in adult mice that were exposed to levels of 0.8mg/kg and above, and these effects were worse with increasing age (Viberg, et al. 2004).

Although PBDEs have been phased out, they have the potential to continue having an effect on humans and the environment. This is not only because the chemicals are persistent, but also because many of the products that contained these flame retardants are still in use. Moving forward, it is important to find new and alternative flame retardants that can be placed in these products while preventing harm to both human health and the environment.

Organophosphate Flame Retardants

OPFRs have emerged as major alternative flame retardants to PBDEs, yet they have not been extensively tested for potential developmental and neurotoxic effects. The use of OPFRs is becoming increasingly popular with the phasing-out of many PBDEs, which is why further testing is necessary to understand their potential effects on human health.

Organophosphates are any organic compound whose molecule contains one or more phosphate ester group (Memidex, 2014). The organophosphates used in this study are all chlorinated organophosphates.

Organophosphates and PBDEs share similar routes of exposure which have led to the questioning of the safety of these new chemicals. In humans, exposure of PBDEs is by direct ingestion of foods containing PBDEs, as well as incidental ingestions of dust contaminated with PBDEs. The most common form of exposure to OPFRs is also through incidental ingestion of dust that has accumulated OPFRs.

Both PBDEs and organophosphates are additive flame retardants, thus they are not chemically bound to the products that they are in. Because of this, the chemicals are more likely to be leached into the environment around them, and eventually into the body. A common source of human exposure to these chemicals is through dust. Dust accumulates on top of products like electronics, and then the dust is inhaled. Infants are particularly at risk for exposure due to the likelihood of them being close to the ground, as well as their tendency to put items in their mouths. This is a concern because organophosphates could possibly be carcinogenic, neurotoxic, and reproductive toxicants (Betts, 2013).

There are also organophosphate pesticides that have contributed to our understanding of the potential toxicities of OPFRs. Chlorpyrifos is an organophosphate insecticide that can interact directly with neurotransmitter receptors, as well as exhibit immediate and delayed-onset effects on cardiac cell signaling (Meyer et al. 2004; Meyer et al. 2003; Ward and Mundy 1995). In a study of pregnant women in New York City, it was found that those with the highest levels of chlorpyrifos exposure were the most likely to give birth to children with attention deficit hyperactivity disorder (ADHD), and who score poorly on tests of cognitive development (Rauh, et al. 2006).

Tris (1,3-dichloro-2-propyl) phosphate

Tris (1,3-dichloro-2-propyl) phosphate (TDCPP) is a chlorinated phosphate ester currently being used as a replacement for PentaBDE in the polyurethane foam of furniture and other products, including baby products. The chemical structure of TDCPP is shown in figure 1a. This chemical was once used in children's pajamas, but was phased

out of this use in the early 1980s due to its previously unidentified mutagenic properties (Stapleton, et al. 2009). Despite this happening nearly forty years ago, in a 2011 survey, TDCPP was the most common flame retardant found in baby products with a mean concentration of 39.22mg/g (2.4-124) (Stapleton, et al. 2011).

In another 2011 study, dust samples were taken from homes in the United States and analyzed for TDCPP. The median value obtained from the dust samples was 1.89µg/g (range not available). This value was higher compared to the values found in Belgium, which was only 0.57µg/g (<0.08-6.64) (Van den Eede, et al, 2011).

In a relevant study using four-month-old zebrafish, TDCPP was studied to determine potential effects on reproduction and endocrine disruption (Choi, 2012). An increase in estrogen, and a decrease in testosterone were noted in the zebrafish. They also reported a decrease in semen production in male fish (Choi, 2012).

In an epidemiological study, similar results were obtained. Betts and coauthors collected samples of dust from the homes of 50 male participants recruited from a Boston infertility clinic. These males also had multiple archived urine samples that were analyzed for flame retardant metabolites. The primary metabolite of TDCPP is bis(1,3-dichloro-2-propyl) phosphate (BDCPP). The urine showed consistent levels of this metabolite over time. This led to the conclusion that it is possible to accurately identify the level of OPFR exposure with a single urine sample (Betts, et al. 2013). This means that taking a sample of urine will show long term exposure to the chemical, rather than just a temporary, recent exposure. Previously, men from the same study who lived in homes with higher levels of TDCPP (maximum level was 56,080ng/g, with a mean concentration of 1890ng/g), were shown to have reduced sperm counts (Betts, et al.

2013). A limitation of this study is that the subjects were recruited from the infertility clinic, thus further investigation would need to be pursued to validate the association of flame retardants to a lowered sperm count in humans.

Tris (1-chloro-2-propyl) phosphate

Tris (1-chloro-2-propyl) phosphate (TCPP) is a chlorinated phosphate triester, and another OPFR. The chemical structure of TCPP is shown in figure 1b. TCPP is used primarily in rigid polyurethane foams, but it is also found in flexible polyurethane foams used in furniture and its upholstery (SinoHarvest, 2013). Data is limited on the potential toxicity of TCPP, but it has been suggested that it may cause reproductive toxicity in female mice (Farhat, et al. 2013).

In the 2011 study examining samples of dust in homes of the United States, the median level of TCPP exposure was 0.572µg/g (range not available). This was significantly lower than the median level found in Belgium which was 1.38 µg/g (0.19-73.7) (Van den Eede, et al, 2011).

In a study using chicken embryos, fertilized chicken eggs were injected with TCPP. They studied the effects of the chemical on pipping success, and embryonic growth and development. Pipping is when the chicken first begins to peck on the egg shell before it hatches. Although pipping was still determined to be successful, TCPP did delay the process. This is likely linked to a decrease in neurological development of the chicken embryo. During the last 20% of development inside of the egg (the time prior to pipping) brain activity should slowly be increasing (Balaban, et al. 2012). Because pipping was still a success, but delayed, this may point to a delayed progression of brain

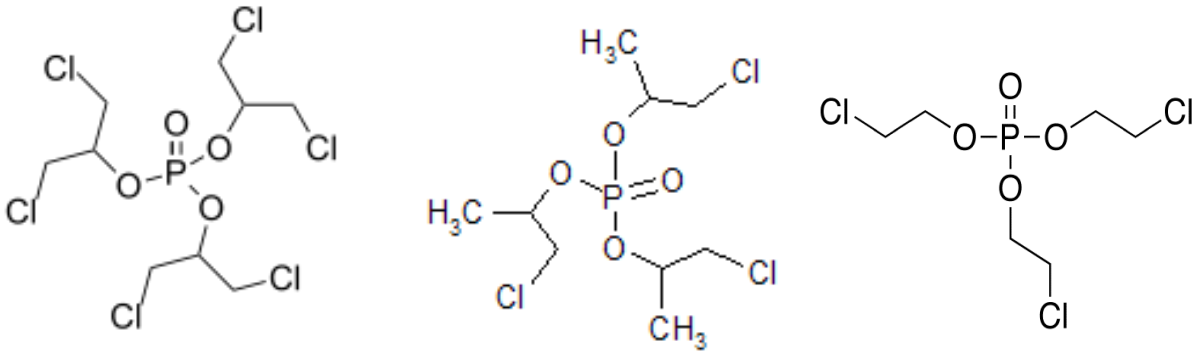
activity as well. TCPP also significantly reduced the length of the chicken's tarsus (part of the leg), as well as played a role in thyroid hormone disruption (Farhat, et al. 2013).

Tris (2-chloroethyl) phosphate

Tris (2-chloroethyl) phosphate (TCEP) is also a chlorinated phosphate triester, and the final OPFR tested in this study. The chemical structure o TCEP is shown in figure 1c. TCEP is used in polyurethane foam, as well as furniture, baby products, and some carpet backing (Putrich, 2013). Exposure to TCEP has been linked to an increase in cancer risk, reproductive effects, and neurotoxicity. It is listed in California as a known carcinogen, and animal studies have found TCEP to lead to tumors in the kidney and liver (California EPA, 2011). As of 2013, TCEP has been banned in Maryland, with other states considering following in their footsteps (MTS, 2013).

There is limited data on the level of TCEP exposure in humans; however, in the 2011 study of dust samples in homes, the median value in Belgium was 0.23µg/g (<0.08-2.65) (Van den Eede, et al, 2011). There was no data for TCEP exposure in the United States in this study.

In a 2008 study, the effects of TCEP were examined using renal proximal tubule cells of rabbits. The results showed that TCEP decreased cell viability, increased lactate dehydrogenase, inhibited expression of many proteins, and decreased DNA synthesis (Ren, et al. 2008). These results suggest that there are potential human health risks from TCEP exposure.



(a) TDCPP (b) TCPP (c) TCEP

Figure 1. Chemical Structures of (a) TDCPP, (b) TCPP, and (c) TCEP

RESEARCH OVERVIEW

This study focused on the effects of three organophosphate flame retardants: TDCPP, TCPP, and TCEP. Through my research, I wanted to determine developmental and behavioral effects that these chemicals would have on zebrafish. The hypothesis was that these effects would have a positive correlation with an increase in chemical exposure.

It is important to research this topic because TDCPP, TCPP and TCEP are all readily used as flame retardants in numerous everyday products. Humans are being exposed to these chemicals on a regular basis, and with knowledge of previous flame retardants having mutagenic effects, it is important to know whether these chemicals will also have these kinds of effects. There is limited data on the specific effects of TDCPP, TCPP, and TCEP, but current evidence suggests toxicity.

To test the hypothesis, the zebrafish were exposed to TDCPP, TCPP and TCEP at doses ranging from 0µM (.64% DMSO) to 64µM. They were then evaluated for developmental and behavioral effects. Developmental effects included yolk sac edema, pericardial edema, bent body axis, caudal fin malformation, pectoral fin malformation, and absent/muted touch response. Behavioral effects were measured as locomotor behavior in response to a light and dark cycle.

The data is limited on the effects of these chemicals, and the hope for this experiment is to determine potential toxicities. Using this information, we can begin to explore how this translates to effects in humans.

Zebrafish Model

In order to study the effects of the selected organophosphates, zebrafish (Danio rerio) were used as the model. Zebrafish are small (adults reaching only 3-4 cm), freshwater fish, and are an ideal model for many reasons. First, they are vertebrates and develop in a similar way to humans. They also have a genome that is comparable to humans (70% gene homology). In addition, 84% of the human genes that are known to be linked to human diseases are also present in zebrafish (Truong, et al. 2013).

Zebrafish are relatively easy to study due to the fact that they mature quickly, and you can observe their development without disturbing the embryos. The embryos are transparent during the first few days of development, and because they are fertilized outside of the female's body, observations can be made without killing either the female or the offspring (as you would have to do in mammalian studies).

METHODS

An overview of the experimental approach can be seen in figure 2.

Zebrafish Husbandry

Tropical 5D wild-type adult zebrafish were housed in tanks with a density of 1000 fish per 100 gallon tank at the Sinnhuber Aquatic Research Laboratory (SARL) of Oregon State University, Corvallis, OR. The tanks were kept under standard laboratory conditions of 28°C on a 14 hour light/ 10 hour dark photoperiod. The water contains reverse osmosis water supplemented with Instant Ocean (commercially available salt). At night, spawning funnels were placed into the tanks, and embryos were collected the following morning (Truong, et al. 2013).

Mature embryos have a chorion—an acellular envelope—surrounding them. To avoid the chorion from presenting a barrier for the chemicals, at 4 hours post fertilization (hpf), the chorion was enzymatically removed using pronase and a custom automated dechorionator.

Chemical Preparation

One initial plate, per chemical, was prepared using a 96 well plate. 40µL of a 2mM stock solution was placed into columns 1 and 7 of plate 1. Using a serial dilution, the resulting concentrations in columns 2-5 (and repeated in columns 8-11) were as follows: 1mM, 0.1mM, 0.01mM, and 0.001mM. Columns 6 and 12 were the control

columns (0mM) and contained 100% DMSO. This plate was stored in a freezer at -20°C until the day of chemical exposure.

A second 96 well plate, plate 2, was then prepared using plate 1 and embryo medium (EM) to dilute. The resulting concentrations in columns 1-6 and 7-12 were then: 640µM, 64µM, 6.4µM, 0.64µM, 0.064µM, and 0µM (6.4% DMSO).

Chemical Exposure

Two 96 well plates, plate A and plate B, were loaded with 90µL of EM per well. At 6 hpf, one viable embryo was placed in each well using a pipette. After confirming that all embryos remained viable in each well, the embryos were exposed to the chemicals. 10µL of the corresponding column in plate 2 was added to both plate A and plate B, being careful not to touch the embryos in order to avoid damage. The final concentrations in columns 1-6, and 7-12, were: 64µM, 6.4µM, 0.64µM, 0.064µM, 0.0064µM, and 0µM (.64% DMSO). With two plates containing this concentration scheme, the total sample size for each concentration was n=32.

Following the chemical exposure, plates A and B each had parafilm placed over them, and the lids put back on. They were then wrapped in foil together, and stored in an incubator maintained at 30°C.

Developmental Toxicity Screening

At 24 hpf, the embryos were screened for two endpoints: mortality, and delayed progression. Delayed progression was classified as any deviation from the normal

phenotype. A normal phenotype at 24 hpf is shown in figure 4a with a well-developed notochord, a body axis that is beginning to straighten, as well as circulation, pigmentation, and fins that are beginning to develop (Kimmel, et al. 1995).

At 120 hpf, zebrafish larvae were screened for six endpoints including yolk sac edema, pericardial edema, body axis malformation, caudal fin malformation, pectoral fin malformation, and a muted/absent touch response. The fish were also analyzed for their locomotor response at 120 hpf. To do this, plates A and B were each placed in a Zebrabox and exposed to intervals of light and dark. A Zebrabox (which can be seen in figure 3) is a device that is connected to the computer and used alongside the software, ViewPoint. This system is utilized for "high throughput tracking and behavioral analysis of zebrafish" (ZebraLab). Within the Zebrabox, there is an initial acclimation period of five minutes, followed by five minutes of light, then ten minutes of dark, and another five minutes of light. ViewPoint tracks the movement of each fish throughout this time. This looks examines potential neurological effects from the chemicals.

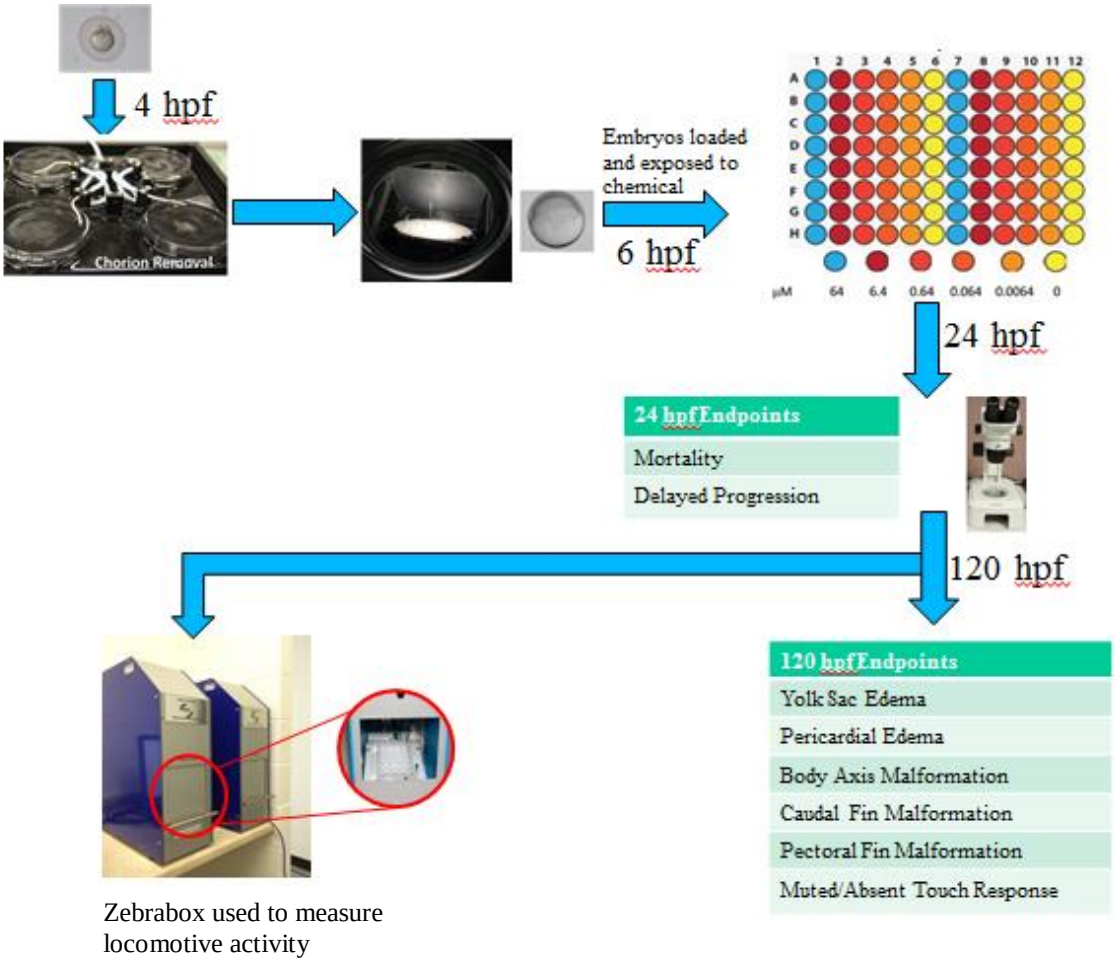
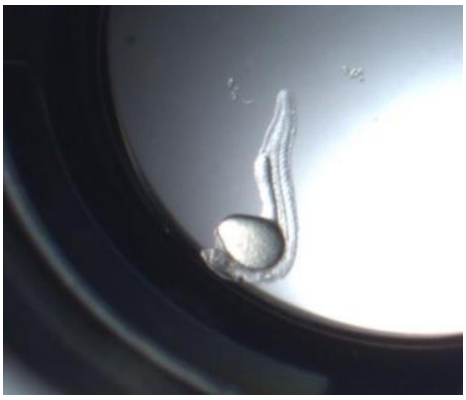


Figure 2. Experimental approach for screening developmental and neurotoxicity for TDCPP, TCPP, and TCEP

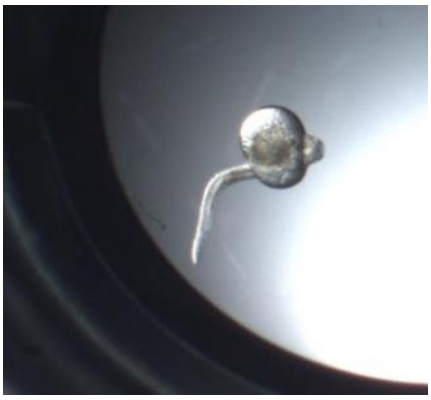
RESULTS

Physical Malformations

At 24 hpf, the embryos are expected to look like the zebrafish in figure 3a. Any variation from this control is considered to be a delayed progression, as seen in figure 3b.



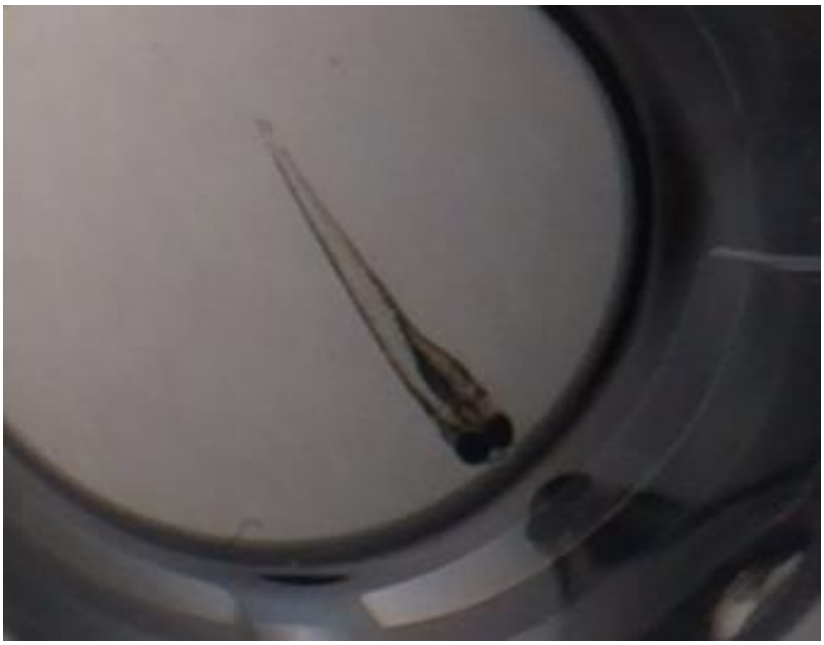
(a) Control embryo



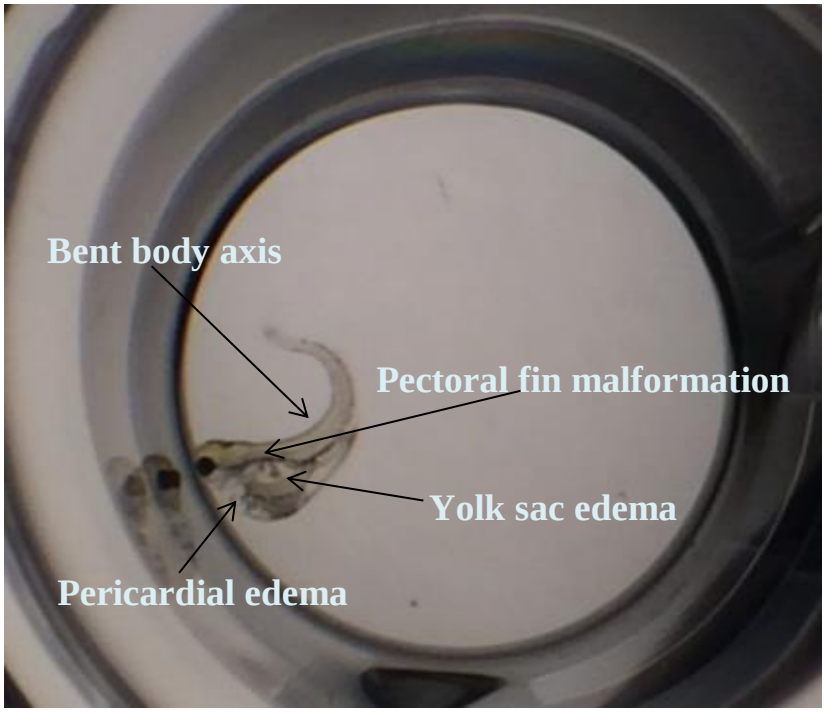
(b) Embryo with noted "delayed progression"

Figure 3. Zebrafish Embryos at 24 hpf

At 120 hpf, the zebrafish are expected to look like the fish in figure 4a. An example of a fish with malformations is in figure 4b. This fish displays a bent body axis, pericardial edema, yolk sac edema, and pectoral fin malformation. Results of number of malformations and mortalities versus no effects are available in table 1. The graphs of these results are shown in figure 5. In TDCPP, mortality increased as dose increased. In TCPP and TCEP, dose didn't seem to change the proportion of mortality or malformations, except in the highest dose tested.



(a) Control zebrafish



(b) Zebrafish with the following malformations: bent axis, pericardial edema, yolk sac edema, and pectoral fin malformation.

Figure 4. Zebrafish at 120 hpf

Table 1. Percent Mortality and Malformations of TDCPP, TCPP, and TCEP at 120 hpf

(a) TDCPP

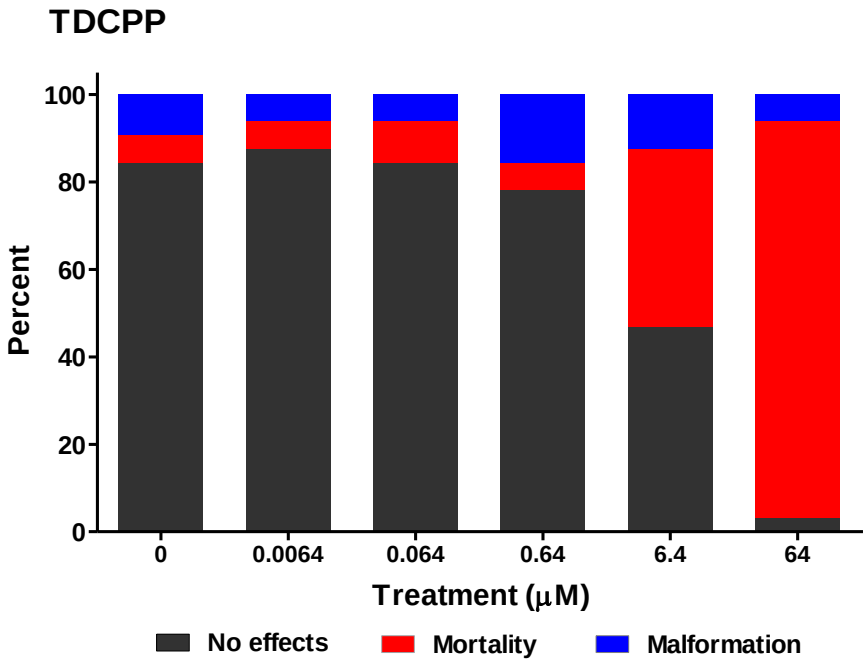
DOSE (µM)	MORTALITY	MALFORMATION	NO EFFECT
0	2 (6.25%)	3 (9.38%)	27 (84.38%)
0.0064	2 (6.25%)	2 (6.25%)	28 (87.50%)
0.064	3 (9.38%)	2 (6.25%)	27 (84.38%)
0.64	2 (6.25%)	5 (15.63%)	25 (78.13%)
6.4	13 (40.63%)	4 (12.50%)	15 (46.88%)
64	29 (90.63%)	2 (6.25%)	1 (3.13%)

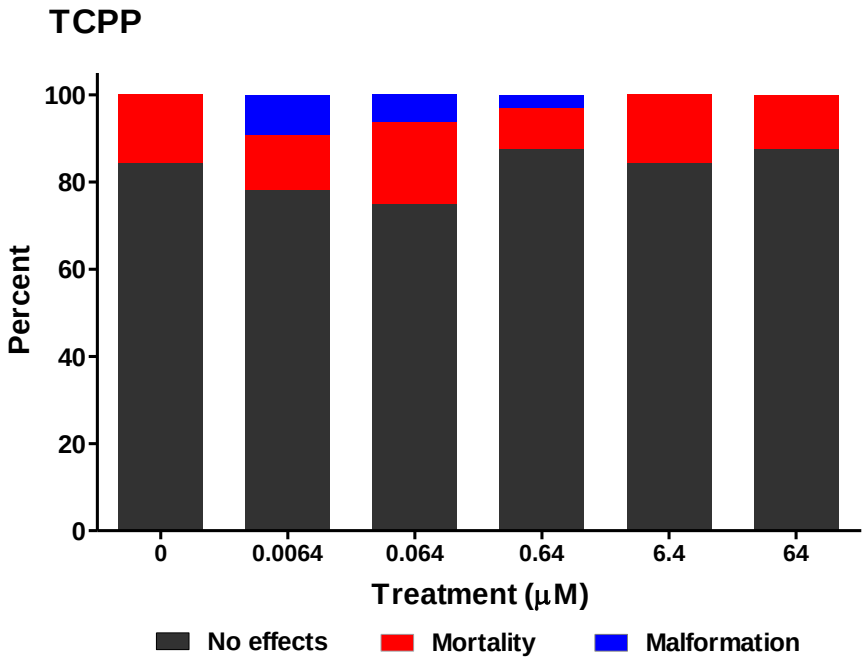
(b) TCPP

DOSE (µM)	MORTALITY	MALFORMATION	NO EFFECT
0	5 (15.62%)	0 (0.00%)	27 (84.38%)
0.0064	4 (12.50%)	3 (9.38%)	25 (78.13%)
0.064	6 (18.75%)	2 (6.25%)	24 (75.00%)
0.64	3 (9.38%)	1 (3.13%)	28 (87.50%)
6.4	5 (15.63%)	0 (0.00%)	27 (84.38%)
64	4 (12.50%)	0 (0.00%)	28 (87.50%)

(c) TCEP

DOSE (µM)	MORTALITY	MALFORMATION	NO EFFECT
0	6 (18.75%)	1 (3.13%)	25 (78.13%)
0.0064	5 (15.63%)	3 (9.38%)	24 (75.00%)
0.064	11 (34.38%)	0 (0.00%)	21 (65.63%)
0.64	6 (18.75%)	2 (6.25%)	24 (75.00%)
6.4	5 (15.63%)	2 (6.25%)	25 (78.13%)
64	15 (46.88%)	16 (50.00%)	1 (3.13%)


TDCPP
Percent
100
80
60
40
20
0
0
0.0064
0.064
0.64
6.4
64
Treatment (µM)
No effects
Mortality
Malformation


TCPP
Percent
100
80
60
40
20
0
0
0.0064
0.064
0.64
6.4
64
Treatment (µM)
No effects
Mortality
Malformation

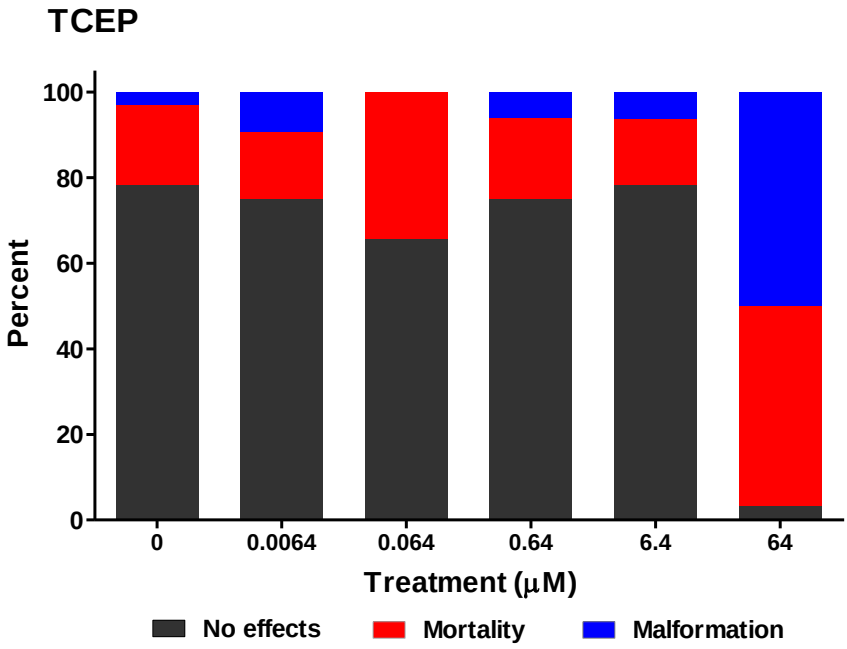


Figure 5. Toxicity Screening Summaries

Locomotor Behavior

Only surviving fish that did not have any noted malformations were included in the analysis of locomotor behavior. Locomotor activity may be indicative of neurotoxicity; however, further testing would need to be performed to determine this. The time series of OPFR impacts on locomotor activity as well as the corresponding Tukey boxplots are presented in figures 6, 7, and 8. The Tukey boxplots show the statistical analysis of the time series data. These boxplots designate the median, 25th and 75th percentiles, along with $\pm$ 1.5 IQRs. The mean is indicated by the plus symbol (+). The red asterisks signify statistical significance where three asterisks denote $p<0.001$, two asterisks denote $p<0.01$, and one asterisk denotes $p<0.05$. The p-values were

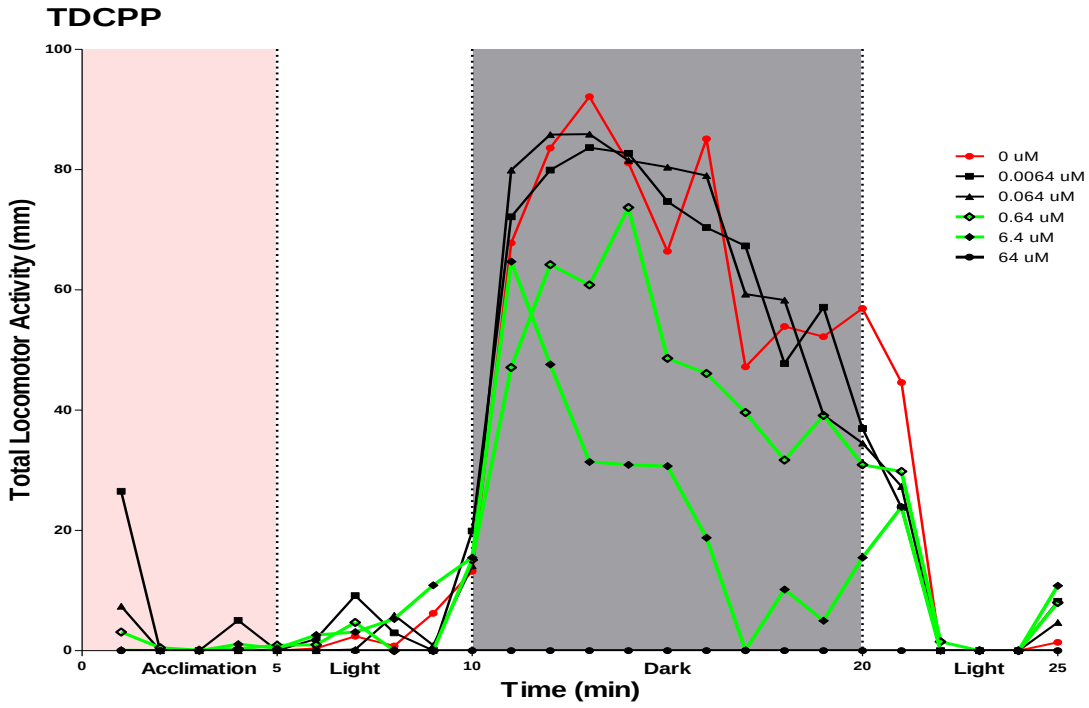
determined using the non-parametric, Kruskal-Wallis test. A red pound sign denotes 100% mortality.

In TDCPP (see figure 6), there is statistically significant deviation ($p<.0.001$), in the form of hypoactivity, from the control in doses of 64µM, 6.4µM, and 0.64µM. However, the 64µM dose had a mortality rate of almost 100% and therefore was unable to be analyzed. The abnormalities occurred during the dark phase.

TCPP (see figure 7) also showed statistically significant abnormalities ($p<0.01$), but they occurred mainly in the first light exposure, and showed hyperactivity. The exposure levels with significant data were 0.064µM, 0.64µM, and 6.4µM. TCPP also had a significant difference ($p<0.001$) in the 64µM dose during the dark phase, and showed hypoactivity.

TCEP did not exhibit much deviation from the control, except for slight hyperactivity ($p<0.05$) in the 0.064µM exposure in the first light phase (see figure 8).

(a)



(b)

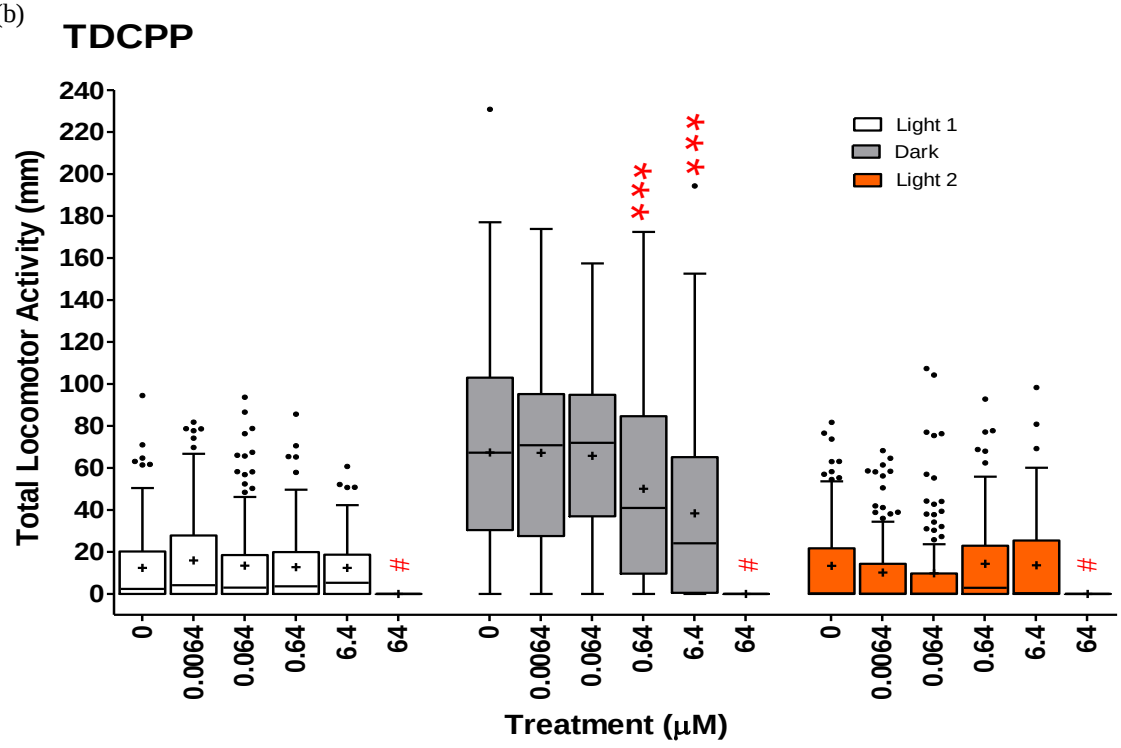
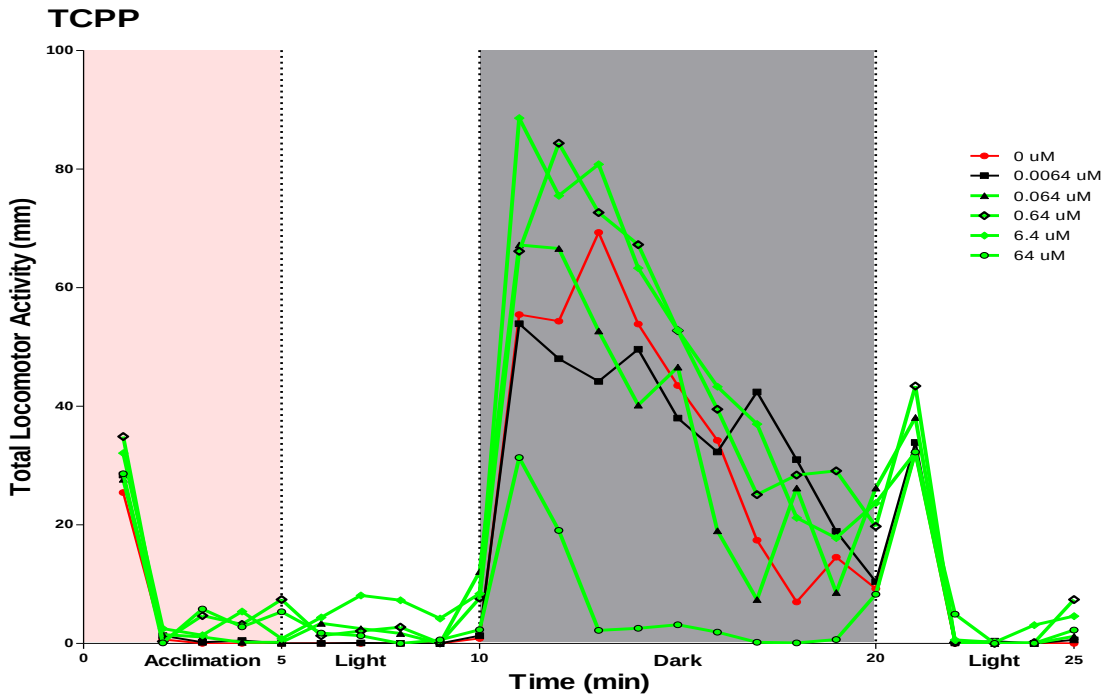


Figure 6. (a) ViewPoint time series measuring locomotor activity in zebrafish exposed to TDCPP and subjected to light and dark stimulation. (b) Tukey boxplots showing statistical significance with exposure to TDCPP

(a)



(b)

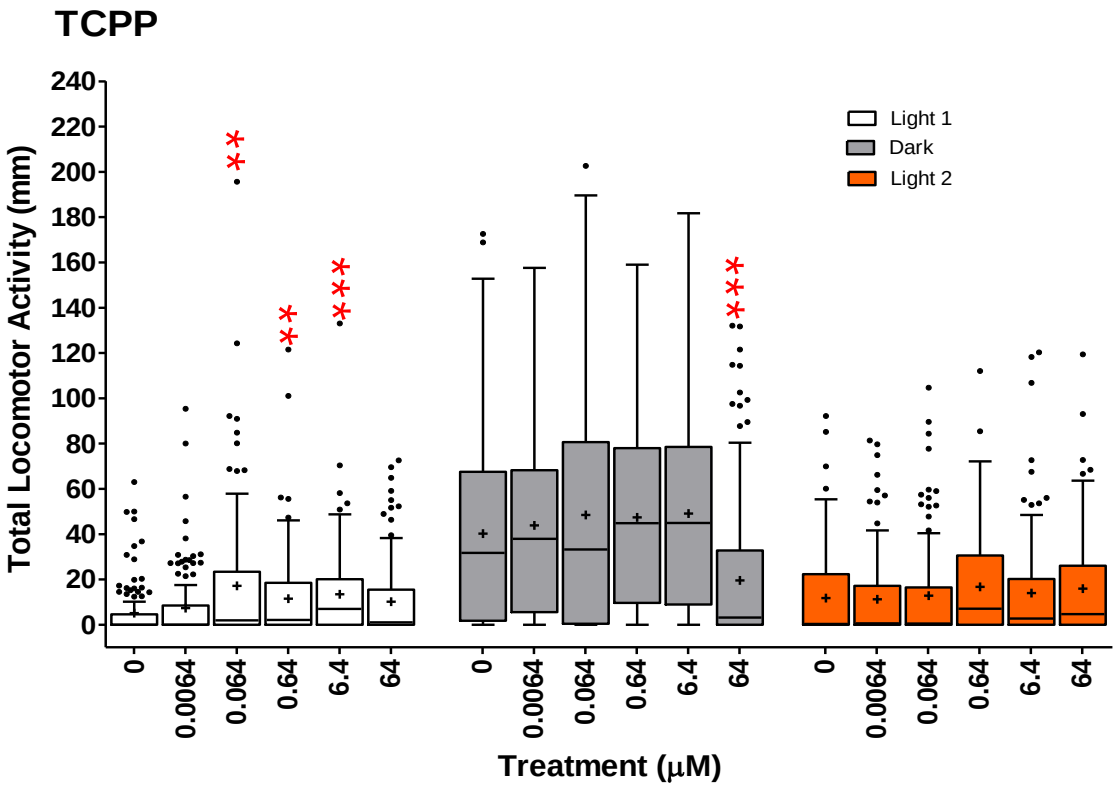
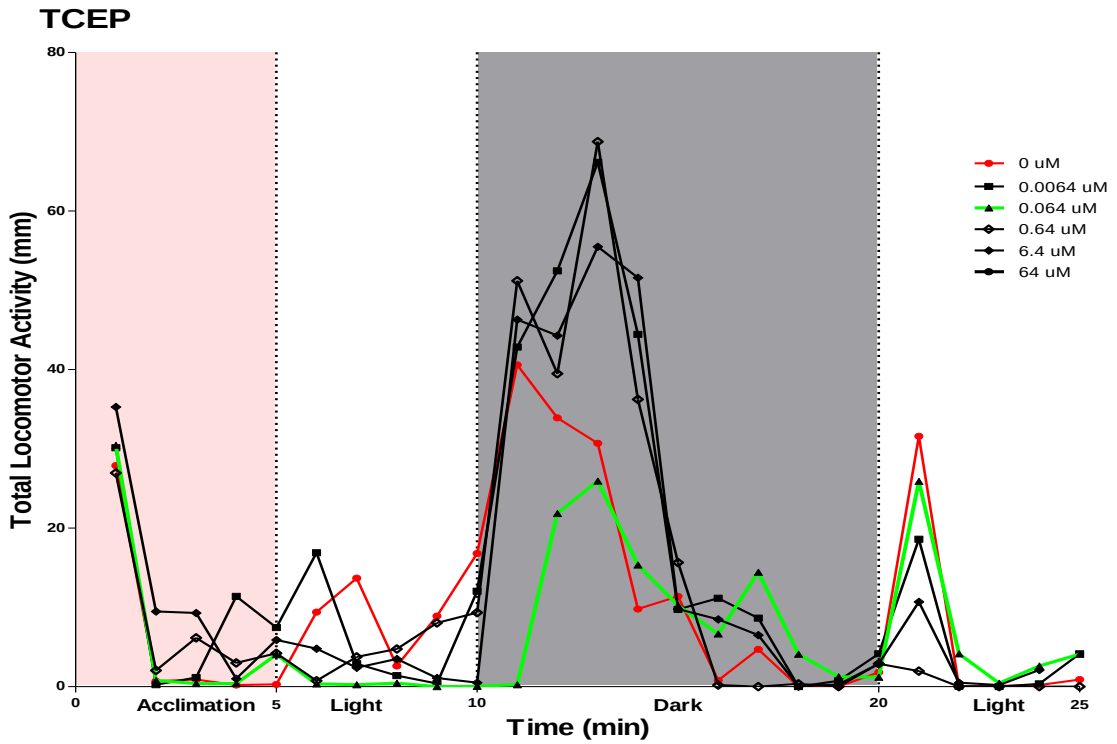


Figure 7. (a) ViewPoint time series measuring locomotor activity in zebrafish exposed to TCPP and subjected to light and dark stimulation. (b) Tukey boxplots showing statistical significance with exposure to TCPP

(a)



(b)

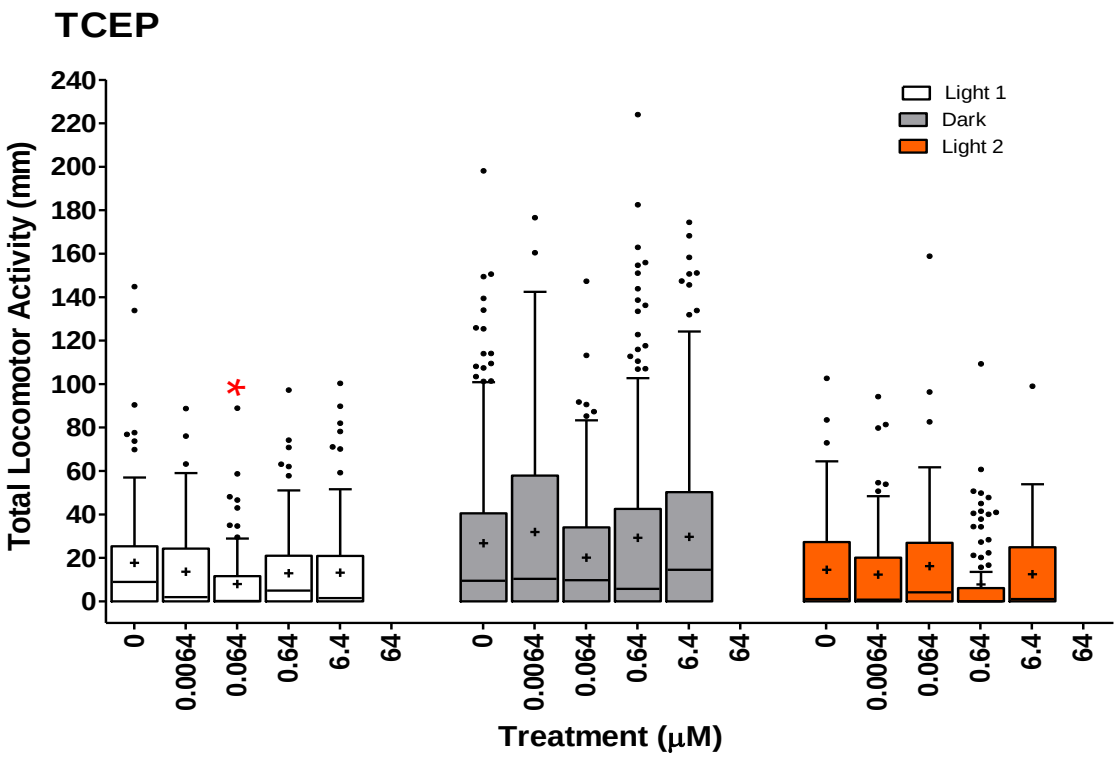


Figure 7. (a) ViewPoint time series measuring locomotor activity in zebrafish exposed to TCEP and subjected to light and dark stimulation. (b) Tukey boxplots showing statistical significance with exposure to TCEP

DISCUSSION

Developmental Toxicity

Other than at the highest doses, TDCPP and TCEP did not cause high mortality. TCPP showed about the same mortality rate regardless of the dose. A limitation of this study was a high background mortality rate. The vehicle control groups (0.64% DMSO) exhibited higher mortality rates than we would have liked to see (6.25% in TDCPP, 15.62% in TCPP, and 18.75% in TCEP), however, the proportions still fall within the accepted range.

When the fish were screened at 120 hpf, they almost always exhibited an all or nothing effect with physical malformations. They either had no physical effect visible, or they exhibited all of the developmental malformations: yolk sac edema, pericardial edema, body axis malformation, caudal fin malformation, pectoral fin malformation, and a muted/absent touch response. This suggests that there is a dose threshold, that when reached, causes all of these developmental malformations to occur, rather than one malformation occurring at one dose and another not being present until a higher dose.

Other studies have also shown no significant effects on embryonic zebrafish survival or development in low doses (doses under 8µM). McGee and coauthors found that zebrafish exposed to TDCPP levels greater than 8µM resulted in a significant increase in mortality and developmental malformations, which is consistent with our data (McGee, et al. 2012). In this same study, no significant effects on mortality or developmental malformations were shown in the highest exposure (50µM) for the chemicals TCPP and TCEP (McGee, et al. 2012). This is also consistent with our data,

and although we had results of high mortality in TCEP, our highest dose was 64µM. This difference may have been enough to elicit the response of a higher mortality rate.

Altered Locomotor Activity

TDCPP, TCPP and TCEP did not have significant impacts on physical development. However, there were significant effects on locomotion, which suggests impaired neurological functioning. Further testing would be necessary to confirm this. Although the fish looked like they were perfectly healthy, they exhibited either hyperactivity or hypoactivity, depending on the chemical.

The expected behavior for a zebrafish is that they will be still in the light, and move in the dark. The hypothesis is that they remain still in the light because they do not want to attract a predator and end up being killed. They then move in the dark when it is more difficult for a predator to detect them.

TDCPP showed significant hypoactive effects on locomotor activity during the dark phase. The fish were not moving around as much as the vehicle controls were when there was no light. Again, further testing would be necessary, but this could have implications in humans. Hypoactivity may be correlated with neurological disorders such as depression or Attention Deficit Disorder (ADD). ADD is a learning disorder that affects how an individual processes and reacts to incoming information or situations in a timely manner. ADD causes affected individuals to react slowly due to the inability to take a thought and quickly convert it into an action (Diamond, 2006). The implications of this include having trouble taking timed tests, having a delay in motor abilities and reflexes, and they may appear to others as being lazy. Diagnosis of hypoactivity can be

made through PET scans, which allows for decreased brain activity to be observed. ADD is associated with an affected frontal lobe of the brain (Diamond, 2006). There are also studies currently being conducted that are looking at a possible association between flame retardants and autism in humans (Redford, 2013).

TCPP showed significant hyperactive effects on locomotor activity during the initial light phase. The fish were moving more than we noted in the vehicle control when light was present. We cannot make a direct translation, without additional testing, to what this means for exposures in humans, but Attention Deficit Hyperactivity Disorder (ADHD) may be correlated with this hyperactive behavior caused by TCPP. ADHD is similar to ADD, except that it has a hyperactivity component as the name suggests. ADHD causes an individual to have trouble focusing, not be able to control behavior, and they are overactive. The hyperactivity leads the affected person to fidget and squirm in their seat, talk excessively, have problems working quietly, and they are often seen as constantly being on the go. Studies of brain scans show that an individual affected by ADHD will have a brain scan that looks different than a scan from a person without ADHD. ADHD is a long-term, chronic condition that can lead to other complications. ADHD is often associated with drug and alcohol abuse, difficulty in school, problems keeping a job, and trouble with the law (Board, 2013).

TCEP had the lowest neurotoxic effects of the three OPFRs in this study. However, relevant studies suggest that TCEP has other toxic effects that were not accounted for in this study—such as cancers, and reproductive effects. Although the exact mechanism is unknown, a potential structure-activity relationship is likely. This is hypothesized due to the fact that both TDCPP and TCPP elicited larger behavioral

effects, and these two chemicals are more structurally similar to each other than they are to TCEP.

Exposure Level in Humans

In the 1980s, TDCPP was detected and measured in human adipose tissue with a maximum level of 257 ng/g (0.5-257) (McGee, et al. 2012). Since the 1980s, TDCPP levels in the environment have been on the rise, which suggests that the current level of TDCPP in the average human is also higher than this value. There is very limited data on the levels of TCPP and TCEP found in the human body. There have been studies that have examined the amount of these chemicals in the surrounding environment, though. In a 2003 study examining levels of OPFRs in dust accumulated on computer screens and computer covers alone, the range of TCPP accumulation was 0.47-73ng/m^2, and the range of TCEP accumulation was 0.19-94ng/m^2 (Marklund, 2003). These values came from various places such as the home, school, and office space. Most people do not stay in one place all day long; therefore, they are likely being exposed to more chemicals everywhere that they go. Although the levels of chemicals in one product may not be sufficient to cause harm, the accumulation of chemicals from multiple products has the potential to be dangerous, and cause both developmental and behavioral effects.

CONCLUSION

Although TDCPP, TCPP, and TCEP have the potential to cause physical malformations, perhaps the bigger issue of this study is the potential neurotoxic effects. Other studies have already shown a link to long term physical effects such as various cancers, but there is now concern for neurological effects as well. This study shows that TDCPP, TCPP, and TCEP elicit behavioral effects, suggesting neurological effects that could be linked to disorders such as ADD and ADHD.

Flame retardants are in multiple products that we are exposed to every day, and they are even in many baby products. Young children are already at an increased risk of exposure because they are often close to the ground where the chemicals accumulate, but babies that are breast fed are at an even higher risk. OPFRs bioaccumulate, and a child that breast feeds will have a level of flame retardant that is three times that of the mother's, which is likely to already be very high (Redford, 2013). With results indicating neurological effects, the question is raised whether these chemicals should still be used on the market.

When deciding if these chemicals should still be used in products, it is important to determine if they are actually doing the job that they are prescribed to do in the first place. Flame retardants are tested in ideal conditions, with only the treated materials (such as the polyurethane foam). Although in these ideal situations studies show that materials treated with flame retardants allow 15 times more time to escape, when you look at these materials in the environment that they are actually used, this is not the case. In the example of couches, only the foam is made with flame retardants. When the couch

is exposed to a flame, the fabric is what will catch on fire first, and although the flame retardants can still slow the process, it is not to the magnitude originally suggested. Other methods have been proposed to slow this process that do not include using flame retardants. For instance, using a thicker fabric that contains a specific groove pattern can affect how fast a product catches on flame. Since flame retardants have been mandated for use in products, it is true that deaths from flames have decreased; however, this is likely due to the new construction legislation requiring sprinklers, an increase in smoke detectors, and the development of self-extinguishing cigarettes (Redford, 2013).

There seem to be few positive effects, yet many negative outcomes of these OPFRs. While flame control is an important issue, we should be spending more time searching for alternative methods that do not include the use of chemicals. Given the ubiquitous nature of OPFRs, it is highly probable that many individuals have some level of exposure to these chemicals and thus have some risk of related adverse health outcomes; this risk may well surpass that of having adverse health outcomes due to a fire.

Future Research

This study has established that there are developmental and neurotoxic effects of TDCPP, TCPP, and TCEP. However, the mechanism remains unknown, and should be further researched. Knowing the mode of action can help determine whether another chemical may be better suited for use as a flame retardant.

The effects of these chemicals were only studied when the zebrafish were exposed at 6 hpf. In the future, studies should be conducted to determine the effects of these chemicals when the zebrafish are exposed at later life stages. This may allow for

predictions of how an adult would react to exposure of these chemicals rather than a child that may be more sensitive to these kinds of chemicals.

Another possibility for future research is changing the animal model. Zebrafish are a great model to use for these types of studies for many reasons. In order to feel even more confident in the comparison of effects to humans, though, studies of these chemicals could be reproduced using other animals such as chimpanzees which share many similarities with humans. Zebrafish are a good model to begin with, but I believe that mammalian models should be the next step now that we have preliminary data. Humans are most closely related to chimpanzees, which is why it would be so beneficial to use them as a model. However, when using higher level vertebrates, such as primates, ethical issues come into play. This is one of the reasons zebrafish have become an increasingly popular model in the first place. Zebrafish are also less expensive than using chimpanzees as a model. The National Institute of Health (NIH) has recently made the decision to retire about 150 of the chimpanzees previously used in research, and that alone has come close to costing thirty million dollars to house them in sanctuaries (Maron, 2013).

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