DEVELOPMENTAL TOXICITY ASSESSMENT OF PERFLUOROALKYL SUBSTANCES (PFAS) USING ZEBRAFISH MODEL SYSTEM

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

School of Health Sciences

West Lafayette, Indiana

August 2022

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To God Almighty, May This work be of use
To The Memory of My Beloved Mother
To My Caring Father, My Husband, and My Lovely Boys,
“Your continuous support made this accomplishment possible”

ACKNOWLEDGMENTS

There are many people who helped me along the way to make this accomplishment true. First and foremost is Dr. Jennifer Freeman. Dr. Freeman, thank you for all you did for me. Your continuous support and guidance were critical throughout my graduate education. Thank you for all the opportunities you gave me to be a better scientist and a competent toxicologist. I couldn't think of a better experience than what I had with you and in your laboratory.

To my committee, Dr. Linda Lee, Dr. Maria Sepulveda, and Dr. Aaron Bowman, thank you for your great support. Your thoughtful feedback and valuable input helped me enhance my research and expand my knowledge.

I am grateful for the help and training I received from Dr. Katharine Horzmann and Kathryn Thompson. I enjoyed being in the lab with you, and learned a lot from you. You both set an ideal model for how cooperative lab mates should be.

A special thank you to my lab mate, Keturah Kiper, thank you for your support through the past couple of years. We passed many of the milestones towards our degrees together. It was pleasure having you in the lab.

I want to thank all Freeman lab members, Brian Sumprer, Arjun Pitchai, Dr. Janiel Ahkin Chin Tai, Athira Nabendhu, Sydney Stradtman, Hanna King, Jenna Nicole Swihart, Karthik Chelladurai, and Vasisht Varsh Sridhar. Thank you all for your cooperation and support.

I wanted to thank Dr. Youn Jeong Choi for her great assistance in the analytical assessments.

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ABSTRACT

Perfluoroalkyl substances (PFAS) are synthetic chemicals that are composed of fluorinated aliphatic chains and widely used in industrial and consumer products. These chemicals are very stable and persist in the environment. Due to concerns linked with longer chain PFAS, shorter chain chemicals are being used as replacements. There are limited human health data regarding the shorter chain chemicals. In addition, these alternatives are persistent in the environment similar to the longer chain PFAS. The main objective of this dissertation was to assess developmental toxicity of the shorter chain PFAS or shorter chain PFAS with chemical modifications represented by perfluorobutanoic acid (PFBA, C4), perfluorohexanoic acid (PFHxA, C6), perfluorobutane sulfonate (PFBS, C4), and perfluoro-2-proxypropanoic acid (GenX, C6).

The first project (Chapter 2) aimed to compare the toxicity of four perfluoroalkyl acids (PFAAs) as a function of chain length and head group (carboxylate versus sulfonate) with in vitro (using zebrafish fibroblasts) and in vivo (zebrafish embryos/larvae). We determined the lethal concentration needed to kill 50% of the fish (LC50) for perfluorooctanoic acid (PFOA), PFHxA, and PFBA to assess effects of chain length. PFBS was included to test for the effects of a sulfonate group. The results revealed that as chain length increases, mortality increases. Also, the presence of a sulfonate group increased toxicity for PFAS of the same chain length, which was indicated by higher mortality observed in PFBS treatments compared to PFBA. The same toxicity trends were observed in vivo and in vitro; however, zebrafish cells were more sensitive to PFOA, PFHxA, and PFBA exposures than the whole animal.

The second study (Chapter 3) focused on assessing chain length on sublethal effects including neurobehavior, morphology, and transcriptomic alterations. We exposed zebrafish embryos to sublethal concentrations at 0, 4, 40, or 400 parts per billion (ppb; µg/L) of PFOA, PFHxA, or PFBA at 1 hour post fertilization (hpf) until the end of embryogenesis (72 hpf). Morphological measurements (whole body length, head width, head length) and transcriptomic analysis were performed at 72 hpf, while neurobehavior was assessed at 120 hpf using the visual motor response assay. Results indicated that PFOA and PFBA exposures induced behavioral changes, while PFHxA did not, indicating that chain length is not the driving factor for the

behavioral alterations observed. PFOA, PFHxA, and PFBA exposure resulted in morphological alterations that were unique for each chemical and were also concentration dependent. Gene ontology analyses via Ingenuity Pathway Analysis (IPA) revealed that each chemical has a unique transcriptomic profile. There were some similarities between the highly enriched altered molecular pathway associated with diseases in the tested exposures to the known effects of these chemicals in other animal models, highlighting the validity of these transcriptomic analysis for predicting toxic effects of PFAS.

The last chapter (Chapter 4) aimed to assess tissue uptake, developmental toxicity, and developmental neurotoxicity of two emerging PFAS: PFBS and GenX. PFBS is a perfluoroalkyl sulfonic acid (PFSA), whereas GenX is a perfluoroalkyl ether carboxylic acid (PFECA). LC50s were determined at 120 hours post fertilization (hpf). PFBS was more acutely toxic than GenX, which indicates that the presence of a sulfonate functional group has more influence in mortality compared to chain length since GenX has 6 perfluorocarbons and PFBS has 4 perfluorocarbons. The tissue uptake analysis showed that PFBS accumulated more than GenX at the same nominal exposure concentration, emphasizing the importance of measuring the internal dose when comparing the toxicity of PFAS. Both GenX and PFBS caused hyperactivity at 120 hpf, but the behavioral alterations were more persistent for PFBS among phases and occurred even at the lowest tested exposure concentration of 4 ppb. Given the current evidence on the potential effects of PFAS exposure on dopaminergic neuron development, dopamine levels and gene expression of genes involved in dopaminergic signaling were assessed after sublethal embryonic exposure to GenX or PFBS. At 72 hpf, an increase in dopamine level was seen at 40 ppb GenX, while a decrease was observed at 400 ppb PFBS; however, no significant changes were observed in expression of monoamine oxidase (*mao*), dopamine transporter *(dat*), or tyrosine hydroxylase (*th*). Further molecular and mechanistic assessment is needed to understand the observed patterns.

Overall, the results showed that chain length and functional group are determinants of toxicity of PFAS. All tested PFAS induced one or more developmental adverse outcome, but the effects of each chemical are unique, warranting further studies to address the toxicity of the replacement PFAS.

CHAPTER 1 : INTRODUCTION

1.1 Zebrafish Model: An Alternative Vertebrate Model

The zebrafish (*Danio rerio*) is a tropical fish native to India and other areas in southern Asia. It is widely used as a complementary vertebrate model for developmental biology and toxicology (de Esch et al. 2012; Scholz et al. 2008). Zebrafish has many unique features, which allows for their use as an alternative model. The development of zebrafish is rapid, where embryos hatch at two-three days post fertilization (dpf), embryogenesis is complete at three dpf, and at five dpf organogenesis of all organs is completed. They are sexually mature by three to four months of age (Kimmel et al. 1995).

The high fecundity rate and *ex vivo* fertilization allows for treating hundreds of embryos with chemicals of interest and assessing developmental toxicity with avoiding confounding effects that may occur through maternal exposures in mammalian models. The zebrafish genome is fully sequenced, and it has been shown that 70% of the human genome has at least one orthologue in zebrafish (Howe et al. 2013). In addition, 82% of genes associated with diseases in humans have at least one zebrafish orthologue (Howe et al. 2013). This similarity makes the zebrafish an ideal model for identifying effects of environmental toxicants on the molecular levels and ability to translate the observed effects to humans. Additional attributes including that the developmental signaling pathways are conserved in vertebrates, the hepatic metabolism in zebrafish is like humans, and similarity of the zebrafish endocrine and central nervous systems to humans are features that have allowed for the use of zebrafish in toxicological assessments (Horzmann and Freeman 2016; Nawaji et al. 2020; Padilla et al. 2022). In addition, zebrafish are small and can easily be maintained in a laboratory setting with the available information on raising them allowing for high-throughput analysis (Westerfield 2000).

1.2 Perfluoroalkyl Substances (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are a class of man-made chemicals that are used in consumer and industrial applications. The perfluoroalkyl substances are composed of fully fluorinated carbon chains, while the polyfluoroalkyl substances are composed of partially

fluorinated carbon chains (Kissa E. 2001). Their unique characteristics of being hydrophobic and hydrophilic allowed for this wide use of PFAS including thousands of chemicals that are composed of fluorinated carbon chains. Due to the strength of the carbon-fluoride bond, these chemicals are resistant to photolysis, biodegradation, photooxidation, and hydrolysis (Gomis et al. 2018), resulting in their persistence in the environment and bioaccumulation in humans and wildlife (Giesy and Kannan 2001; Li et al. 2018). PFAS were detected in humans in 98% of the tested samples in US (Calafat et al. 2007).

PFAS chemicals vary in their chain length, presence of side chain, and the functional group. The long chain PFAS include perfluoroalkyl carboxylic acids that have more than 6 carbons and the perfluoroalkyl sulfonic acids that have more than 7 carbons. Perfluorooctanoic acid (PFOA) and perfluoroalkyl octane sulfonic acid (PFOS) are the most commonly known PFAS. PFOA and PFOS have long half lives in humans (Olsen et al. 2007). PFAS are linked with many adverse health effects in humans including thyroid disease, increased cholesterol levels, breast cancer, liver damage, kidney cancer, testicular cancer, pregnancy induced hypertension, and suppression of immune response. In addition, many developmental effects are linked to PFAS exposure in the unborn child including lower birth weight, early onset of puberty, low sperm count and mobility, obesity, and reduced response to vaccines [reviewed by (Fenton et al. 2021)].

To address the concern regarding the persistence of PFAS and their potential adverse effects on humans, chemicals with shorter alkyl chains [(e.g., perfluorohexanoic acid (PFHxA, C6), perfluorobutanoic acid (PFBA, C4), and perfluorobutane sulfonic acid (PFBS)] or compounds with one or more ether group [e.g., perfluoro-2propoxypropanic acid (GenX, C6)] replaced long chain chemicals (e.g., PFOA and PFOS). The goal of the replacement strategy was to increase degradability of these chemicals by shortening the chain length or introducing a weaker point (ether linkage) to reduce their persistence. Unfortunately, these replacements are also being detected in the environment (Scher et al. 2018; Strynar et al. 2015). However, they do have shorter half-lives in the body compared to the long chain PFAS, but are still resistant to degradation similar to PFOA and PFOS (Wang et al. 2015).

Relatively limited information is available about the safety of these replacements and there is evidence that indicates they have adverse health effects similar in nature to those elicited by long chain PFAS. For example, animal studies showed that PFHxA is associated with hematopoietic effects, hepatotoxicity, and developmental toxicity (Usepa and Iris 2022); GenX

caused hepatoxicity and immunotoxicity in rats; and PFBS is associated with thyroid hormone disruption (US EPA 2018). These results highlight the importance of studying these chemicals further to better understand their toxicity profile. Studies on neurotoxicity and developmental neurotoxicity for these chemicals are lacking. Given the evidence that PFOA and PFOS, and other long chain PFAS may cause neurotoxicity and developmental neurotoxicity (reviewed in Foguth et al. 2020; Mariussen 2012), it is highly important to address the neurotoxicity of the short chain PFAS.

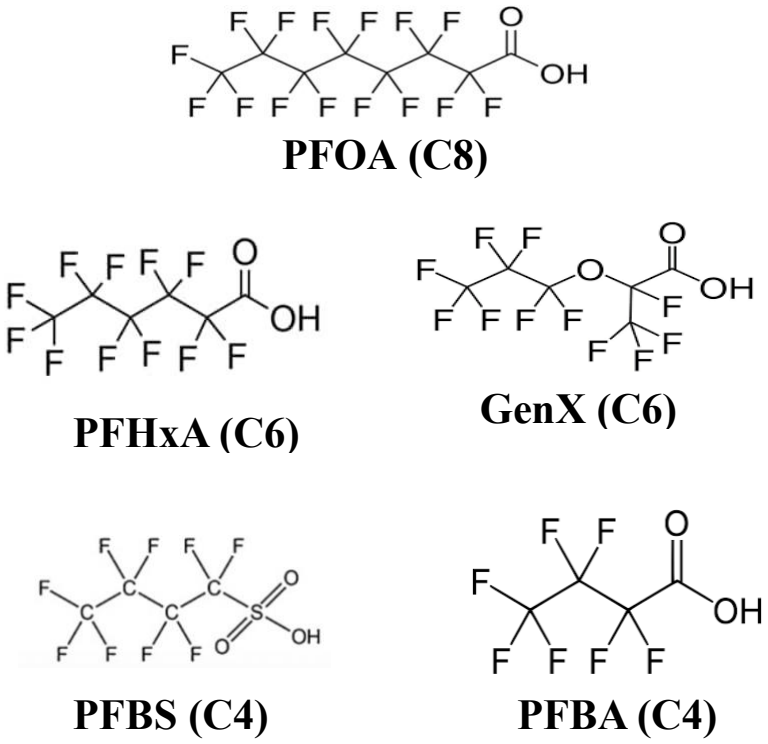

Figure 1-1: Chemical structure of PFOA, PFHxA, PFBA, PFBS and GenX.

1.3 Neurobehavior Assessment in Zebrafish

There are differences in the anatomical structure of the brain in zebrafish, humans, and other mammals, but there is a high degree of functional homology that allows for the use of zebrafish to be used in the field of neuroscience (Rink and Wullimann 2004). Zebrafish larvae develop mature swimming behavior at 5 dpf, when the swim bladder development is completed

(Kimmel et al. 1995b). Larval and adult zebrafish behavioral responses are well characterized (Kalueff et al. 2013), Assessing neurobehavior in zebrafish is a useful tool to evaluate neurotoxicity of environmental chemicals and drugs. In addition, assessing larval behavior can indicate abnormal development. A study reported that behavior evaluation of some chemicals showed abnormal behavior at lower concentrations than that, which causes morphological changes (Reif et al. 2016), indicating that behavioral analysis is a sensitive tool to predict developmental toxicity. This advantage is very important to evaluate toxicity of chemicals with environmentally relevant concentrations. Behavioral assays using zebrafish were used to assess multiple behavioral endpoints such as thigmotaxis, scototaxis, geotaxis, habituation, sleep/awake behavior, and startle response (reviewed by Basnet et al. 2019).

The visual motor response test is used to assess locomotor activities in response to a visual stimulus. In this test, zebrafish larvae are exposed to alternative periods of dark and light. Normal larvae have a unique pattern of movements during these lighting phases, where the movement of the larvae decreases when transitioning from dark to light and movements increases in light to dark transitioning. Exposing zebrafish larvae to neuroactive drugs causes altered responses in the visual motor response test similar to the known effects of these drugs in humans. For example, a study was performed to compare the effects of dopamine (DA) receptor agonists and antagonists on zebrafish to their effects in mammals (Irons et al. 2013). Larval zebrafish exposed to DA agonist, SKF-38393 (D1 agonist), quinpirole (D2-agonist), or apomorphine at 144 hpf showed hyperactivity in the light/dark locomotor activity test, while exposure to DA antagonists, haloperidol (D2-antagonist), SCH-23,390 (D1-antagonist), or butaclamol caused a decrease in locomotor activity. Selective receptor agonists (SKF-38393, quinpirole) and selective antagonists (haloperidol, SCH-23,390) showed a dose dependent decrease in locomotor activity, while non-selective receptor agonists and antagonists caused a biphasic behavioral pattern. These results showed that zebrafish behavior can predict adverse responses to a chemical exposure. The main limitation of behavioral analysis using the zebrafish is the absence of a standardized method for conducting the experiments, which adds a challenge in comparing results between studies.

1.4 PFAS Neurotoxicity

There are limited data on neurotoxicity of PFAS. Two epidemiological studies showed a protective effect of PFAS exposure to cognition functions in adult humans (Gallo et al. 2013; Power et al. 2013). These unexpected results were explained by poor assessment (most results were self-reported), confounding factors such as kidney diseases and fish consumption, and potential non-monotonic dose response of PFAS (Park et al. 2021). It is important to note that PFAS are endocrine disruptors. A non-monotonic dose response is often seen for endocrine disruptors and may be observed in PFAS studies, which can complicate findings in epidemiological studies (Vandenberg et al. 2012). As such, epidemiological studies assessing behavioral and learning outcomes in children report conflicting results. One study showed an increase in risk of attention deficient/hyperactivity disorder (ADHD) and reduced executive functioning with PFAS exposure (Oulhote 2016), while another study showed no association between PFAS exposure and behavioral outcomes and motor coordination (Liew et al. 2018). These findings, along with studies showing that PFAS accumulate in the brain of humans and other animals substantiate a need to further address risks of PFAS neurotoxicity. For example, a study reported accumulation of PFOA, PFHxA, and PFHxS in the brain stem of autopsy samples from residents of a PFAS polluted area in Italy (di Nisio et al. 2022). This study also reported decreased expression of tyrosine hydroxylase, neurofilament heavy, and dopamine transporter in human dopaminergic neurons obtained by differentiating human induced pluripotent stem cells (hiPSCs) that were exposed to 10 ng/ml PFOA for 24 h indicating PFAS may target the dopaminergic system (di Nisio et al. 2022).

Animal studies provide a more controlled option to study PFAS neurotoxicity and further assessment of potential non-monotonic dose response. In rodent studies, mice exposed to 0.3 mg/kg of PFOA or PFOS during pregnancy resulted in accumulation of PFOS and PFOA in the brain of the offspring. Furthermore, PFOS exposure was associated with decreased locomotion in a novel environment in male offspring, while PFOA exposure was associated with alterations in exploratory behavior in male and female offspring (Onishchenko et al. 2011). Another study showed that exposure of neonatal mice to 21 mol/kg of PFOA or PFOS caused a significant increase in proteins that are important in normal brain development such as CaMKII, GAP-43, and synaptophysin (Johansson et al. 2009). These studies demonstrate potential for PFAS exposure to result in altered behavior and brain development.

To date, alterations in intracellular calcium in neurons and alterations in neurotransmitters are the two main studied potential mechanisms of PFAS-induced neurotoxicity (Li et al. 2021). Perturbations to dopaminergic gene transcription (tyrosine hydroxylase and dopamine transporters) in the mouse cerebral cortex and hippocampus with a postnatal PFOS exposure in mice was reported (Hallgren and Viberg 2016), while PFOS exposure in male rats to 0.5, 1, 3, or 6 mg/kg/day for 28 days caused changes in dopamine levels and alteration in dopamine turnover due to alterations in expression of dopamine receptors D1 and D2. These changes were found to be brain-region dependent with alterations in hypothalamus-pituitary-adrenal (HPA) axis activity also suggested (Salgado et al. 2016). In addition, PFAS exposure affected neuronal differentiation in PC12 cells with specific alterations for each PFAS including shorter chain PFAS. PFOS exposure induced differentiation of cholinergic neurons at the expense of the differentiation of dopaminergic neurons, PFBS suppressed differentiation of both neurons, and PFOA exposure had no effect on differentiation (Slotkin et al. 2008).

Multiple studies using the zebrafish model system showed that PFAS exposure led to behavioral changes in larvae and that these changes were associated with dopaminergic alterations. Developmental exposure to 1 mg/L PFOS caused alterations in spontaneous swimming activity in 6 day old larvae were less frequent, but more intense bouts of activity were observed (Spulber et al. 2014). In addition, treatment of PFOS-exposed larvae to 1 μm dexamfetamine, a catecholamine reuptake inhibitor, caused an increase in the frequency of bouts of spontaneous activity and reduced the activity within the bout. Additionally, in the 1 mg/L PFOS-exposed larvae treatment co-exposed with apomorphine, a non-specific dopamine agonist, caused activation of D1 and D2 receptors; treatment with quinpirole, a specific agonist for D2 receptors, increased the frequency of spontaneous swimming bouts; and treatment with skf-81297, a specific agonist for D1 receptors, reduced the within-bout activity. Altogether, this study showed that the observed alterations in swimming activity was associated with dopaminergic deficits. Similarly, another study showed that developmental exposure to 1, 10, or 100 μg/L PFOA decreased distance travelled with this decrease in activity associated with changes in expression of dopaminergic-related genes and genes involved in dopaminergic neurons development (Yu et al. 2021).

Additional studies in other models also report changes in the dopaminergic system. Northern leopard frogs with a developmental exposure to PFOA (1000 ppb) or PFOS (100 and 1000 ppb) were observed to have decreased dopamine and dopamine turnover (Foguth et al. 2019).

Furthermore, alterations in neurotransmitter levels were reported with PFOS exposure in planarians (Yuan et al. 2018). Dopaminergic neuropathology in C. elegans with a 72 hour PFOS exposure was also noted (Sammi et al. 2019). This study showed that dopaminergic neurons were more sensitive to neurodegeneration following PFOS exposure (i.e., dopaminergic neurodegeneration was observed at 25 ppm PFOS) compared to GABAergic, serotonergic, or cholinergic neurons (which were only affected at 100 ppm PFOS). Interestingly, this study showed that PFOA exposure did not affect dopaminergic neurodegeneration, while GenX exposure caused dopaminergic neurodegeneration at 200 ppm treatment. This study highlighted the potential selective effect of PFAS exposure on dopaminergic neurons and potential neurotoxicity of short chain PFAS such as GenX. The majority of studies have focused only on PFOA and PFOS, ignoring the potential neurotoxicity of emerging PFAS. The few studies that have included the shorter chain PFAS indicate they can cause similar adverse health effects that legacy longer chain PFAS induce; thus, neurotoxicity of shorter chain PFAS should be investigated. Furthermore, from the aforementioned studies, effects of shorter chain PFAS on the dopaminergic system is an important endpoint to investigate.

1.5 Dopaminergic Neurons in Zebrafish

Neurotransmitter systems are conserved between zebrafish and mammals allowing translation of mechanisms of neurotransmission alterations and associated developmental and disease pathways (Horzmann and Freeman 2016). The dopaminergic system in zebrafish is well characterized and completely developed by 96 hours post fertilization (hpf) (Rink and Wullimann 2002). DA neuronal populations in zebrafish larvae that resemble the human substantia nigra have been identified, supporting the zebrafish as a model to predict effects of toxicants on the dopaminergic system.

1.5.1 Dopamine Signaling in Zebrafish

DA is a catecholamine neurotransmitter synthesized by oxidation of the amino acid tyrosine. Tyrosine hydroxylase (TH) converts tyrosine to dihydroxyphenylalanine (L-DOPA), which is converted to dopamine by the action of aromatic amino acid decarboxylase (AADC) (Figure 1.2). DA is a precursor of other catecholamine neurotransmitters, noradrenaline and

adrenaline. Oxidation of DA with DA β hydroxylase results in formation of noradrenaline. There are five families of dopaminergic receptors. Those receptors are G-protein coupled receptors and are classified into two types according to their effect on adenylate cyclase. D1-like receptors (D1 and D5) activate adenylate cyclase and the downstream targets, while D2-like receptors (D2, D3, and D4) inhibit adenylate cyclase and the downstream targets (Missale et al. 1998). In the central nervous system, DA is transported from the cytoplasm into secretory vesicles by vesicular monoamine transporter (VMAT2) [also known as solute carrier family 18 member 2 (SLC18A2)] (Figure 1.2) (Benarroch 2013). Dopamine uptake from the synapse occurs by presynaptic dopamine transporters called solute carrier family 6 member 2 (SLC6A2) [also called dopamine transporter (DAT)]. SLC6A2 transports dopamine from the synaptic cleft back to the cytosol, which is influenced by an ion concentration gradient generated by the plasma membrane Na^+/K^+ ATPase (Torres and Gainetdinov 2003). Then, dopamine can be packed in the synaptic vesicles or metabolized. Dopamine is metabolized by monoamine oxidase (MAO) in the cytosol into 3,4-dihydroxyphenylacetic acid (DOPAC) (Figure 1.2). Mammals have two isozymes, MAO-A and MAO-B. MAO in zebrafish is similar to MAO-A (Sallinen et al. 2009a). MAO-B is mainly responsible for degradation of serotonin. In mammals, dopamine can be degraded in the synaptic cleft by catechol-O-methyltransferase (COMT) into 3 methyltyramine (3MT) and converted to homovanillic acid by MAO. There are two forms of *COMT* genes in zebrafish, *comta* and *comtb*. The role of COMT in metabolizing dopamine in zebrafish is not well understood, but Sallinen et al. (2009) suggested that the presence of 3MT, the product of dopamine methylation, is evidence of the role of COMT in metabolizing dopamine. Another study showed that *comta* mRNA expression was abundant in the gut, gills, and spleen, while *comtb* mRNA expression was abundant in the liver and brain, highlighting that *comtb* is more relevant to central nervous system than *comta* (Semenova et al. 2017). Although COMT inhibition didn't result in an increased level of dopamine in larval zebrafish, an increase in DOPAC was observed, indicating activation of oxidative metabolism (Semenova et al. 2017). D2 autoreceptor plays a role in inhibiting neurotransmission by a negative feedback mechanism. Horzmann and Freeman (2018) discussed in detail the comparison between genes involved in catecholamine neurotransmission in zebrafish and human genes (Horzmann and Freeman 2016). Neurons that express *th* and don't express *β hydroxylase* are considered dopaminergic neurons (Guo et al. 1999). Holzeschuh et al. (2001) suggested that *dat* expression distinguishes dopaminergic neurons from noradrenergic and

adrenergic neurons (Holzschuh et al. 2001). This study showed that neurons that express *th* and don't express *dat* are adrenergic and noraderengic and are found in the locus coeruleus and medulla. It was proposed that zebrafish have another TH-encoding gene, *th2*, but it was found that this gene encodes for tryptophan hydroxylase and should be used as a marker for serotonin neurons (Ren et al. 2013).

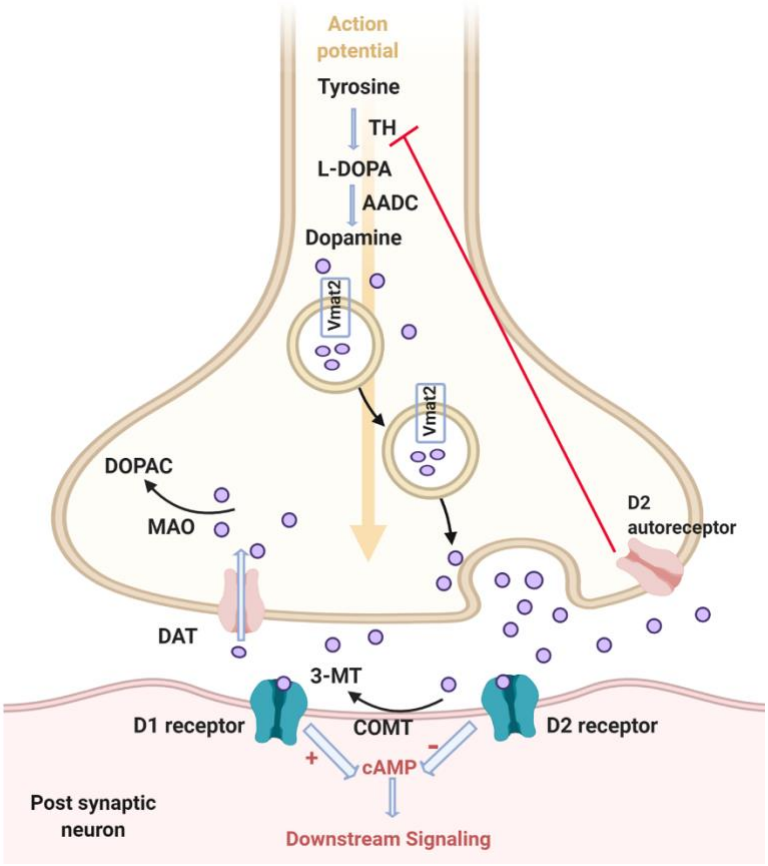

Figure 1-2: Dopamine signaling pathway. Tyrosine is converted to L-DOPA by the rate limiting step enzyme, tyrosine hydroxylase (TH). Then dopamine is synthesized by action of the aromatic amino acid decarboxylase (AADC). Dopamine is packed in the cytosol via vesicular monoamine transporter 2 (Vmat2). Dopamine is then released from vesicles into the synaptic cleft in response to an action potential. Dopamine can either bind to D1 receptor and activate adenylate cyclase and consequently activate downstream signaling through cAMP or bind D2 receptor and inhibit adenylate cyclase and downstream signaling. Reuptake of dopamine from the synaptic cleft to the cytosol occurs through the dopamine transporter (DAT). Then dopamine can either be degraded to 3,4-dihydroxyphenylacetic acid (DOPAC) via monoamine oxidase (MAO) or repacked in vesicles via Vmat2. Dopamine can also be degraded in the synaptic cleft via catechol-O-methyltransferase (COMT) to 3 methyltyramine (3MT). Extracellular dopamine can bind to the D2 autoreceptor, which inhibits the synthesis of dopamine. Created with BioRender.com.

1.5.2 Distribution of Dopaminergic Neurons in the Zebrafish Brain

Catecholaminergic (CA) populations in zebrafish larvae were determined by several groups, but different nomenclature was used to identify them (Schweitzer et al. 2012). Rink and Wullimann (2002) classified CA populations in different brain areas in larval zebrafish. The telencephalon contains CA populations in the olfactory bulb (OB), and subpallium (SP).

According to this study, the CA populations in the diencephalon were identified to 8 populations, DC1-DC8. The rhombencephalon has CA in the preoptic area (PO), locus coeruleus (LC), medulla oblongata (MO), and area postrema (AP). Sallinen et al. (2009) classified CA populations into 17 populations (populations 1-17). The midbrain of zebrafish doesn't have any DA neurons, in contrast to the mammalian brain. It has been found that larval DA neurons in DC1, DC2, and DC4 (ventral thalamic and posterior tuberculum in adult brain) are sending projections to the subpallium in adult zebrafish. This pathway is hypothesized to be homologous to DA neurons in the substantia nigra and ventral tegmental area (DA groups A9-A10, respectively) that projects to the striatum in the mammalian brain(Rink and Wullimann 2001). Du et al. (2016) found that axons from the periventricular nucleus of the posterior tuberculum projected to the subpallium in 120 hours post fertilization (hpf) larvae(Du et al. 2016). Lam et al. (2005) observed that an embryonic exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreased the number of DA neurons in the diencephalon in zebrafish embryos. Also, 48 hpf embryos treated with MPTP showed a weak reflex in response to touch stimuli compared to control fish. In addition, the decrease in DA neurons in the diencephalon are reversed in MPTP-treated embryos by deprenyl, a monoamine oxidase β inhibitor that inhibits conversion of MPTP to its metabolite MPP^+ (1-methyl-4-phenylpyridinium) (Lam et al. 2005). Sallinen et al. (2009) showed that populations 5, 6, and 11 (DC2 and DC4, according to Rink and Wullimann's nomenclature) in 120 hpf zebrafish larvae were sensitive to MPTP and MPP^+, which induced a transient decrease in tyrosine hydroxylase-positive cells and a decrease in swimming activity (Sallinen et al. 2009b). Those studies confirm that populations 5, 6, and 11 (preoptic, thalamic, and posterior tuberculum DA neurons in the adult zebrafish brain) in the diencephalon represent the mammalian midbrain DA neurons that are sensitive to MPTP and the loss of DA neurons caused motor deficits. Another study suggested that DA clusters DC2 and DC4 in zebrafish larva (periventricular posterior tuberculum and posterior tuberculum in adult brain) are corresponding to DA in the caudal diencephalon of mammals (A11) (Tay et al. 2011). This study showed that differentiation of DA neurons in the periventricular posterior tuberculum and posterior tuberculum was dependent on the transcription factor Orthopedia (*otp*) and sent ascending projections to the ventral diencephalon and subpallium and descending projections to the pretectum, tectum, thalamus, hypothalamus, hindbrain, and spinal cord. Mammalian population A11 is also dependent on *OTP* expression and sends ascending neocortical and descending diencephalospinal projections (Ryu 2007). Lambert et al. (2012) found

that the dopaminergic otp-dependent neurons are mediating the development of locomotor activity via spinal dopamine receptor D4 (D_4R) signaling (Lambert et al. 2012). This study agrees with Thirumalai et al. (2008) who saw differential effects of dopamine during development. They reported that dopamine suppressed spontaneous swimming via D2 receptors at 72 hpf, but this inhibition of activity didn't occur in 120 hpf larvae (Lambert et al. 2012). It was observed that the number of neurons in the periventricular of posterior tuberculum increased from 24 to 72 hpf and then decreased from 72 to 120 hpf (Du et al. 2016).

1.6 Central Hypothesis and Specific Aims

The central hypothesis tested in this dissertation is that an embryonic exposure to PFAS can cause developmental toxicity and developmental neurotoxicity. This toxicity depends on the chemical structure of PFAS (length of the carbon chain, presence of ether linkage and the functional head group). To test this central hypothesis, three specific aims were developed.

Specific Aim 1 (Chapter 2) was designed to compare the toxicity of PFOA, PFHxA, PFBA, and PFBS in a zebrafish embryonic cell line and in whole larvae. This aim tests the hypothesis that mortality rate will increase with increase in carbon chain length, numbers of perfluorocarbons, and presence of sulfonate group and toxicity ranking will be similar in zebrafish cells and larvae. As the number of fluorinated carbon increases, bioaccumulation of these chemicals increases, thus causing more toxicity. In addition, PFAS-containing sulfonate group accumulate more than PFAS-containing carboxylate group, which also may lead to higher toxicity.

Specific Aim 2 (Chapter 3) was designed to compare the developmental neurotoxicity and transcriptomic alterations of PFOA, PFHxA, and PFBA in zebrafish eleuthero-embryos and larvae. This aim tests the hypothesis that embryonic exposure to PFOA, PFHxA, or PFBA results in developmental neurotoxicity that is dependent on the carbon chain length. Developmental neurotoxicity will be assessed by behavior changes.

Specific Aim 3 (Chapter 4) was designed to assess developmental neurotoxicity of PFBS and GenX. This aim tests the hypothesis that embryonic exposure to PFBS or GenX, PFOS and PFOA replacements, respectively, results in developmental neurotoxicity and alterations in the development of dopaminergic neurons. The majority of studies that assessed neurotoxicity of PFAS focused on long chain PFAS. This aim will assess developmental neurotoxicity and effects

on dopaminergic neuron development after exposure to GenX or PFBS, two emerging short chain PFAS due to the current evidence that the dopaminergic system can be selectively affected by PFAS exposure.

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CHAPTER 2 : COMPARISON OF ZEBRAFISH *IN VITRO* AND *IN VIVO* ASSESSMENTS OF PERFLUOROALKYL SUBSTANCES (PFAS) DEVELOPMENTAL TOXICITY[1]

2.1 Abstract

Perfluoroalkyl acids (PFAAs) are persistent environmental contaminants that are associated with various adverse health outcomes. Perfluorooctanoic acid (PFOA) is one of the most prominently detected PFAAs in the environment, which is now replaced with shorter chain carbon compounds including perfluorohexanoic acid (PFHxA) and perfluorobutyric acid (PFBA). Here, we compared the toxicity of four PFAA as a function of chain length and head group (carboxylate versus sulfonate) with in vitro and in vivo zebrafish assessments, which we then compared to other cell and aquatic models. Mortality increased with chain length (PFOA > PFHxA >> PFBA) in both whole embryo/larvae and embryonic cell models. The sulfonate group increased toxicity with perfluorobutane sulfonate (PFBS) showing higher toxicity than PFBA and PFHxA in both the larvae and cells. Toxicity trends were similar among different aquatic models, but sensitivities varied. Discrepancies with other zebrafish studies were confirmed to be a lack of neutralization of acidic pH of dosing solutions in these other studies, demonstrating the need for rigor in reporting pH of exposure solutions in all studies. The zebrafish embryonic cell line was also found to be similar to most other cell lines regardless of exposure length. Overall, results agree with findings in other cell lines and organisms where longer chain length and the sulfonate group increase toxicity, except in studies not neutralizing the exposure solutions for these acidic compounds.

[1]This chapter was originally published as: Wasel, Ola, Kathryn M. Thompson, Yu Gao, Amy E. Godfrey, Jiejun Gao, Cecon T. Mahapatra, Linda S. Lee, Maria S. Sepúlveda, and Jennifer L. Freeman. 2021. "Comparison of Zebrafish in Vitro and in Vivo Developmental Toxicity Assessments of Perfluoroalkyl Acids (PFAAs)." *Journal of Toxicology and Environmental Health - Part A: Current Issues* 84 (3): 125–36.

2.2 Introduction

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals that contain fully or partially fluorinated carbon chain and a polar terminal group. Due to their unique structure, PFAS are water and oil repellant, and have properties of surfactants. These properties allowed for wide industrial PFAS use since the 1950s. PFAS are used in firefighting foams, coating papers, carpets, textile, food packaging, and production of fluoropolymers (Kissa 2001; Lemler 2005). PFAS are persistent in the environment and bioaccumulate in wildlife and humans because they are resistant to hydrolysis, photolysis, and biodegradation (Wang et al. 2015). Some PFAS can degrade microbially, but their terminal metabolites are perfluoroalkyl acids (PFAAs), which include the perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonic acids (PFSAs) (Liu and Mejia Avendano 2013). With over 4,000 PFAS manufactured and used in the world (including over 600 PFAS used commercially in the USA), PFAS are detected in air, surface water, drinking water, and soil, as well as in wildlife and humans (Lau et al. 2007; U.S. EPA 2020). PFAS are even detected in the air of the arctic region and brain of polar bears in East Greenland (Pedersen et al. 2015).

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) are the most well -known PFAAs that were previously produced in large amounts, are extremely stable, resistant to biological degradation, and a terminal product in the microbial degradation of precursor PFAS (Lau et al. 2007). PFOA and PFOS are extensively studied in cell, animal, and human epidemiological studies demonstrating hepatoxicity, immunotoxicity, neurotoxicity, thyroid disruption, cardiovascular injury, renal toxicity, reproductive toxicity, and carcinogenicity (Zeng et al. 2019). Starting in the 2000s, the longer chain PFAAs were phased out and replaced by shorter chain PFAAs, such as perfluorohexanoic acid (PFHxA) and perfluorobutyric acid (PFBA) for PFOA and perfluorobutane sulfonic acid (PFBS) for PFOS (US EPA 2017). As part of the PFOA Stewardship Program, there was an initiative to replace PFOA use by 2015 with these shorter derivatives, which are predicted to be less toxic. However, the amount of literature covering the toxicity of these shorter perfluoroalkyl carboxylic acids (PFCAs), such PFHxA and PFBA, is still limited.

PFAA toxicity varies with carbon chain length, number of fluorinated carbons, and functional group type. In general, PFAA lethality and cytotoxicity follows the rule that longer chain lengths are more toxic than shorter carbon chain length PFAA (Buhrke et al. 2013;

Gorrochategui et al. 2014; Kleszczyński et al. 2007; Mahapatra et al. 2017), but differential effects are observed in vitro and in vivo (Godfrey et al. 2017; Gomis et al. 2018; Hagenaars et al. 2011; Slotkin et al. 2008; Zheng et al. 2012). Overall, PFHxA and PFBA are reported to be less acutely toxic than PFOA (Buhrke et al. 2013; Gorrochategui et al. 2014; Hoover et al. 2019; Kleszczyński et al. 2007; Mahapatra et al. 2017). The toxicity and risk characterization of PFHxA was recently reviewed (Anderson et al. 2019; Luz et al. 2019). In animal studies, PFHxA exposure resulted in hepatic peroxisomal beta-oxidation and hepatic and thyroid changes similar to PFOA (Chengelis et al. 2009). Similarly, liver and hematological parameters are also affected by PFBA exposure (Bijland et al. 2011; Butenhoff et al. 2012). PFBS bioaccumulates less compared to PFOS, but it has been shown that PFBS bioaccumulates in cetaceans (Lam et al. 2016). Animal and in vitro studies indicate that PFBS induces reproductive toxicity, thyroid alterations, and adipogenesis (Chen et al. 2018; Feng et al. 2017; Qi et al. 2018), but overall questions on the toxicity of the shorter chain PFAA alternatives remain.

Zebrafish (*Danio rerio*) offer many advantages as a model species for environmental and developmental toxicity studies (Hill et al. 2005; Horzmann and Freeman 2018). Adult zebrafish are small, can be mated once a week, and produce 100-200 embryos per mating pair. This permits affordable husbandry and larger sample sizes within a shorter period. The embryos develop ex vivo, thus allowing for easy chemical exposure and monitoring. Embryonic development also occurs rapidly over 72 hours post fertilization (hpf). Furthermore, the zebrafish genome is mapped and has 70-80% homology with the human genome with greater homology in genes linked to disease (Barbazuk et al. 2000; Howe et al. 2013). The great majority of the fish PFAS toxicity studies have been conducted with zebrafish. To exemplify a few, Hagenaars et al. (2011) showed that exposure to PFOA, PFOS, or potassium perfluorobutane sulfonate (K-PFBS) caused significant alteration in heart rate at 48 and 72 hpf. Interestingly, PFBA didn't cause any change in heart rate suggesting a chain length effect among the carboxylates with shorter chain PFCAs being less toxic. In addition, embryonic exposure to PFOS or PFOA, but not K-PFBS or PFBA, were reported to cause a decrease in total body length at 120 hpf (Hagenaars et al. 2011). Furthermore, Zheng et al. (2012) showed that exposure to PFOS, perfluorononanoic acid (PFNA), or PFOA caused developmental abnormalities such as pericardial edema and spine malformation. In addition, PFOA tissue distribution following exposure was described facilitating the estimation of tissue dose from external exposure concentrations (Ulhaq et al. 2015).

The purpose of the present study was to evaluate chain length effects on toxicity using PFOA, PFHxA, and PFBA in a zebrafish whole larva and in a zebrafish embryonic cell line. To evaluate the role of the functional group (carboxylate versus sulfonate), K-PFBS was included for comparison with PFBA with both being C4 PFAAs. We hypothesized that 1) mortality rate will increase with increasing carbon chain length; 2) sulfonate-containing PFAA will be more toxic compared to carboxylate-containing PFAA for a given chain length; and 3) toxicity profiles will be similar in zebrafish larvae and cells. In addition, when comparing the data attained in this study with some others, differences in values were observed. As these chemicals are acids, we hypothesized differences were due to a lack of the other studies adjusting solutions to neutral pH. The health of the zebrafish and other fish species is dependent on pH and pH is an important variable to consider in experiments as acidic chemicals can influence the water parameters of the test system. Lastly, results from our study representing the zebrafish model were compared to other aquatic organisms and other cellular models.

2.3 Methods and Materials

2.3.1 Chemicals and Test Solutions

PFOA (CAS No. 335-67-1), PFHxA (CAS No. 307-24-4), PFBA (CAS No. 375-22-4), and K-PFBS (CAS No. 29420-49-3) of >99% purity were purchased from Sigma-Aldrich 22 (St. Louis, MO, USA). Physico-chemical properties of these PFAAs are listed in Table 2.1 (ATSDR 2018; US EPA 2016). Stock solutions were prepared by first dissolving each of the chemicals in reverse osmosis (RO) water. Buffered solutions were neutralized by adding sodium bicarbonate to neutral pH. For every 500 mL of exposure solution, 0.1 mL of embryo medium containing 13-14% calcium, 1% magnesium, 0.12% potassium, and 0.6-0.7% sodium was added (Replenish, Seachem Laboratories Inc., Madison, GA, USA). For the zebrafish embryonic cell exposure, stock solutions and exposure solutions were prepared by dissolving each of the chemicals in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with AmnioMax, Fetal Bovine Serum (FBS) (Atlanta biologicals, Norcross, GA, USA), and Pen/Strep Amphotericin B (Lonza BioWhittaker, Basel, Switzerland). MTT (298-93-1,3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) was used to determine cell viability.

Table 2-1. Physico-chemical properties of PFOA, PFHxA, PFBA, and K-PFBS.

Chemical	Perfluorooctanoic acid	Perfluorohexanoic acid	Perfluorobutyric acid	Potassium perfluorobutane sulfonate
Acronym	PFOA, C8	PFHxA, C6	PFBA, C4	K-PFBS, C4
CAS registry no.	335-67-1	307-24-4	375-22-4	29420-49-3
Chemical formula	$CF_3(CF_2)_6COOH$	$CF_3(CF_2)_4COOH$	$CF_3(CF_2)_2COOH$	$CF_3(CF_2)_3SO_3K$
Molecular weight (g/mol)	414.07	314.05	214.04	338.19
Solubility in water	9.5×10^3 mg/L at 25°C	15,700 mg/L at 25°C	2.14×10^3 mg/L at 25°C	46.2 mg/L at 20°C
pKa	2.8	-0.16	0.08	Not Applicable

2.3.2 Toxicity Tests with Zebrafish Embryos/Larvae

Adult AB wild-type zebrafish were maintained at the Aquatic Ecology Laboratory (Purdue University) with a photoperiod of 14 h with light and 10 h in the dark and a temperature of 28 ± 1°C in a flow-through system. Fish were fed twice daily with a combination of hatched *Artemia* nauplii and commercial food (Tetramin). Zebrafish were bred as described by Gao et al. (2015). Gastrula-stage embryos (5-6 hpf) were exposed to a range of concentrations of PFOA, PFHxA, PFBA, or K-PFBS in neutralized solutions through 96 hpf with half of the exposure solutions renewed daily. Mixed clutches of zebrafish embryos were used over multiple days with one replicate represented by 20 embryos per plate ($N \geq 3$ for each treatment). For each replicate, water without chemical treatment was considered as 0 parts per million (ppm; mg/L) negative control. Embryos were incubated in an environmental chamber at the same temperature and photoperiod as described above. Concentration ranges used to calculate lethal concentrations needed to cause 50% mortality (LC50) were as follows: 0.01 – 1,000 ppm (0.024-2,415 μM) for PFOA; 10 – 9,000 ppm (31.8-28,657 μM) for PFHxA; 10 – 15,000 ppm (46.7-70,080 μM) for PFBA; and 500 – 2,000 ppm (1,478.4-5,913.8 μM) for K-PFBS. Mortality was recorded daily and at the end of the

exposure. All protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely regarding prevention and alleviation of suffering.

2.3.3 Influence of pH on PFCA Toxicity on Zebrafish Embryos/Larvae

Embryos were attained and exposed from 5-96 hpf in PFCA solutions as described above (20 embryos per plate; $N \geq 3$ for each treatment) with or without addition of sodium bicarbonate to achieve the buffered (neutralized) and unbuffered solutions and mortality recorded. pH of the PFCA solutions were determined. In a second experiment, different pH value solutions (6.8, 6.5, 6, 5.5, 4.5, 4.0, 3.5, and 3.0) were prepared by adding HCl into zebrafish embryo medium and developing zebrafish exposed to the solutions as described above through 96 hpf (20 embryos per plate; $N \geq 3$ for each treatment) and mortality recorded.

2.3.4 In Vitro Toxicity Tests with Zebrafish Embryonic Fibroblast Cells

The MTT Assay was used to quantify cell survival using an AB zebrafish embryonic fibroblast cell line (Freeman et al. 2007) maintained at 28°C and 5.0% CO_2 in DMEM media supplemented with AmnioMax, Fetal Bovine Serum (FBS), and antibiotics. Stock solutions of 6,000 ppm (19,105 μM for PFHxA and 19.993 μM for K-PFBS) were prepared in media for both PFHxA and K-PFBS treatments. Stock solutions of 2,000 ppm (4,830 μM) and 8,000 ppm (37,376 μM) were prepared in media for PFOA and PFBA treatments, respectively. Cells were counted using a Coulter counter. 96-well plates were seeded at a density of 7,000 cells/well and cells treated with PFOA, PFHxA, PFBA, or K-PFBS at a range of concentrations. Cells with regular media, without chemical treatment, were considered as 0 ppm negative control. After 96 hours of treatment, 10 μL of 5 mg/mL MTT was added to each well and incubated for 4 hours. A volume of 100 μL of Solubilization Solution (SS) of 10% Triton in isopropanol at pH 4.8 was then added to each well and the plate was allowed to shake for 30 min at room temperature to solubilize the crystals formed. Living cells will produce insoluble purple color by reducing MTT by mitochondrial succinate dehydrogenase (Mosmann 1983). The reduction of MTT to a purple formazan product was determined with a SpectraMax® M2e Microplate Reader at 570 nm. The absorbance was recorded and expressed as percent of the control.

2.3.5 Statistical Analyses

LC50 and associated 95% confidence limits at 96 hpf in zebrafish larvae was determined using nonlinear regression with a hill slope curve fitting using GraphPad Prism 8.4.1. EC50 and associated 95% confidence limits in zebrafish fibroblast cells were determined using the Probit method with SPSS 23.0. Unless otherwise noted, values presented are means ± standard error (SE). In all cases, $p<0.05$ was considered statistically significant.

2.4 Results

2.4.1 Toxicity Tests with Zebrafish Embryos/Larvae

The 96 hpf-LC50 of the zebrafish embryos/larvae test confirmed our hypothesis that mortality followed the increase in carbon chain length (PFOA > PFHxA > PFBA) (Table 2.2, Figure 2.1). The 96 hpf-LC50 of neutralized solutions of PFOA, PFHxA and PFBA were 487.4 ppm, 6,486 ppm, and 9,703 ppm, respectively. In addition, the LC50 of K-PFBS was 1,394 ppm, which indicates it is more toxic than both PFBA and the longer chain PFHxA (Table 2.2, Figure 2.1). This result confirmed that the presence of the sulfonate group increased toxicity compared to the carboxylic group-containing PFAA, even those with longer chain length.

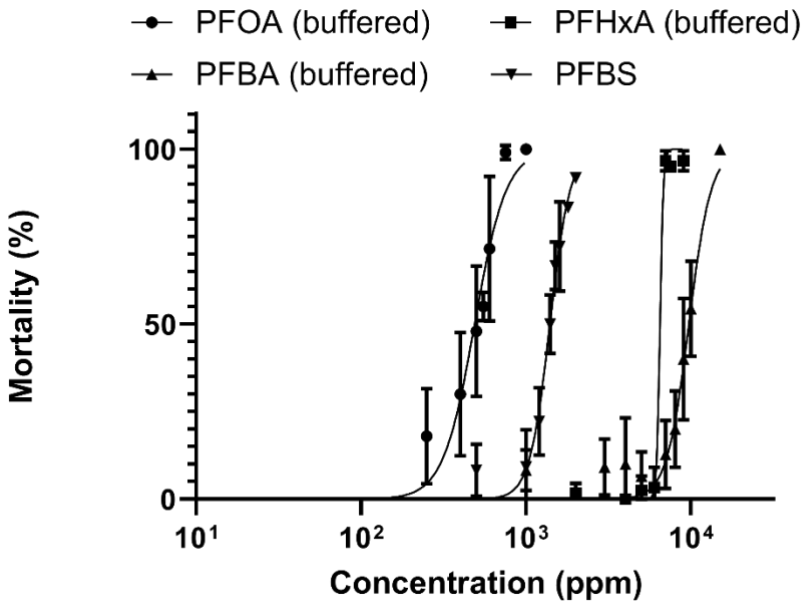

Figure 2-1: LC50 curves of zebrafish embryos/larvae exposed to PFAAs from 5–96 hpf. The toxicity ranking aligned with carbon chain length among the PFCAs with PFOA being more toxic than the shorter chain PFCAs. The sulfonate is also more toxic for a given chain length. N=3-12 with 20 fish per treatment per replicate. Error bars are standard error.

Table 2-2. LC50 and 95% confidence intervals of zebrafish larvae exposed to PFAAs from 5-96 hpf

Chemical	LC50 (ppm)	95% CI
PFOA	487.4	(457.8-523.8)
PFHxA	6,486	NC
PFBA	9703	(9386-10,097)
K-PFBS	1,394	(1354 - 1433)

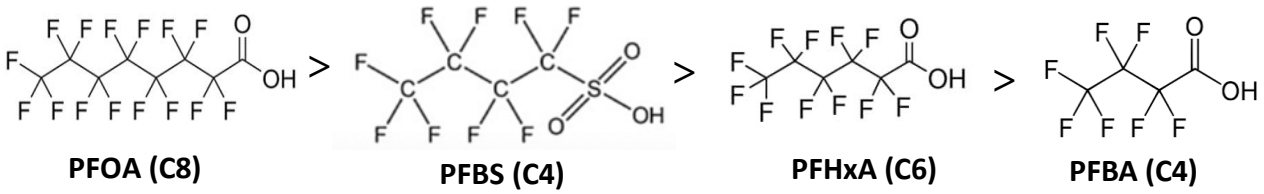

Figure 2-2: Acute toxicity ranking of PFAAs in zebrafish embryos/larvae exposed from 5–96 hpf.

2.4.2 Influence of pH on PFCA Toxicity on Zebrafish Embryos/Larvae

When comparing the results of our study to others, major differences were noted for some PFCAs. We hypothesized these differences were due to the other studies not neutralizing their PFCA solutions and thus, LC50s reported in other studies were reflective of pH influences and not the PFCA dose. To test this hypothesis, we compared buffered (neutralized pH) PFCA to unbuffered (pH influenced by PFCA). As hypothesized a higher acute toxicity was observed in unbuffered PFCA exposures compared to neutralized PFCA exposure (Table 2.2, Figure 2.2). Overall, a similar trend of toxicity was observed among the unbuffered PFCA exposures (96 hpf-LC50s: PFOA: 57.6 ppm, PFHxA: 76.3 ppm, and PFBA: 83.6 ppm) as the neutralized PFCA exposures, but the LC50s among the unbuffered PFCA exposures clustered much closer compared to the neutralized PFCA exposures (Table 2.2, Figure 2.2).

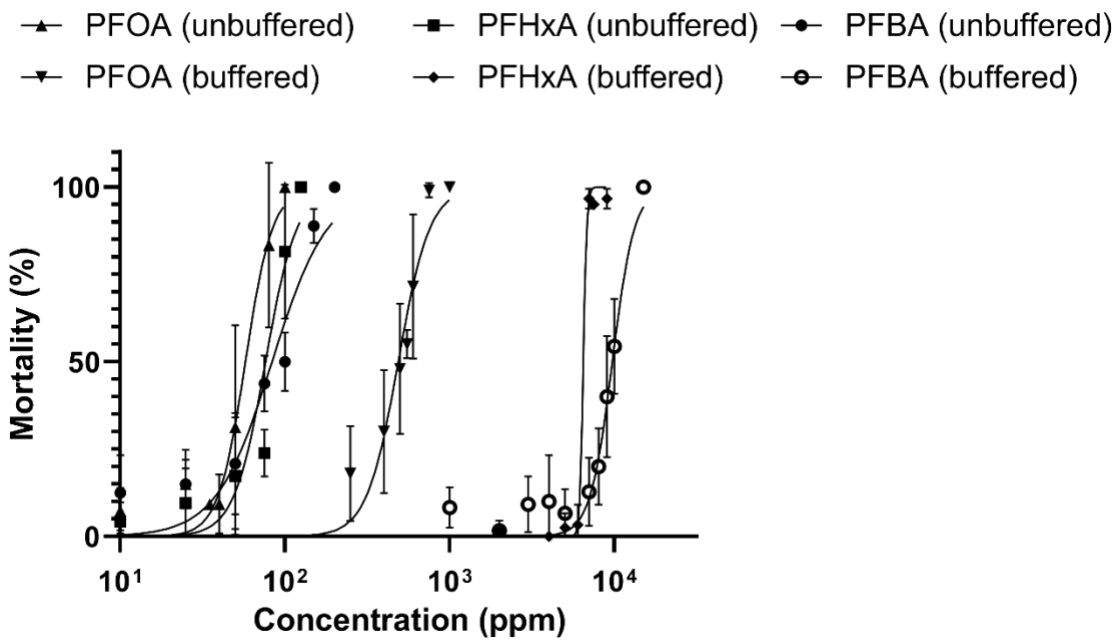

Figure 2-3: LC50 curves of zebrafish embryos/larvae exposed to unbuffered and buffered (neutralized) PFCAs from 5–96 hpf. Toxicity of the unbuffered PFCAs were greater than all the buffered PFCA solutions. N=3-12 with 20 fish per treatment per replicate. Error bars are standard error.

Table 2-3. Mean + SE pH values for PFOA, PFHxA, and PFBA tested in zebrafish embryo media

Dose (ppm)	PFOA	PFHxA	PFBA
0	6.92 ± 0.009	6.89 ± 0.01	6.96 ± 0.02
0.1	6.79 ± 0.07	6.52 ± 0.01	6.50 ± 0.04
0.5	6.71 ± 0.05	6.51 ± 0.02	6.56 ± 0.007
1	6.68 ± 0.05	6.49 ± 0.01	6.51 ± 0.003
10	6.38 ± 0.009	6.11 ± 0.02	5.91 ± 0.007
25	5.54 ± 0.01	4.80 ± 0.02	4.31 ± 0.01
50	4.53 ± 0.006	4.03 ± 0.003	3.74 ± 0.003
100	3.70 ± 0.009	3.51 ± 0.006	3.32 ± 0.006

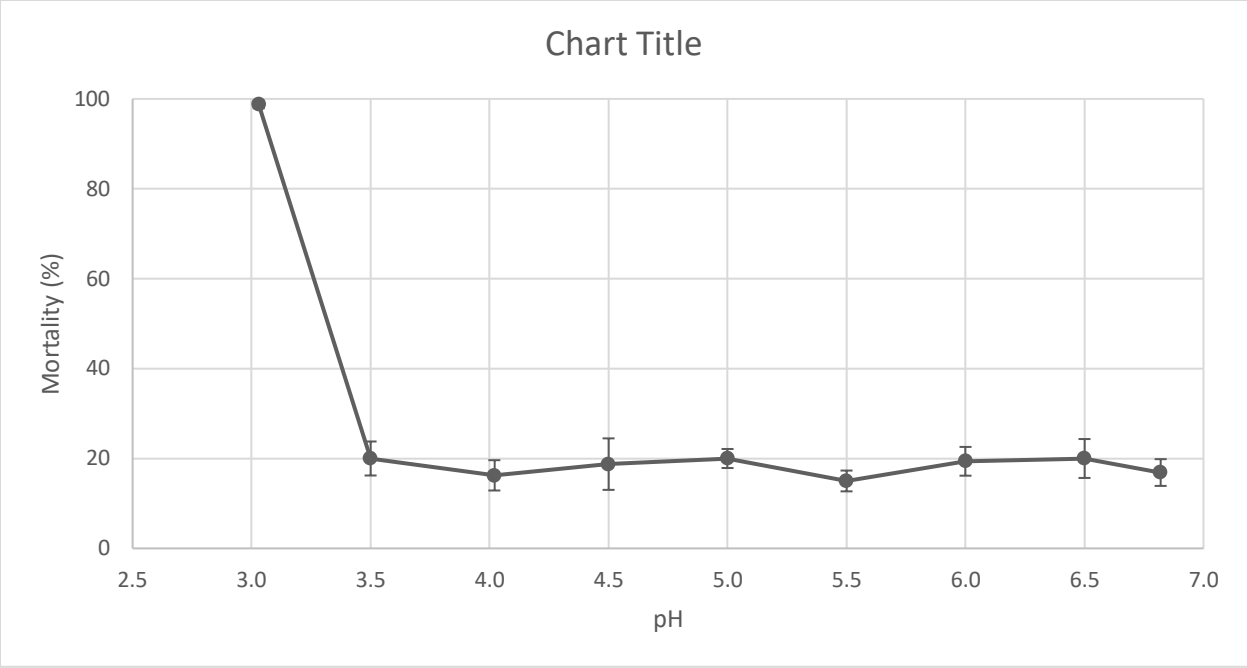

Figure 2-4: Mortality of zebrafish larvae exposed to different pH levels in embryo media from 5- 96 hpf. A drastic increase in mortality occurred at pH levels less than 3.5. N=3 with 20 fish per treatment per replicate. Error bars are standard error.

2.4.3 In Vitro Toxicity Tests with Zebrafish Embryonic Fibroblast Cells

To compare in vivo and in vitro toxicity assays, an embryonic zebrafish cell line was exposed to a range of concentrations of the PFAAs and MTT assay completed. A similar response was observed following increase in carbon chain length (PFOA > PFHxA > PFBA) with 96 hpf-EC50 of 148 ppm (357 μM), 2,079 ppm (6,619.96 μM) and 2,212 ppm (10,334 μM), respectively. The EC50 of K-PFBS (1,444 ppm, 4269 μM) was more toxic than PFHxA and PFBA (Table 2.4, Figure 2.4). Among the PFAAs, the MTT assay EC50 values are at lower concentrations than the LC50s obtained in the in vivo zebrafish experiments, except for K-PFBS which is comparable to the LC50 obtained for the larvae.

Table 2-4. EC50 and confidence intervals (CI) of zebrafish embryonic fibroblast cells exposed to PFAAs for 96 hr

Chemical	EC50 (ppm)	95% CI
PFOA	148	(121 - 175)
PFHxA	2,079	NC
PFBA	2,212	(1,061 - 6,762)
K-PFBS	1,444	NC

[NC] Not calculable

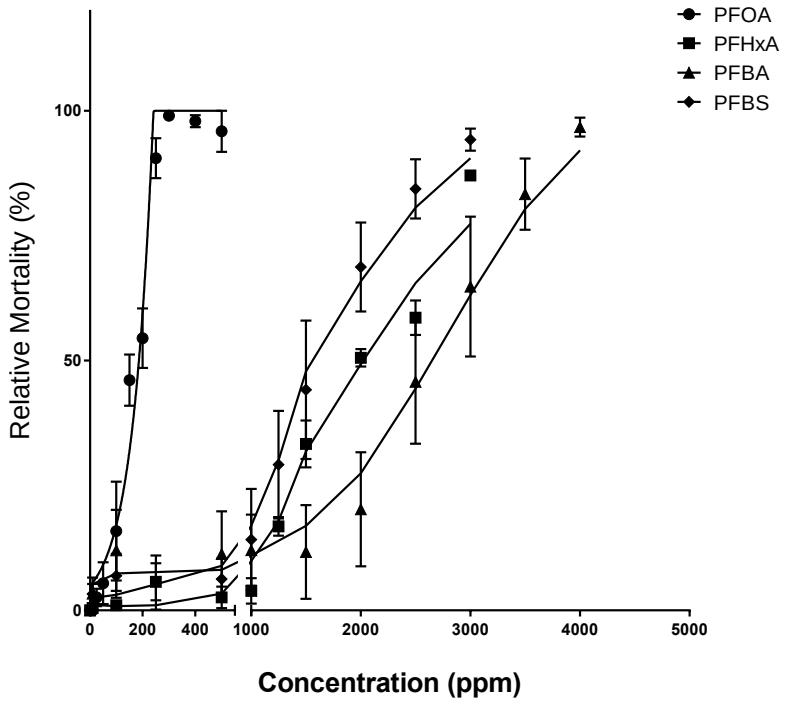

Figure 2-5: EC50 curves of zebrafish embryonic fibroblast cells exposed to PFAAs for 96 h. The toxicity ranking aligned with carbon chain length among the PFCAs with PFOA being more toxic than the shorter chain PFCAs. The sulfonate is also more toxic for a given chain length. Overall toxicity ranking is as follows: PFOA > K-PFBS > PFHxA > PFBA and agrees with that of the zebrafish larvae. N=4, 96 well plate with 7000 cells per well per treatment per replicate. Error bars are standard error.

2.5 Discussion

The LC50 of selected PFAAs: PFOA, PFHxA, PFBA, and K-PFBS were determined. Chain length is reported as a driver for differential toxicity of PFAS and our data confirm that the LC50 at 96 hpf for zebrafish reflect chain length (PFOA > PFHxA > PFBA). The LC50 of PFOA in the present study (487.4 ppm) was similar to that noted in past investigations using similar developmental exposure periods, except for a recent study by Gebreab et al. (2020) (Table 2.5). In addition, the LC50 of PFBA in developing zebrafish at 96 or 144 hpf was observed to be >3,000 ppm (Hagenaars et al. 2011; Ulhaq et al. 2013) or 13,795 ppm (Godfrey et al. 2017), respectively; which is similar to the 96 hpf-LC50 found in the present study (9,703 ppm). The 96 hpf-LC50 for K-PFBS (1,394 ppm) was also comparable to that reported by Ulhaq et al. (2013) at 144 hpf (1,500 ppm) for PFBS, but there were discrepancies between our study and those reported by Annunziato et al. (2019) for PFHxA LC50 of 91 ppm. These discrepancies may be attributed to the different length of exposure and age of embryo at the beginning of exposure, but the pKa of PFOA, PFHxA, and PFBA are low, indicating that these are strong acids. As such, pH of exposure solutions might be influenced by these acids.

In our study all solutions were initially neutralized to eliminate the effect of low pH on mortality rates, but when compared to results in that of Gebreab et al. (2020) and Annunziato et al. (2019) one questions whether the disparate findings were due to PFOA and PFHxA dosing solution not being neutralized in their experiments, respectively. Both studies dissolved the PFCAs in deionized water, but neither investigation reported on pH of their dosing solutions. As such, the influence of pH on PFCA toxicity was then examined. First, mortality rate in buffered (neutralized) PFCA solutions and unbuffered PFCA solutions were compared, where pH was not neutralized. Data demonstrated that while the same toxicity ranking was observed for the unbuffered PFCA solutions, the unbuffered PFCA solutions were more toxic than the neutralized solutions and that the three PFCA 96 hpf-LC50s clustered closer together. The pH of the unbuffered PFCA solutions near the LC50 value was approximately 3.5 and in a second study determining pH on mortality frequency it was found that the pH at which 50% of larval death occurred was between a pH of 3–3.5 at 96 hpf. Gebreab et al. (2020) reported a 120 hpf-LC50 of 82 ppm for PFOA and Annunziato et al. (2019) a 120 hpf-LC50 of 91 ppm for PFHxA leading to the conclusion that the LC50 noted in these two papers are due to pH influence of the acids and not PFCA concentration. In addition, this might account for the discrepancy between the LC50 in our study versus these two studies.

Our findings along with the other studies demonstrated that acute toxicity depends upon the carbon chain length as well as functional group. The functional group plays an important role in determining the toxicity as exemplified for K-PFBS versus PFCA with the same carbon chain length (PFBA, C4) in zebrafish. K-PFBS was also more toxic than longer the C6 PFCA (PFHxA). Ng and Hungerbühler (2014) noted there are differential affinities of PFAAs with different chain length and PFSAs and PFCAs of same chain length toward binding to proteins and phospholipids, the two common mechanisms involved in PFAA bioaccumulation. Overall, while the trend was consistent among species for chain length and influence of functional group there were differences in sensitivities with zebrafish as there was more sensitive than the water flea (Daphnia magna) (Boudreau 2002), but less sensitive than the planktonic rotifer (Brachionus calyciflorus) (Wang et al. 2014; Zhang et al. 2013) (Table 2.5).

The *in vitro* EC50 results for the zebrafish embryonic cells in this study were also found to be similar to other observations in zebrafish liver (ZFL) cells (Cui et al. 2015; Mahapatra et al. 2017). For example, the EC50 of PFOA in this study was 148 ppm [which was lower than the LC 50 in the larvae (487.4 ppm)] but similar to the 48 and 96 hr exposure at 85 ppm and 90 ppm in ZFL cells, respectively (Cui et al. 2015; Mahapatra et al. 2017) (Table 2.6). While length of incubation may influence cell survival, this value is also similar to that reported with other cell lines (HCT116, C6, IPC-81, and JEG-3) for exposure from 24 to 72 hr (Table 2.6) (Ding et al. 2013; Gorrochategui et al. 2014; Kleszczyński et al. 2007). Greater differences were observed for HepG2 cells (Buhrke et al. 2013) and an amphibian cell line (Hoover et al. 2019). Similarly, the EC50 of PFHxA (2,079 ppm) and PFBA (2,212 ppm) in zebrafish embryonic cells are lower than observed in larvae, but higher than in ZFL cells (500 ppm for PFHxA and 563 ppm for PFBA) (Mahapatra et al. 2017). PFHxA in the present study was also similar to treatments ranging from 24 to 72 hr in HCT116 (Kleszczyński et al. 2007), C6 (Mulkiewicz et al. 2007), IPC-81 (Mulkiewicz et al. 2007), and an amphibian cell line (Hoover et al. 2019), but again differed from that of HepG2 cells (Rappazzo et al. 2017) (Table 2.6).

Table 2-5. Toxicity of PFOA, PFHxA, PFBA, and K-PFBS in aquatic models

CAS Number	Chemical Name	Species	Organism Age (hpf)	Length of Exposure (Days)	LC50 (mg/L)	Reference
335-67-1	PFOA	*Daphnia magna*	NR	2	268,732	Boudreau 2002
		Danio rerio	1	3	262	Zheng et al. 2012
			1	3	600	Ding et al. 2013
			1	4	371	Ding et al. 2013
			2	4	157	Kalasekar et al. 2015
			4.5	4	473	Godfrey, Abdel-Moneim, and Sepùlveda 2017
			Table 2- 5: Continued			
			1	4	759	Stengel, Wahby, and Braunbeck 2018
			1	4	>500	Hagenaars et al. 2011
			5.5	**4**	**487.4**	**Present study**
			1	5	>500	Hagenaars et al. 2011
			NR	6	430	Ulhaq et al. 2013
			4	5	<82	Gebreab et al. 2020
		Brachionus calyciflorus	< 2	1	150	Zhang et al. 2013
307-24-4	PFHxA	*Brachionus calyciflorus*	< 2	1	140	Wang et al. 2014
		Danio rerio	**5.5**	**4**	**6486**	**Present study**
			3	5	91	Annunziatio et al. 2019
375-22-4	PFBA	*Daphnia magna*	NR	2	> 1,005,986	Boudreau 2002
		Danio rerio	4.5	4	13,795	Godfrey, Abdel-Moneim, and Sepùlveda 2017
			1	4	>3000	Hagenaars et al. 2011
			5.5	**4**	**9703**	**Present study**
			1	5	>3000	Hagenaars et al. 2011
			NR	6	>3000	Ulhaq et al. 2013
29420-49-3	K-PFBS	*Danio rerio*	1	4	>3,000	Hagenaars et al. 2011
			5.5	**4**	**1,363**	**Present study**

NR = Not Reported; hpf = hours

Table 2-6. Comparison of PFAA toxicity in *in-vitro* models

CAS Number	Chemical Name	Cell line	Length of Exposure (hours)	EC_{50}/IC_{50} (µM)	Reference
335-67-1	PFOA	HCT116	24 hr	937 µM	Kleszczyński et al. 2007
		JEG-3	24 hr	594 µM (Alamar Blue), 647 µM (CFDA-AM)	Gorrochategui et al. 2014
		IPC-81	44 hr	457µM	Ding et al. 2013
		C6	44 hr	676 µM	Ding et al. 2013
		Amphibian cells ("Speedy")	48 hr	1210 µM (501 mg/L)	Hoover et al. 2019
		ZFL	48 hr	205 µM (85 ppm)	Cui et al. 2015
		HCT116	72 hr	312 µM	Kleszczyński et al. 2007
		HepG2	72 hr	47 µM	Buhrke, Kibellus, and Lampen 2013
		ZFL	96 hr	217 µM (90 ppm)	Mahapatra et al. 2017
		Zebrafish embryonic fibroblast	**96 hr**	**357 µM (148 ppm)**	**Present study**
307-24-4	PFHxA	HCT116	24 hr	4153 µM	Kleszczyński et al. 2007
		JEG-3	24 hr	None up to 500 µM	Gorrochategui et al. 2014
		IPC-81	44 hr	3715 µM	Mulkiewicz et al. 2007
		C6	44 hr	7943 µM	Mulkiewicz et al. 2007
		Amphibian cells ("Speedy")	48 hr	7059 µM (2217 mg/L)	Hoover et al. 2019
		HCT116	72 hr	1157 µM	Kleszczyński et al. 2007

Table 2-6: Continued

		HepG2	72 hr	344 µM	Buhrke, Kibellus, and Lampen 2013
		ZFL	96 hr	1592 µM (500 ppm)	Mahapatra et al. 2017
		Zebrafish embryonic fibroblast	**96 hr**	**6620 µM (2079 ppm)**	**Present study**
375-22-4	PFBA	JEG-3	24 hr	None up to 500 µM	Gorrochategui et al. 2014
		HepG2	72 hr	>1000 µM	Buhrke, Kibellus, and Lampen 2013
		ZFL	96 hr	2630 µM (563 ppm)	Mahapatra et al. 2017
		Zebrafish embryonic fibroblast	**96 hr**	**10,334 µM (2212 ppm)**	**Present study**
29420-49-3	K-PFBS	JEG-3	24 hr	None up to 500 µM	Gorrochategui et al. 2014
		Zebrafish embryonic fibroblast	**96 hr**	**4269 µM (1444 ppm)**	**Present study**

2.6 Conclusion

Mortality increased with an increase in chain length in both whole embryo/larva and embryonic cell models, which agrees with previous studies. The cell line was more sensitive to exposure to PFOA, PFHxA, and PFBA compared to the whole animal. These differences may be attributed to tissue/intracellular dose or absence/presence of metabolic activity. When comparing the in vivo and in vitro findings to other published studies, zebrafish embryo/larval studies are similar among multiple studies regardless of the time point when exposure was initiated or length of exposure when PFAA dosing solutions are neutralized; however, sensitivity between species varies. Zebrafish showed greater sensitivity than *Daphnia magna* and lower sensitivity than *Brachionus calyciflorus*, especially to PFHxA. The zebrafish embryonic cell line was also found to be similar to most other cell lines regardless of exposure length, except for HepG2 cells for PFOA and PFHxA and the amphibian speedy cells for PFOA. In addition, our study highlights the

importance of reporting detailed methods of preparing PFAS solutions that should indicate pH of working solution to allow proper interpretation of the results. Overall, these results are in agreement with trends observed for other aquatic and in vitro models when comparing the acute toxicity of PFAS with chain length and functional group driving toxicity. Further studies are needed to show the differential effects of PFAS of different chain length and functional group at sublethal concentrations on different organs of biological systems.

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CHAPTER 3 : ASSESSMENT OF UNIQUE BEHAVIORAL, MORPHOLOGICAL, AND MOLECULAR ALTERATIONS IN THE COMPARATIVE DEVELOPMENTAL TOXICITY PROFILES OF PFOA, PFHXA, AND PFBA

3.1 Abstract

Perfluoroalkyl substances (PFAS) are a class of synthetic chemicals that are persistent in the environment. Due to concerns linked with longer chain PFAS, shorter chain chemicals were used as replacements, but toxicity and human health risk data of the shorter chain chemicals are limited. The aim of this study was to compare the developmental toxicity of three perfluoroalkyl acids (PFAAs): perfluoroctanoic acid (PFOA), composed of 8 carbons (C8), perfluorohexanoic acid (PFHxA, C6), and perfluorobutanoic acid (PFBA, C4) using the zebrafish (*Danio* rerio) model. Lethal concentration needed to kill 50% of the fish (LC50s) were determined to assess of the potency after an acute toxicity these PFAAs by exposing zebrafish larvae to a range of concentrations of each chemical. In sublethal assessments, zebrafish were exposed to 0, 4, 40, or 400 parts per billion (ppb; µg/L) of individual PFAAs throughout embryonic development (72 hours post fertilization, hpf). Effects of the embryonic exposure on locomotor activities was completed with the visual motor response test at 120 hpf. At 72 hpf, morphological changes including total body length, head length, and head width were assessed. In addition, transcriptomic profiles of PFOA, PFHxA, and PFBA were also determined at 72 hpf. The LC50 ranking was PFOA > PFHxA > PFBA, which followed the trend expected with increasing chain length. PFOA caused hyperactivity and PFBA induced mainly hypoactivity, while PFHxA did not result in behavioral changes. PFOA, PFHxA, and PFBA caused morphological and transcriptomic alterations that were unique for each PFAA and were concentration dependent. Cancer was a top enriched disease for PFOA, while FXR/RXR activation was a top canonical pathway in all PFBA exposures. Overall, an embryonic exposure to PFOA, PFHxA, or PFBA resulted in morphological alterations in zebrafish eleuthero-embryos; however, only PFOA and PFBA induced neurobehavioral alterations. Transcriptomic profile of each chemical is unique indicating different toxicity mechanisms.

3.2 Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of man-made chemicals that have been used since the 1940s in a wide range of consumer and industrial products (OECD 2018). These substances are composed of alkyl chains of partially or fully fluorinated carbons. Although this chemical structure provides some unique characteristics (e.g., low surface tension, hydrophobicity, and lipophobicity) the strength of the carbon-fluoride bond leads to their persistence in the environment. PFAS are resistant to degradation by oxidizing agents, strong acids, alkalis, and photolysis (Wang et al. 2015). Some PFAS are biodegradable, but their biodegradation leads to the formation of other PFAS that are stable in the environment or accumulate in biological organisms (Nilsson et al. 2013; Giesy and Kannan 2002).

The most studied PFAS are perfluorooctanic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). Epidemiological studies suggest association between PFOA and PFOS exposure and many adverse health effects such as pregnancy-induced hypertension, increased serum hepatic enzymes, increased lipids, decreased immune response, and decreased birth weight (Fenton et al. 2021; ATSDR, 2020). In 2002, 3M, the main manufacturer of PFOA and PFOS announced voluntary phase out in the United States, but based on the persistence of PFOA, PFOS, and other longer chain PFAS, these substances remain in the environment presenting continual risk of exposure. Moreover, these chemicals are still used in other countries (EPA 2020). Recently, the United States Environmental Protection Agency (US EPA) proposed regulations to control the entry of imported products containing long chain PFAS [≥ 6 perfluorocarbons which translates to perfluoroalkyl carboxylic acids (PFCAs) with alkyl chains of ≥ 7 carbons and perfluoroalkyl sulfonic acids (PFSAs) with alkyl chains of ≥ 6 carbons] ITRC 2017).

Alternatives were introduced into industry to replace the use of these longer chain PFAS. Those alternatives include PFAS with chemical modifications to their alkyl chain where one or more oxygen atoms have been inserted leading to perfluoroalkyl ether carboxylic acids (PFECAs). These ether alternatives have lower bioaccumulation potential compared to legacy PFAS (ATSDR, 2020); however, unfortunately, the next generation are also stable and persist in the environment similar to the legacy PFAS. Thus, there is a concern that these PFAS alternatives can bioaccumulate leading to toxicity; however, with given toxicity data are too limited to know if they indeed represent safer alternatives in regard to both human and ecological health (Wang et al. 2015).

Additional alternatives include shorter chain PFAS that are more water soluble and partition less to solids, thus have higher mobility in the environment compared to PFOA and PFOS (Wang et al. 2015). Shorter chain PFAS may also bioaccumulate but in different tissues compared to PFOA or PFOS (Ghisi, Vamerali, and Manzetti 2019). Furthermore, a study showed that perfluorobutanoic acid (PFBA, C4) was detected in higher levels in dust, soil, and produce in several cities in Minnesota, compared to the longer chain compounds, such as PFOA and PFOS (Scher et al. 2018). Interestingly, this study was completed six years after installation of carbon filter systems that were used to remove these substances (Scher et al. 2014). Altogether, the alterative shorter chain PFAS have shorter half lives in organisms but are still being detected in the environment presenting risk of continual exposure through contaminated water or food sources. Therefore, thorough toxicity investigations into the safety of the shorter chain alterative PFAS are needed especially during development, a susceptible life stage.

Developmental toxicity is one of the main concerns regarding PFAS exposure. PFOA and PFOS have been detected in the serum of pregnant women, in cord blood, and in breast milk, indicating risk for in utero and early developmental PFAS exposure (Barbarossa et al. 2013; Kim et al. 2011). Animal studies showed that developmental PFAS exposure results in many adverse health effects including a reduction in neonate weight, altered bone development, delays in mammary gland differentiation, delays in eye opening, and neurodevelopmental alterations (ATSDR, 2020; Vuong et al. 2021).

Overall, there is a need to increase our knowledge on the developmental toxicity of the shorter chain PFAS replacements. To this end, the zebrafish presents as a complementary vertebrate model to investigate developmental toxicity outcomes. Strengths of the zebrafish model include ease in husbandry, high fecundity, and ex vivo development. In addition, the great similarity in gene function through vertebrate evolution (i.e., 82% similarity in disease-related genes) has secured their widespread use in developmental biology and toxicology studies allowing for translation of results observed in zebrafish to human health (Howe et al. 2013). Additionally in recent years, there has been an expansion of behavioral assays in the zebrafish resulting in application of this animal model in neuropharmacology and neurotoxicity research (Basnet et al. 2019; de Esch et al. 2012; Irons et al. 2010).

In this study, we compared the developmental toxicity of legacy PFAS exposure, represented by PFOA, to the shorter chain PFAS, represented by perfluorohexanoic acid (PFHxA)

and perfluorobutyric acid (PFBA) using the zebrafish. The US EPA is in the process of conducting an IRIS toxicological review for PFHxA (US EPA, 2022) and PFBA (US EPA, 2021). In addition, currently there are no federal regulatory levels for PFAS in drinking water (i.e., no Maximum Contaminant Level, MCL). In the interim, most recently the US EPA has set health advisory levels of exposure at 70 parts per trillion (ppt, ng/L) in drinking water (EPA, 2021). However, some states adopted more strict policies due to high concerns of PFAS. For example, Michigan Department of Environment, Great Lakes, and Energy (EGLE) has set regulatory limits for seven PFAS, including PFOA at 8 ppt and PFHxA at 400,000 ppt (or 400 parts per billion, ppb, µg/L) (EGLE, 2020). Globally, the European Chemicals Agency (ECHA) proposed restricted use of PFHxA in textile, food-contact product, and firefighting foam due to its mobility and persistence in the environment (ECHA, 2019). Given limited developmental toxicity information, a comparative toxicity study was completed to first compare lethality. Next, an assessment of additional outcomes at a wide range of sublethal concentrations including behavioral, morphological and growth, and transcriptome alterations were completed to address observed similarities and differences for the different chain lengths of PFOA, PFHxA, and PFBA. The study hypothesized that greater lethality, along with more significant impacts to behavior, morphology, and molecular alterations would be observed with increased chain length. In addition, it was hypothesized that perturbed biological and disease pathways for PFOA would align with adverse health outcomes reported in epidemiological and animal studies such as cancer, thyroid alterations, changes in liver function, altered cholesterol pathways, and immunotoxicity.

3.3 Materials and Methods

3.3.1 Zebrafish Husbandry

Zebrafish (*Danio rerio*) of the wild-type AB strain were housed in a Z-Mod system (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle. Water was maintained at 28±1°C, pH at 7.0–7.2, and conductivity at 550 µS/cm. Water quality was assessed twice a day. Fish were fed a mixture of brine shrimp (Artemia International LLC., Fairview, Texas), Golden Pearls 500–800 µm (Artemia International LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, PA). Adult fish were bred in spawning tanks according to established protocols

(Peterson et al. 2011, 2013). Embryos were collected within 1 hour post fertilization (hpf). The embryos were rinsed with embryo water (filtered reverse osmosis water of pH 7.2 and conductivity at 550 µS/cm) and randomly distributed in groups of 50 embryos into 100 mm X 20 mm polystyrene Petri dishes for each treatment group within each biological replicate. All fish used in experiments were incubated at 28°C through 72 or 120 hpf. Protocols were approved by the Purdue University Animal Care and Use Committee (PACUC protocol # 1110000088) and all fish treated humanely with regard to prevention and alleviation of suffering.

3.3.2 Chemical Treatments for Zebrafish Assays

PFOA (CAS# 335-67-1, 95% purity, Sigma, St. Louis, MO), PFHxA (CAS# 307-24-4, ≥ 97.0% purity, Sigma, St. Louis, MO), and PFBA (CAS# 375-22-4, 98% purity, Sigma, St. Louis, MO) were used in the study (Table 3.1). The chemicals were solubilized in reverse osmosis water all within solubility limits. Stock solutions of PFOA, PFHxA, and PFBA were neutralized to pH 7 with 5 M sodium hydroxide (Wasel et al. 2021).

Table 3-1. Physico-chemical properties of test chemicals

Chemical	**Perfluorooctanoic acid**	**Perfluorohexanoic acid**	**Perfluorobutyric acid**
Acronym	PFOA, C8	PFHxA, C6	PFBA, C4
CAS Registry No.	335-67-1	307-24-4	375-22-4
Chemical Formula	$CF_3(CF_2)_6COOH$	CF3(CF2)4COOH	$CF_3(CF_2)_2COOH$
Molecular Weight (g/mol)	414.07	314.05	214.04
Solubility in Water (at 25°C)	9.5 x 10^3 mg/L	15,700 mg/L	2.14 x 10^3 mg/L
pK_a	2.8	-0.16	0.08

3.3.3 Acute Developmental Toxicity Assay

To assess the lethal concentration at which 50% mortality was observed (LC50) of the test chemicals, three biological replicates (embryos from 3 different clutches) of 50 embryos per treatment were placed into a Petri dish and dosed with 20 mL of a range of concentrations of each PFAS within one hour after spawning (Weber et al. 2013; Peterson et al. 2011). Selected concentrations were 0, 300, 500, 600, 700, and 900 parts per million (ppm; mg/L) (724.5-2173.5

μM) of PFOA; 0, 5000, 7000, 8000, 9000, or 10,000 ppm (15,921.03-31,842.06 μM) of PFHxA; and 0, 5000, 7000, 8000, 9000, and 10,000 ppm (23,360-46,720 μM) of PFBA. All solutions were adjusted to a neutral pH (Wasel et al. 2021). The developing zebrafish were exposed to the test chemicals until 120 hpf. The negative control was filtered water only. Mortality rates were monitored every 24 hours. Mortality rates of treatment groups were normalized to the control treatment group.

3.3.4 Visual Motor Response Behavior Assay

Larval locomotion activities were assessed to evaluate if the developmental exposure to sublethal concentrations of test chemicals caused behavioral alterations. A visual motor response test was performed using Noldus DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands). In each biological replicate, 50 zebrafish embryos were exposed to 0, 0.4, 4, 40, 400, or 4000 parts per billion (ppb, μg/L) of PFOA (0.0009- 9.66 μM), PFHxA (0.00127-12.737 μM), or PFBA (0.0018-18.688 μM) within 1 hpf. Concentrations were chosen to encompass a log scale of sublethal concentrations with inclusion of and concentrations above an earlier US EPA provisional health advisory limit for PFOA at 0.4 ppb (US EPA, 2009). At the time, there were no health advisory levels for PFHxA or PFBA. The maximum contaminant level for PFHxA in drinking water set by the state of Michigan is 400 ppb (EGLE, 2020). 0 ppb represents the negative control treatment consisting of filtered water.

At 72 hpf, exposure to the chemicals was terminated by rinsing the fish with filtered water. Fish were then incubated in water only at 28°C until 120 hpf when behavioral analysis was completed similar to our past studies (Horzmann et al. 2018). Subsamples from each replicate were placed in separate wells in a 96-well plate (i.e., 16 subsamples of each treatment). Grossly malformed or dead larvae were excluded. The wells were filled with 500 μl filtered water, the plate placed in the DanioVision observation chamber, and temperature maintained at 28°C throughout the experiment using the Noldus temperature control unit. After a 10-minute dark acclimation period, the test was started by exposing larvae to 10 minutes of alternating periods of dark and light for a total of 50 minutes (dark-light-dark-light-dark) (Horzmann et al. 2018). Infrared light that is not visible to zebrafish larvae was used for tracking the movement. During the light phase, a 5000-lux light was activated from underneath the DanioVision observation chamber. The

infrared movement traces were recorded at a rate of 25 frames per second with a Basler GenICam acA 1300-60gm camera. Tracks were smoothed via a minimum distanced moved profile set to > 0.2 mm. The exposure to dark or light was controlled by EthoVision 12 software. All behavioral experiments were performed at 11 am-2 pm to minimize circadian variability in movement. Total distance moved, mean velocity, and cumulative time spent moving were calculated using EthoVision 12 software. A total of 8 biological replicates were performed for each chemical with 16 subsample fish per treatment in each replicate to total up to 128 fish per treatment group.

3.3.5 Morphological Growth Assessment

Morphological growth evaluation of zebrafish at 72 hpf was performed to assess abnormalities in PFAS treatment groups compared to the control group. Zebrafish embryos were collected within 1 hpf. For each replicate, 50 embryos were dosed with 0, 4, 40, or 400 ppb PFOA, PFHxA, or PFBA through the end of embryogenesis (72 hpf). Twenty eleuthero-embryos were randomly selected from each replicate for the morphological analysis, euthanized using 0.4 mg/mL buffered tricaine-S (ethyl m-amino benzoate methane sulfonate; Western Chemical Inc., Ferndale, WA), and analyzed using a Nikon SMZ1500 dissecting microscope with NIS Elements imaging software (Melville, NY) to obtain measurements. Endpoints included body length, head width, and head length. The body length was measured as the distance from snout to tail, head length was measured as the distance from snout to operculum, and head width (intraocular space) was measured as the length between the midpoint of each eye (Horzmann et al. 2017, 2020). Ratios of head length to body length and head width to body length were calculated to assess changes in overall eleuthero-embryo size. Three biological replicates were included with 20 subsample fish per treatment per replicate for a total 60 fish assessed per treatment group.

3.3.6 Analysis of Eleuthero-Embryo Transcriptome

Zebrafish embryos were exposed to 0, 4, 40, or 400 ppb PFOA, PFHxA, or PFBA within 1 hpf through 72 hpf (end of embryogenesis) in groups of 50 in a Petri dish. At 72 hpf, fish were rinsed, pooled, homogenized in TRIzol (Life Technologies), and flash-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Limburg) and quality confirmed by an Agilent Bioanalyzer (RIN >9.0) and spectrometry. Transcriptome changes were

assessed using the one-color hybridization strategy on a microarray containing 180,000 probes interrogating 36,000 gene targets with 3-5 probes per target based on Ensembl and UCSC genome databases (Agilent Technologies, Santa Clara, CA) (Horzmann et al. 2018; Wirbisky et al. 2016a). Each microarray slide contained 4 arrays allowing comparison of gene expression profiles between the control treatment (0 ppb) and the three PFAS treatments (4, 40, 400 ppb) on the same slide to block and account for any potential slide to slide variation. Four biological replicates were included for each PFAS treatment. Microarray procedures and analyses were performed as previously described (Horzmann et al. 2018, 2021; Wirbisky et al. 2016a). Briefly, RNA was hybridized to the array, arrays were washed in buffer solutions, and scanned using an Agilent Technologies SureScan Microarray Scanner (Agilent Technologies, Santa Clara, CA). Array image data was extracted and normalized using Agilent Feature Extraction software 12.0. Microarray analysis was performed according to MIAME guidelines (Brazma er al. 2001) using GeneSpring 14.9 (Agilent Technologies) for statistical analysis. Gene lists were imported into Ingenuity Pathway Analysis (IPA) and core analysis was performed for gene ontology and molecular pathway analysis. IPA identifies human orthologs of zebrafish genes, which are subsequently used for pathway analysis. Thus, genes mentioned are the human nomenclature.

3.3.7 Statistical Analyses

For the acute toxicity assessment, the LC50 and associated 95% confidence limits at 120 hpf in zebrafish larvae was determined using non-linear regression with a hill slope curve fitting using GraphPad Prism 8.4.1. A Grubb's outlier test was used to detect outliers within a treatment group for each outcome of the behavioral and morphological analyses. The experimental design was a randomized complete block design with block (biological replicate) by day (i.e., embryos collected and dosed at the same time on the same day for each treatment group). The experiments were repeated on multiple days to attain separate biological replicates. In the visual motor response assay, total distance moved, mean velocity, and time spent moving were analyzed using a two-way repeated measures analysis of variance (ANOVA) using SAS 94 software (α=0.05). The phase (dark or light) and chemical treatments were the independent variables and locomotor activity was the dependent variable. Differences among the treatment groups were only assessed if the treatment group and/or interaction of treatment group*phase was $p<0.05$ by evaluating the

dependent variable treatment group least square means table for the treatment groups at $p<0.05$. A one way ANOVA with SAS 94 software was used to analyze survival rates and morphological outcomes with a least significant difference (LSD) post hoc test when a significant ANOVA was observed ($\alpha=0.05$). Similarly, the LSD tables were only evaluated when the treatment group variable was $p<0.05$ for the ANOVA (p-values reported are the ANOVA p-value). The LSD post hoc treatment group differences are set at $p<0.05$ within SAS, which allows comparisons among treatment groups following the significant ANOVA ($p<0.05$). Significance of transcriptome results was determined using GeneSpring 14.9 (Agilent Technologies) with an ANOVA ($\alpha=0.05$) and a Tukey's post hoc test. A 1.5-fold change in gene expression criterion was also included for biological significance. All data are presented as mean ± standard deviation.

3.4 Results

3.4.1 Acute Developmental Toxicity Comparison of PFOA, PFHxA, and PFBA

Survival rates were monitored every 24 hours after exposure to a range of PFOA, PFHxA, or PFBA concentrations (Figure 3.1) to serve as anchors for sublethal experiments and for comparison of findings with other studies that used slightly different exposure conditions. By 24 hpf, significant mortality was observed in the 600, 700, and 900 ppm PFOA treatment groups (ANOVA: $p<0.0001$) (Figure 3.1A). In addition, at 48 hpf a significant decrease in mortality was also seen in the 500 ppm PFOA treatment group (ANOVA: $p=0.0002$), which continued for these four treatment groups through the end of the experiment (ANOVA: 72 hpf: $p=0.0002$; 96 hpf: $p=0.0004$; 120 hpf: $p= 0.0004$) (Figure 3.1A). No significant decrease in mortality was observed in the lowest treatment of 300 ppm (Figure 3.1A). As a result, the PFOA 120 hpf-LC50 was 561.05 ppm (95% CI: 465.58-659.17) (Figure 3.2).

In the PFHxA treatment groups, no significant increase in mortality occurred in the lowest treatment group of 5000 ppm (Figure 3.1B), while decreased survival occurred in all other treatment groups (7000, 8000, 9000, and 10,000 ppm) beginning at 24 hpf (ANOVA: $p<0.0001$) though the end of the exposure period (ANOVA: 48 hpf: $p<0.0001$; 72 hpf: $p=0.0001$; 96 hpf: $p<0.0001$; 120 hpf: $p<0.0001$). The PFHxA 120 hpf-LC50 was calculated at 8394.5 ppm (95% CI: 7925.01-871.56) (Figure 3.2).

No significant change in lethality occurred for the PFBA treatment groups up to 10,000 ppm throughout the exposure period ($p>0.05$ at all developmental time points) (Figure 3.1C) and thus, the 120 hpf-LC50 is greater than 10,000 ppm (Figure 3.2).

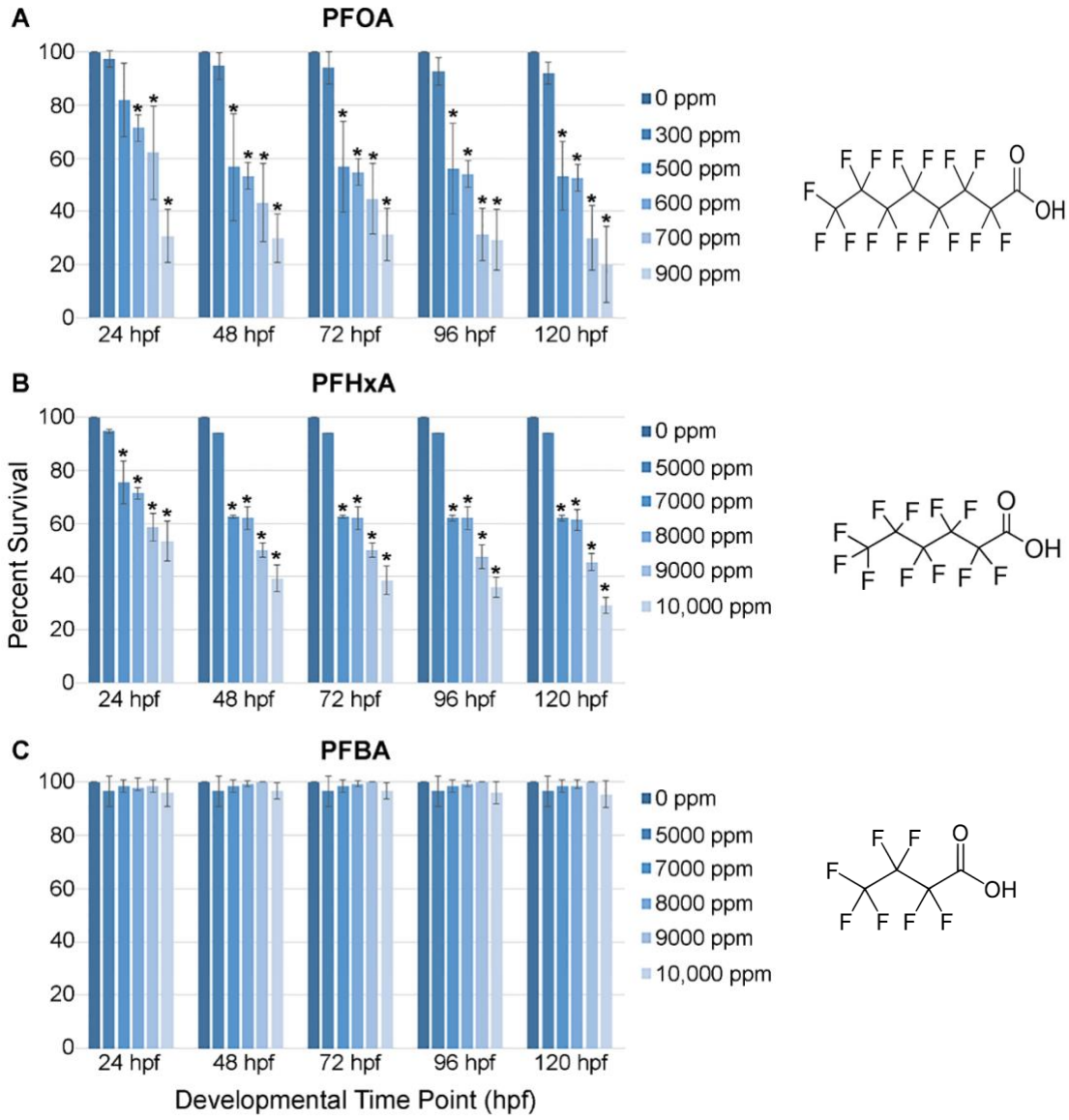

Figure 3-1: Percent survival of developing zebrafish exposed to (A) PFOA, (B) PFHxA, or (C) PFBA at 24, 48, 72, 96, and 120 hours post fertilization (hpf). Exposures were initiated at 1 hpf. N=3 with 50 subsamples per treatment in each biological replicate. Error bars are standard deviation. *$p<0.05$ compared to the control treatment group at each developmental time point

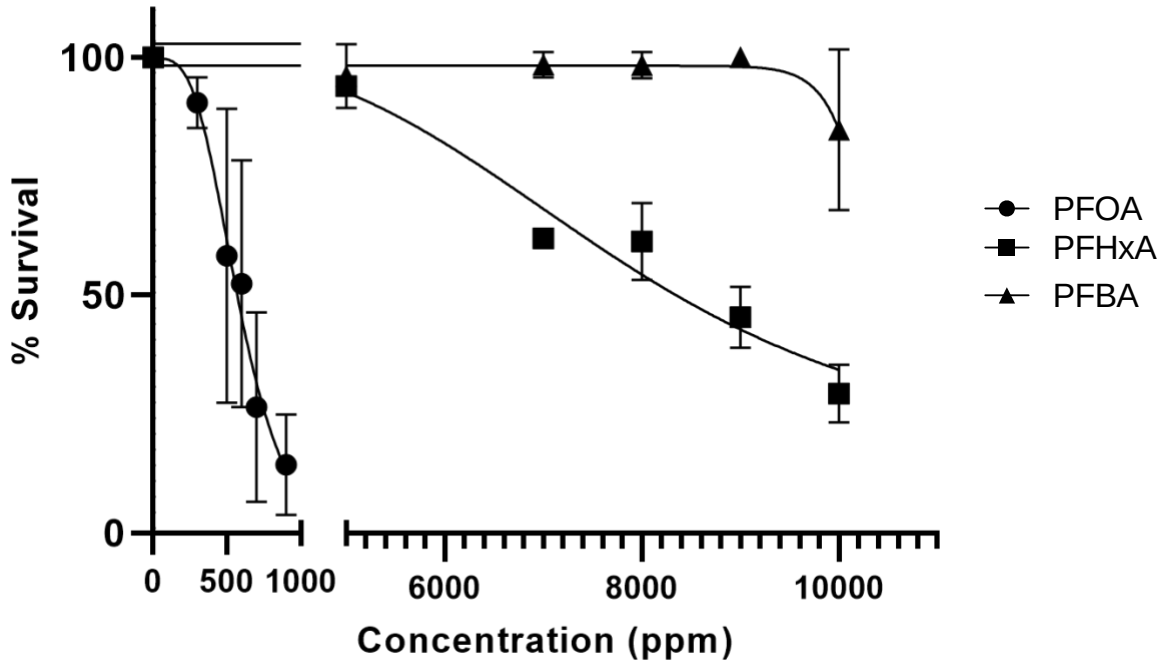

Figure 3-2: 120 hpf-LC50 curves of developing zebrafish exposed to PFOA, PFHxA, or PFBA from 1-120 hpf. The overall toxicity ranking is PFOA > PFHxA > PFBA. The LC50 for PFOA is 561.01 ppm, for PFHxA is 8394.5 ppm, and for PFBA is >10,000 ppm. N=3 with 50 fish per treatment per replicate. Error bars are standard deviation.

3.4.2 Locomotor Behavior Activity of Zebrafish Larvae Exposed to PFOA, PFHxA, Or PFBA During Embryogenesis

As expected, zebrafish larvae were more active in dark phases and less active in light phases as measured in all parameters. Two-way repeated measure ANOVA was used to analyze the data, because the locomotor activities of the same sample was measured multiple times (during different lighting phases). PFOA exposure resulted in a significant overall effect of treatment [$(F5,743) = 2.54$, $p=0.02$)] on the total distance moved (Figure 3.3A) and mean velocity (Figure 3.3D). In the first and second dark phase, exposure to 400 or 4000 ppb PFOA caused an increase in total distance moved and velocity, while hyperactivity was also observed in the first light phase at 400 ppb only (Figure 3.3A, 3.3D). Although trends of hyperactivity were observed for time spent moving there was no significant difference in treatment [$(F5,743)=1$, $p=0.4$)] or in the interaction of treatment and time [$(F5, 743)=1.14$, $p=0.29$)] (Figure 3.3G).

No significant effects were seen in the PFHxA exposure experiment for treatment [(F5, 754)=0.58, p=0.7)] or for interaction of treatment and time [(F20, 3016)=1.11, p=0.3)] for total distance moved or mean velocity (Figure 3.3B, 3.3E). Similarly, no significant effect of treatment [(F5,854)=0.18, p=0.18)] or interaction between treatment and time [(F20, 3016)=1.26, p=0.19)] was observed for time spent moving (Figure 3.3H).

There was a significant effect for PFBA exposure for treatment [(F5, 754)=2.18, p= 0.05)] and also a significant interaction between treatment and time [(F20, 3016)=2.67, p<0.0001)] for total distance moved and mean velocity (Figure 3.3C, 3.3F). Specifically, hyperactivity was observed in the first dark phase at 4 ppb and hypoactivity at 4000 ppb. Additionally, hypoactivity was seen in the first light phase at the lowest treatment concentration included in this study (0.4 ppb), along with hypoactivity at 400 and 4000 ppb in the second dark phase, which persisted at 4000 ppb in the third dark phase. In addition, there was a significant effect for treatment [(F5, 754)=2.56, p=0.02)] for time spent moving (Figure 3.3I). A similar pattern was observed; however, hypoactivity was observed in the three dark phases in the 4000 ppb treatment group only and in the 0.4 ppb treatment group in the first light phase. Overall, these results indicate a more complex behavioral effect including a biphasic response among the treatment group concentrations.

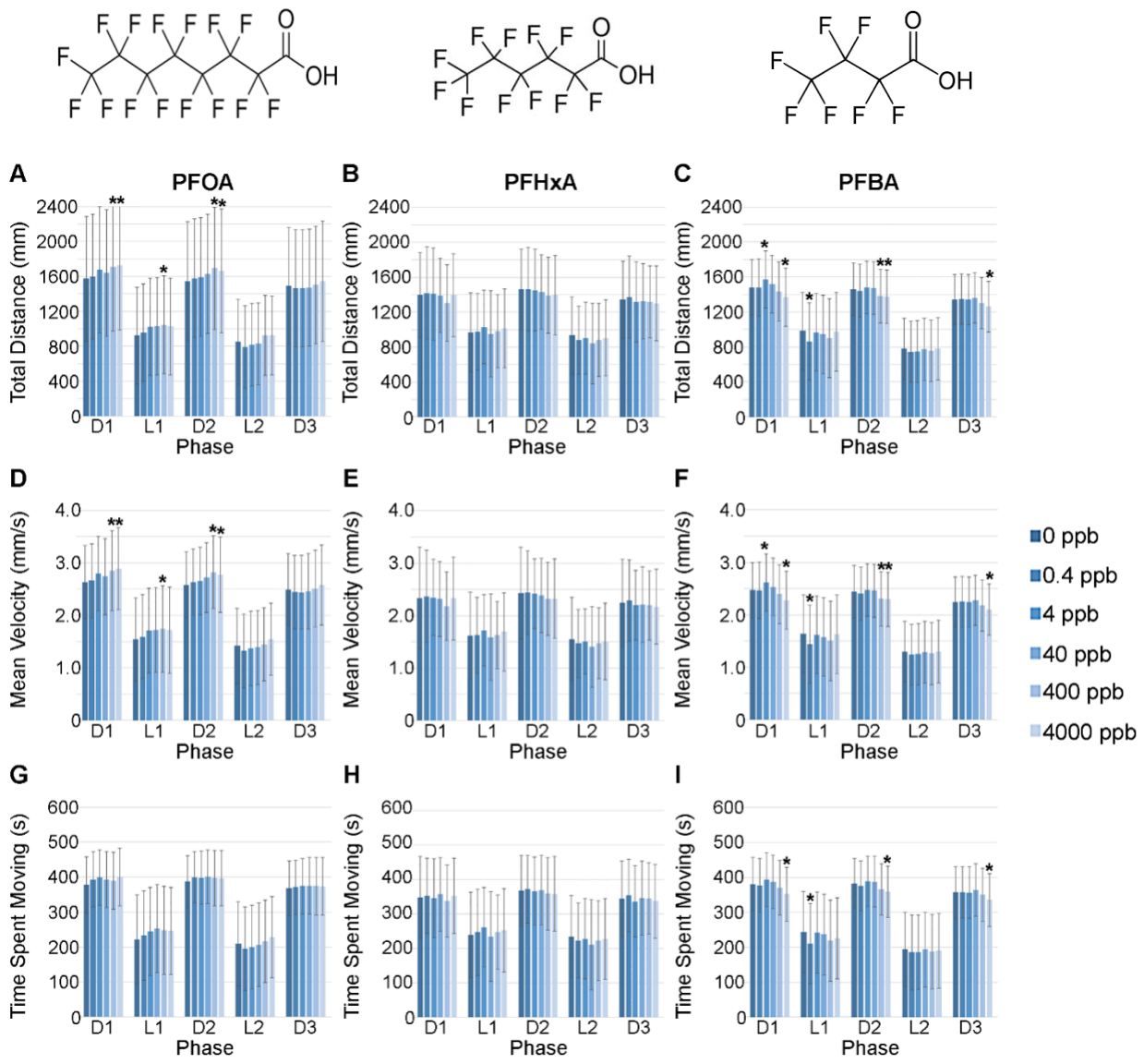

Figure 3-3. Visual motor response behavior assay in zebrafish larvae at 120 hpf following an embryonic exposure (1-72 hpf) to PFOA (A, D, G), PFHxA (B, E, H), or PBFA (C, F, I). N=8 biological replicates with 13-16 subsamples per treatment per replicate to total 122-128 fish per treatment group. Error bars represent standard deviation. *p<0.05 compared to the control treatment group within each phase. (D1: first dark phase; L1: first light phase; D2: second dark phase; L2: second light phase; D3: third dark phase)

3.4.3 Morphological Assessment of Eleuthero-Embryos after PFOA, PFHxA, or PFBS Exposure

Impacts to developmental growth were non-monotonic as measured at 72 hpf immediately following chemical exposure (Table 3.2). A significant effect of treatments on body length (ANOVA: p=0.02), head width (ANOVA: p<0.0001), and head length (ANOVA: p<0.0001) were

observed. Specifically, a significant decrease in body length and head width occurred in the 4 ppb PFOA treatment group compared to control with no significant differences observed in measurement ratios ($p>0.05$). In addition, a significant effect of PFOA treatment on head length to body length ratio was seen (ANOVA: $p<0.0001$) with the 400 ppb treatment group being different from the control.

Also, PFHxA treatment had a significant effect on body length (ANOVA: $p<0.0001$), head length (ANOVA: $p=0.01$), and head width (ANOVA: $p=0.04$). In the PFHxA 40 ppb treatment group, a decrease in body length, head length, and head width were observed with no differences in the ratio of measurements ($p>0.05$). In addition, fish in the 400 ppb PFHxA treatment group had a significant decrease in head length, along with a significantly lower head length to body length ratio.

PFBA treatment had a significant effect on body length (ANOVA: $p=0.0004$), head width (ANOVA: $p=0.02$), and head width (ANOVA: $p=0.04$). The effects of PFBA included decreased body length in the 4 and 400 ppb treatment groups and an increased head width in the 400 ppb exposure group. In addition, a significant increase in the head width to body length ratio was detected in all three treatment groups (ANOVA: $p<0.0001$).

Table 3-2. Morphological alterations at 72 hpf after an embryonic exposure to PFOA, PFHxA, or PFBA[a]

Concentration	Whole Body Length (μM)	Head Length (μM)	Head Width (μM)	Head Length / Body Length	Head Width / Body Length
PFOA					
0 ppb	3878.3 ± 133.0	650.3 ± 31.1	630.9 ± 34.1	0.168 ± 0.008	0.163 ± 0.006
4 ppb	3813.5 ± 152.4*	639.4 ± 33.3	616.4 ± 37.6*	0.168 ± 0.006	0.162 ± 0.006
40 ppb	3860.2 ± 176.4	642.0 ± 32.8	636.8 ± 32.9	0.166 ± 0.007	0.165 ± 0.006
400 ppb	3857.7 ± 197.5	623.0 ± 48.2*	619.4 ± 45.3*	0.162 ± 0.009*	0.161 ± 0.008
PFHxA					
0 ppb	3877.5 ± 105.9	663.9 ± 27.6	650.3 ± 24.8	0.173 ± 0.007	0.168 ± 0.006
4 ppb	3753.4 ± 104.4	656.7 ± 34.2	642.3 ± 28.9	0.171 ± 0.007	0.167 ± 0.006
40 ppb	3753.4 ± 101.9*	644.1 ± 36.2*	634.8 ± 30.7*	0.172 ± 0.001	0.169 ± 0.007
400 ppb	3891.4 ± 209.8	651.4 ± 47.9*	643.1 ± 39.9	0.168 ± 0.001*	0.165 ± 0.007
PFBA					
0 ppb	3782.5 ± 126.9	620.9 ± 38.9	624.9 ± 20.9	0.164 ± 0.008	0.165 ± 0.005
4 ppb	3706.4 ± 106.6*	607.3 ± 34.6	623.7 ± 26.3	0.163 ± 0.008	0.169 ± 0.007*
40 ppb	3773.5 ± 98.9	617.3 ± 32.4	632.7 ± 25.2	0.164 ± 0.008	0.168 ± 0.006*
400 ppb	3731.9 ± 115.1*	615.0 ± 36.1	634.7 ± 23.4*	0.165 ± 0.007	0.170 ± 0.005*

[a]Data are presented as mean ± standard deviation, *$p<0.05$ compared to the control group (0 ppb). N=3 with 20 subsamples per replicate to total 60 fish per treatment group.

3.4.4 Transcriptome Alterations of Eleuthero-Embryos Exposed to PFOA, PFHxA, or PFBA During Embryogenesis

Transcriptomic analysis of developing zebrafish exposed to PFOA identified 36 mapped genes with altered expression in the 4 ppb PFOA exposure group, 77 genes with altered expression in the 40 ppb PFOA exposure group, and 66 genes with altered expression in the 400 ppb PFOA exposure group (Figure 3.4). There were 9 common genes (*BATF, CIT, DOCK7, JDP2, NEDD4, Nedd4-2, PLCB2, PLCG1,* and *TASL)* altered in all three treatment groups.

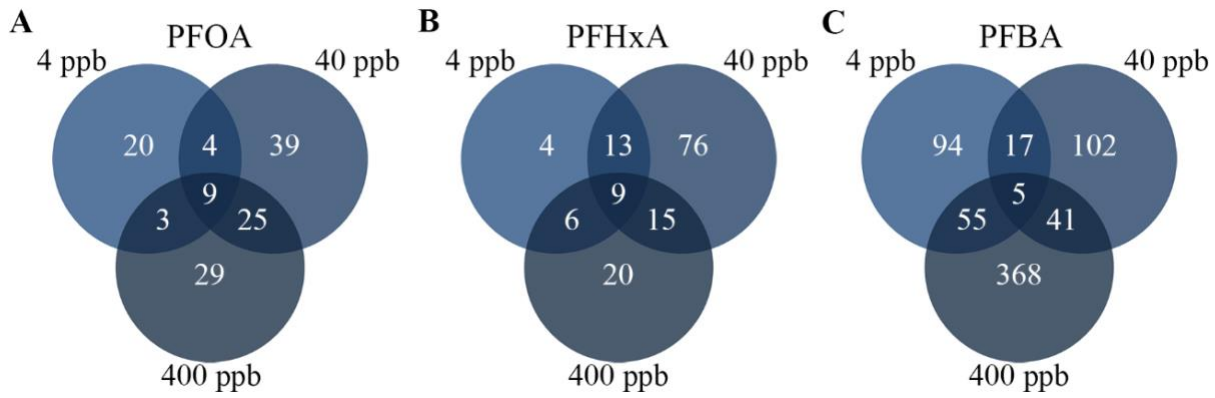

Figure 3-4: Venn diagram of the number of mapped genes changed in (A) PFOA, (B) PFHxA. and (C) PFBA at 72 hpf following embryonic exposure

Gene ontology and molecular pathway analyses with IPA revealed the most enriched pathways associated with diseases and disorders, physiological system development and function, and molecular and cellular functions based on gene expression changes for each treatment of each chemical (Tables 3.3, 3.4, and 3.5). Cancer, organismal injury and abnormalities, and reproductive system disease were among the top five enriched diseases and biological functions for the embryonic exposure to 4, 40, and 400 ppb PFOA (Table 3.3). In addition, immune cell trafficking, cell-mediated immune response, and hematological system development and function were among the top enriched physiological system development and function pathways in all PFOA treatment groups (Table 3.4). Concentration-dependent pathways included endocrine diseases for the embryonic exposure to 40 ppb and 400 ppb PFOA, while neurological diseases were one of the top enriched pathways in the 4 ppb PFOA exposure group (Table 3.3). Glioma was the most enriched disease for 4 ppb PFOA exposure group under neurological disease category. Molecular and cellular function groups were more variable among the PFOA treatment groups with

alterations associated with cell cycle; cellular development, growth, proliferation, movement, signaling, function, maintenance, death, and survival (Table 3.5), with the ERBB pathway specifically identified (Figure 3.5).

Table 3-3. Disease and disorder enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA[a,b]

PFOA			PFHxA			PFBA		
4 ppb								
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Cancer	4.40E-02 - 6.33E-04	29	**Endocrine System Disorders**	3.85E-02 - 1.58E-04	3	**Nutritional Disease**	1.05E-02 - 6.67E-05	16
Neurological Disease	1.37E-02 - 6.33E-04	10	**Gastrointestinal**	4.35E-02 - 1.58E-04	22	**Cardiovascular Disease**	1.67E-02 - 1.06E-04	21
Organismal Injury and Abnormalities	4.40E-02 - 6.33E-04	31	**Hereditary Disorder**	4.60E-02 - 1.58E-04	7	**Developmental Disorder**	1.31E-02 - 1.06E-04	17
Reproductive System Disease	4.19E-02 - 7.88E-04	17	**Metabolic Disease**	4.35E-02 - 1.58E-04	5	**Hereditary Disorder**	1.31E-02 - 1.06E-04	24
Hematological Disease	4.34E-02 - 1.21E-03	12	**Organismal Injury and Abnormalities**	4.97E-02 - 1.58E-04	27	**Metabolic Disease**	1.57E-02 - 1.06E-04	23
40 ppb								
Cancer	4.92E-02 - 4.52E-04	68	**Hematological Disease**	2.42E-02 - 1.73E-04	13	**Gastrointestinal Disease**	2.07E-02 - 1.34E-04	107
Organismal Injury and Abnormalities	4.92E-02 - 4.52E-04	68	**Immunological Disease**	3.21E-02 - 1.73E-04	12	**Organismal Injury and Abnormalities**	2.07E-02 - 1.34E-04	136
Reproductive System Disease	4.65E-02 - 4.52E-04	48	**Dermatological Diseases and Conditions**	3.21E-02 - 1.93E-04	72	**Neurological Disease**	2.07E-02 - 2.20E-04	21
Infectious Disease	8.37E-03 - 5.99E-04	5	**Organismal Injury and Abnormalities**	3.21E-02 - 1.93E-04	90	**Endocrine System Disorders**	2.07E-02 - 2.38E-04	57

Table 3-3: Continued								
Endocrine System Disorders	4.12E-02 - 9.31E-04	34	**Infectious Diseases**	2.44E-02 - 3.85E-04	8	**Renal and Urological Disease**	2.07E-02 - 3.59E-04	20
400 ppb								
Cancer	4.76E-02 - 8.99E-05	57	**Endocrine System Disorders**	4.60E-02 - 6.49E-04	12	**Cancer**	1.66E-02 - 3.38E-06	422
Endocrine System Disorders	3.82E-02 - 8.99E-05	32	**Gastrointestinal Disease**	4.60E-02 - 6.49E-04	33	**Dermatological Diseases and Conditions**	1.25E-02 - 3.38E-06	281
Gastrointestinal Disease	4.19E-02 - 8.99E-05	50	**Hereditary Disorder**	3.47E-02 - 6.49E-04	9	**Organismal Injury and Abnormalities**	1.69E-02 - 3.38E-06	430
Organismal Injury and Abnormalities	4.97E-02 - 8.99E-05	57	**Metabolic Disease**	4.97E-02 - 6.49E-04	13	**Nutritional Disease**	1.90E-03 - 1.46E-05	52
Reproductive System Disease	4.76E-02 - 3.31E-04	33	**Organismal Injury and Abnormalities**	4.97E-02 - 6.49E-04	39	**Hematological Disease**	1.69E-02 - 2.38E-05	135

[a]p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.
[b]Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 3-4. Physiological system development and function enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA[a,b]

PFOA			PFHxA			PFBA		
4 ppb								
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Renal and Urological System Development and Function	3.97E-02 - 1.41E-05	3	**Organismal Functions**	2.71E-02 - 1.49E-03	3	**Endocrine System Development and Function**	1.73E-02 - 9.22E-05	10
Cell-mediated Immune Response	3.32E-02 - 9.65E-04	6	**Connective Tissue Development and Function**	4.97E-02 - 1.96E-03	5	**Organ Development**	1.11E-02 - 9.22E-05	19
Hematological System Development and Function	4.40E-02 - 9.65E-04	9	**Embryonic Development**	3.28E-02 - 1.96E-03	3	**Behavior**	1.62E-02 - 4.22E-04	29

Table 3-4: Continued								
Immune Cell Trafficking	3.75E-02 - 9.65E-04	8	**Hematological System Development and Function**	4.03E-02 - 1.96E-03	5	**Hematological System Development and Function**	1.48E-02 - 6.29E-04	15
Connective Tissue Development and Function	2.66E-02 - 2.25E-03	3	**Hematopoiesis**	3.66E-02 - 1.96E-03	1	**Immune Cell Trafficking**	1.04E-02 - 6.29E-04	3
40 ppb								
Cell-mediated Immune Response	3.71E-02 - 3.39E-03	5	**Embryonic Development**	3.21E-02 - 1.93E-04	19	**Nervous System Development and Function**	2.07E-02 - 2.09E-05	14
Hematological System Development and Function	4.92E-02 - 3.39E-03	17	**Organismal Development**	3.21E-02 - 1.93E-04	24	**Digestive System Development and Function**	2.07E-02 - 1.34E-04	8
Immune Cell Trafficking	3.71E-02 - 3.39E-03	11	**Tissue Morphology**	3.21E-02 - 1.93E-04	20	**Organ Morphology**	2.07E-02 - 1.34E-04	25
Connective Tissue Development and Function	4.92E-02 - 4.19E-03	7	**Hematological System Development and Function**	3.21E-02 - 2.22E-04	26	**Organismal Developmental**	2.07E-02 - 1.34E-04	33
Digestive System Development and Function	2.08E-02 - 4.19E-03	1	**Humoral Immune Response**	3.21E-02 - 3.18E-04	14	**Organ Development**	2.07E-02 - 3.59E-04	18
400 ppb								
Hematological System Development and Function	4.76E-02 - 5.78E-04	19	**Connective Tissue Development and Function**	4.97E-02 - 8.79E-05	10	**Tissue Morphology**	1.51E-02 - 1.28E-05	104
Immune Cell Trafficking	4.09E-02 - 5.78E-04	10	**Embryonic Development**	4.97E-02 - 2.42E-03	18	**Hematological System Development and Function**	1.63E-02 - 2.16E-04	86
Lymphoid Tissue Structure and Development	4.76E-02 - 1.81E-03	17	**Hematological System Development and Function**	4.97E-02 - 2.42E-03	11	**Connective Tissue Development and Function**	4.85E-03 - 2.61E-04	10
Hematopoiesis	3.68E-02 - 2.45E-03	8	**Hematopoiesis**	4.97E-02 - 2.42E-03	8	**Skeletal and Muscular System Development and Function**	1.07E-02 - 2.61E-04	12
Cell-mediated Immune Response	3.64E-02 - 2.70E-03	5	**Humoral Immune Response**	4.97E-02 - 2.42E-03	4	**Tissue Development**	7.66E-03 - 2.61E-04	31

[a]p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b]Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 3-5. Molecular and cellular function enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA[a,b]

PFOA			PFHxA			PFBA		
4 ppb								
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Cell Cycle	4.14E-02 - 1.41E-05	12	**Molecular Transport**	4.97E-02 - 2.11E-05	4	**Cell Cycle**	1.91E-02 - 4.95E-06	14
Cellular Development	4.19E-02 - 2.63E-04	16	**Nucleic Acid Metabolism**	1.55E-02 - 2.11E-05	2	**Cellular Development**	1.79E-02 - 9.22E-05	50
Cellular Growth and Proliferation	3.63E-02 - 2.63E-04	13	**Small Molecule Biochemistry**	4.97E-02 - 2.11E-05	5	**Cellular Growth and Proliferation**	1.67E-02 - 9.22E-05	47
Cellular Movement	4.34E-02 - 2.75E-04	18	**Cell Cycle**	2.13E-02 - 1.96E-03	2	**Cell Morphology**	1.31E-02 - 6.29E-04	18
Cell Death and Survival	4.40E-02 - 3.07E-04	17	**Cell Morphology**	3.85E-02 - 1.96E-03	5	**Cell-to- Cell Signaling and Interaction**	1.46E-02 - 6.29E-04	13
40 ppb								
Cell Cycle	4.52E-02 - 2.52E-04	8	**Cell Morphology**	3.21E-02 - 1.93E-04	18	**Cell-to- Cell Signaling and Interaction**	2.07E-02 - 2.09E-05	13
Cell Death and Survival	4.76E-02 - 7.47E-04	10	**Cellular Function and Maintenance**	2.77E-02 - 2.22E-04	29	**Cellular Assembly and Organization**	2.07E-02 - 2.09E-05	16
Cellular Movement	3.85E-02 - 3.39E-03	11	**Protein Synthesis**	2.91E-02 - 3.18E-04	12	**Lipid Metabolism**	2.07E-02 - 2.09E-05	37
Amino Acid Metabolism	4.19E-03 - 4.19E-03	1	**Cell Death and Survival**	3.21E-02 - 3.85E-04	15	**Molecular Transport**	2.07E-02 - 2.09E-05	34
Carbohydrate Metabolism	4.92E-02 - 4.19E-03	5	**Cellular Development**	3.21E-02 - 3.85E-04	27	**Small Molecule Biochemistry**	2.07E-02 - 2.09E-05	46
400 ppb								
Cellular Development	4.59E-02 - 5.78E-04	24	**Cell Morphology**	4.97E-02 - 8.79E-05	12	**Drug Metabolism**	1.04E-02 - 3.56E-07	18
Cell Signaling	4.59E-02 - 7.17E-04	10	**Cellular Development**	4.97E-02 - 8.79E-05	15	**Lipid Metabolism**	1.56E-02 - 4.82E-07	99

Table 3-5: Continued								
Molecular Transport	4.59E-02 - 7.17E-04	11	**Cellular Growth and Proliferation**	4.97E-02 - 2.42E-03	11	**Molecular Transport**	1.35E-02 - 4.82E-07	94
Vitamin and Mineral Metabolism	4.59E-02 - 7.17E-04	10	**Carbohydrate Metabolism**	4.22E-02 - 3.91E-03	9	**Small Molecule Biochemistry**	1.56E-02 - 4.82E-07	126
Cellular Function and Maintenance	4.59E-02 - 1.19E-03	16	**Cell Cycle**	4.60E-02 - 3.91E-03	6	**Vitamin and Mineral Metabolism**	1.41E-02 - 1.97E-06	50

[a]p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b]Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

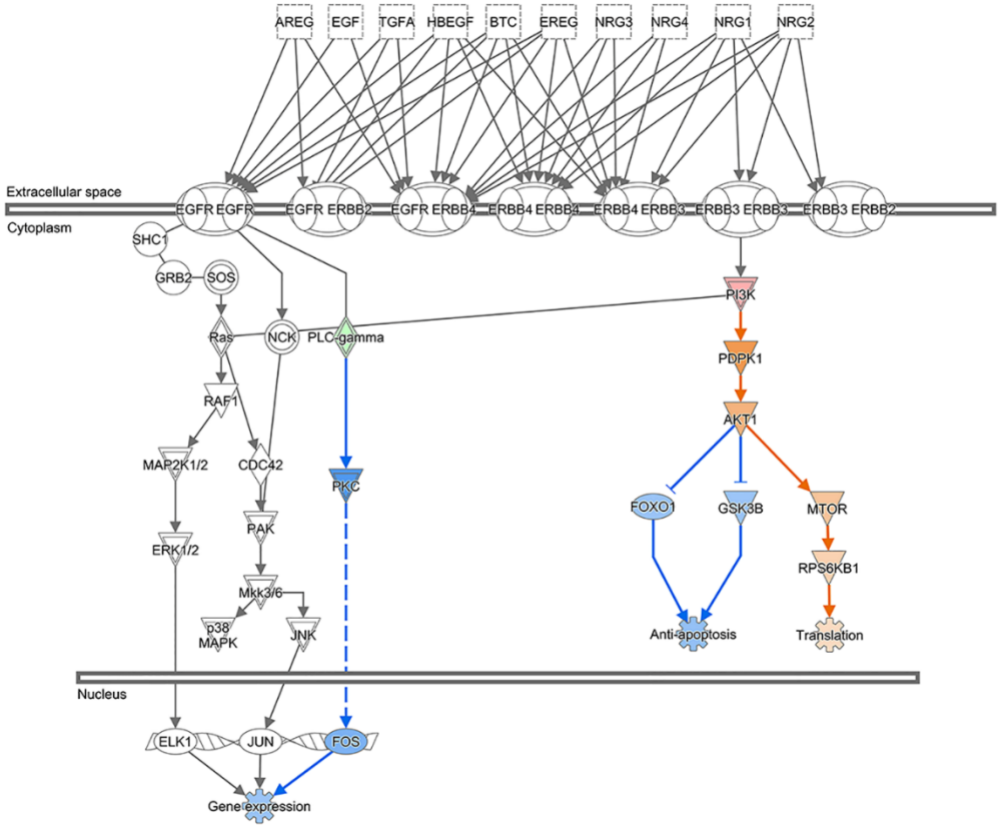

Figure 3-5: ERBB signaling pathway alterations associated with 4 ppb PFOA treatment group. Triangle indicates kinase, oval shape indicates transcription regulator, and diamond shape indicates enzyme. Red indicates upregulation, while green indicates down regulation. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates a direct interaction and dashed line indicates an indirect interaction. A dotted line indicates that it is predicated but not confirmed in literature.

There were 32 mapped genes with altered expression in the 4 ppb PFHxA exposure, 113 genes with altered expression in the 40 ppb PFHxA exposure, and 50 genes with altered expression in the 400 ppb PFHxA exposure (Figure 3.4). In addition, there were 9 common genes altered in all three PFHxA treatment groups including *DUSP29, EXOSC6, KLF9, LRRC3, PFKB4, PHLPP2, RHCG, TLN2,* and *UCP3.* The embryonic exposure to 4 and 400 ppb PFHxA resulted in the same disease and disorder pathways including endocrine system disorders, gastrointestinal disease, hereditary disorder, metabolic disease, and organismal injury and abnormalities, while exposure to 40 ppb PFHxA resulted in disease and disorder pathways related to organismal injury and abnormalities and hematological, immunological, dermatological, and infectious diseases (Table 3.3). For the physiological system development and function pathways, hematological system development and function was identified to be altered in all three PFHxA treatment groups similar to PFOA (Table 3.4). In addition, the embryonic development pathway was enriched in all three PFHxA treatment groups (Table 3.4). Other similarities included connective tissue development and function and hematopoiesis pathways in the 4 and 400 ppb PFHxA treatment groups and the humoral immune response pathway in the 40 and 400 ppb PFHxA treatment groups (Table 3.4). Similar to PFOA, there was more variability in the top enriched pathways associated with molecular and cellular functions with some similarities in cell cycle and cell development, growth, proliferation, morphology, function, maintenance, death, and survival (Table 3.5). The top canonical pathways in the three PFHxA treatment groups indicated immune system-related pathways such as the cross talk between dendritic cells and natural killer cells at 4 ppb PFHxA; altered T cell and B cell signaling in Rheumatoid Arthritis, Toll-like receptor signaling, and communication between innate and adaptive immune cells at 40 ppb PFHxA; and primary immunodeficiency signaling at 400 ppb PFHxA. Significant expression changes of B-cell linker protein (BLNK) and CD40 was observed in 40 and 400 ppb PFHxA treatment groups. The BLNK gene encodes a protein that plays a role in B-cell development, while CD40 is a receptor protein on antigen-presenting cells of the immune system.

Transcriptomic analysis of 72 hpf zebrafish with embryonic exposure to PFBA identified substantially more genes compared to PFOA and PFHxA with 171 mapped genes with altered expression in the 4 ppb PFBA exposure, 165 genes with altered expression in the 40 ppb PFBA exposure, and 469 genes with altered expression in the 400 ppb PFBA exposure (Figure 3.4). There were only 5 common genes altered in all three treatment groups, which were *ACE2, BC01, C3,*

CEL, and *ENPP7*. There were no similar disease and disorder pathways for all three PFBA treatments group, but the nutritional disease pathway was identified for the 4 and 400 ppb PFBA treatment groups and the organismal injury and abnormalities pathway for the 40 and 400 ppb PFBA treatment groups (Table 3.3). All other pathways were only present in a single PFBA treatment group including neurological diseases in the 40 ppb PFBA exposure groups with genes specifically associated with neurodegenerative diseases such as Alzheimer's disease, dementia, onset of amyotrophic lateral sclerosis, and schizophrenia. Cancer was the top enriched disease for the 400 ppb PFBA exposure group (Table 3.3) with results showing genes associated with skin cancer, specifically cutaneous tumor which was among the top significantly altered cancer-related genes in the 400 ppb PFBA and PFOA treatment groups (Table 3.3). For the pathways related to physiological system development and function, again there were no pathways common to all three PFBA treatment groups with only organ development in the 4 and 40 ppb PFBA treatment groups and hematological system development and function in the 4 and 400 ppb PFBA treatment groups (Table 3.4). All other pathways were concentration-dependent with endocrine system development and function and behavior in the 4 ppb PFBA treatment group; nervous system development and function along with organ morphology and organismal development in the 40 ppb PFBA treatment group; and tissue morphology and development along with connective tissue and skeletal and muscular system development and function in the 400 ppb PFBA treatment group (Table 3.4). There were more similarities in the pathways related to molecular and cellular function in the PFBA treatment groups compared to PFOA and PFHxA (Table 3.5). Cell-to-cell signaling and interaction pathway was identified in the 4 and 40 ppb PFBA exposure groups; while multiple pathways including lipid metabolism, molecular transport, and small molecule biochemistry were in both the 40 and 400 ppb PFBA treatment groups (Table 3.5) with several regulators and functions associated with lipid oxidation, absorption, uptake, and transport (Figure 3.6). In addition, FXR/RXR activation was among the most enriched canonical pathway in all three PFBA treatment groups with several pathways showing activation and/or inhibition including the role of FXR in lipoprotein and lipid metabolism, trans-repression of FXR during acute phase response, role of FXR in bile acid homeostasis, and role of FXR in glucose homeostasis (Figure 3.7).

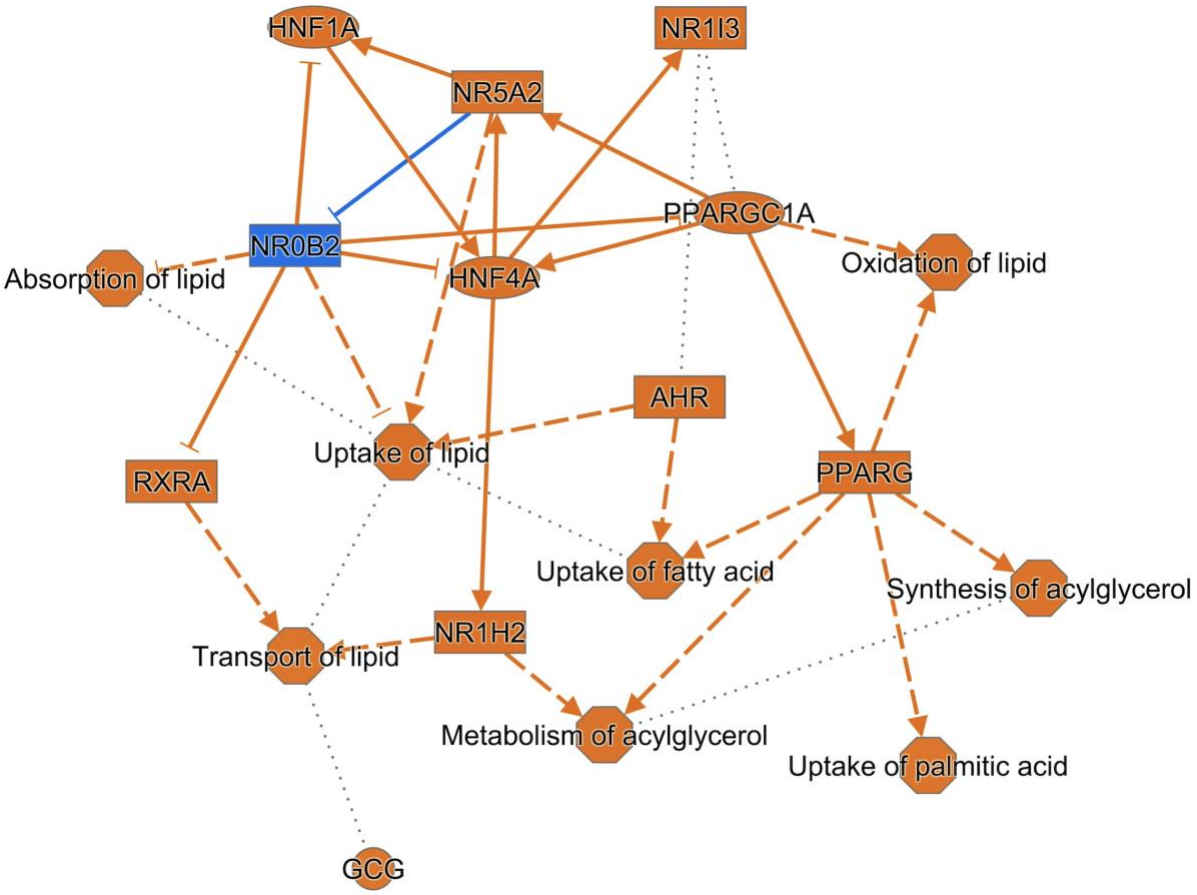

Figure 3-6: Graphical summary of the top altered regulators and functions in zebrafish exposed to 400 ppb PFBA during embryogenesis. The top regulators and functions are primarily associated with lipid pathways. Rectangles represent ligand-dependent nuclear receptor, hexagons represent function, ovals represent transcription regulator, and circles represent other molecules. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates direct interaction and a dashed line indicates indirect interaction. A dotted line indicates that the association is predicated but not confirmed in literature.

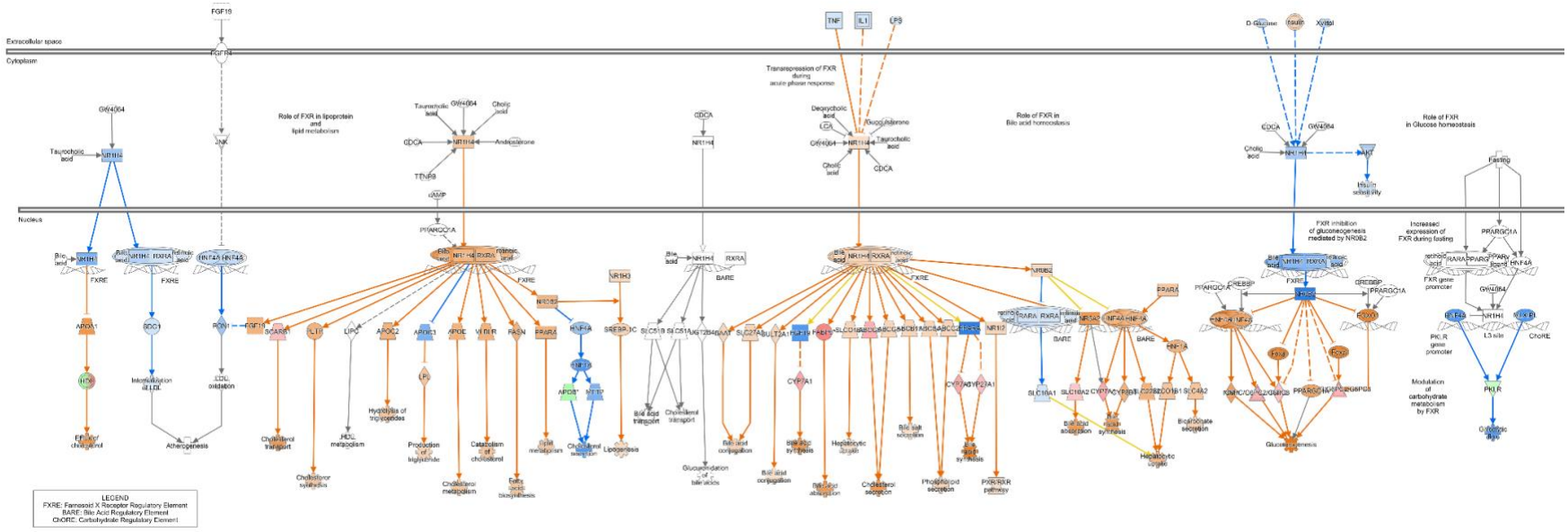

Figure 3-7: Multiple FXR pathways are activated and/or inhibited in zebrafish exposed to 400 ppb PFBA during embryogenesis. Rectangles represent ligand-dependent nuclear receptor, hexagons represent function, ovals represent transcription regulator, and circles represent other molecules. Red indicates upregulation of a molecule and green indicates down regulation. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates a direct interaction, and a dashed line indicates an indirect interaction. A dotted line indicates that the association is predicated but not confirmed in literature.

3.4.5 Comparison of Transcriptome Alterations with an Embryonic Exposure to PFOA, PFHxA, or PFBA

Comparative enrichment pathway analysis among the three PFAS overall identified unique signatures for each chemical and were also sometimes limited to a single treatment group for that chemical. For example, altered genes associated with neurotransmitter and nervous system signaling were primarily limited to the 4 ppb PFOA exposure group showing changes in multiple related canonical pathways (Figure 3.8). On the other hand, the 4 ppb PFHxA treatment group had association with netrin signaling and the 4 ppb PFBA treatment group affected dopamine-DARPP32 feedback in cAMP signaling (Figure 3.8). Interestingly, all of these neurological associated signaling pathways were only observed in the lowest exposure group (4 ppb) of each chemical tested in this study.

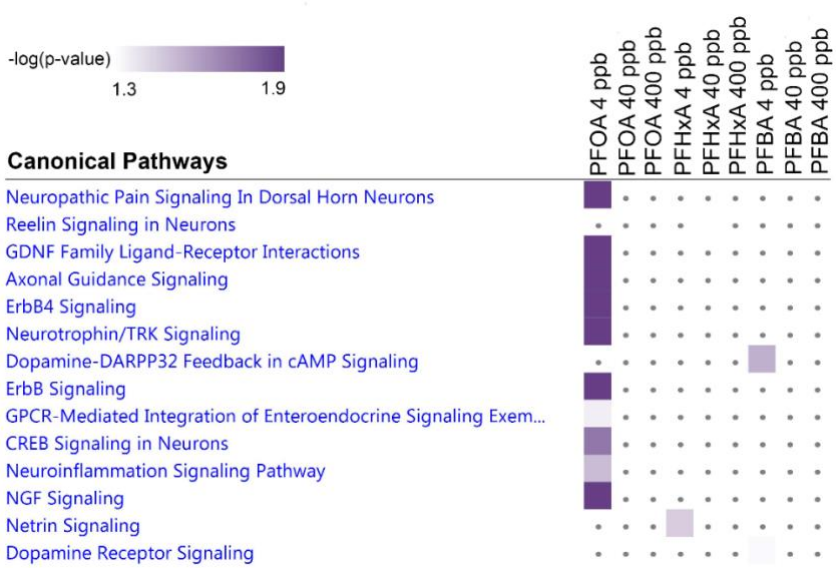

Figure 3-8: Comparison analysis showing canonical pathways related to neurotransmitter and nervous system signaling following embryonic exposure to PFOA, PFHxA, or PFBA. Purple color indicates statistical significance (-log (p-value) = 1.3). Intensity of color correspond to – log (p-value).

The comparison analysis also showed that the FXR/RXR and LXR/RXR activation pathways were mainly limited to the PFBA treatment groups (Figure 3.9). In addition, TR/RXR and PXR/RXR activation and LPS/IL-1 mediated inhibition of RXR function was limited to only the 400 ppb PFBA exposure group (Figure 3.9). Alternatively, alterations in aldosterone signaling in epithelial cells pathway was only observed in the PFOA treatment groups (Figure 3.9).

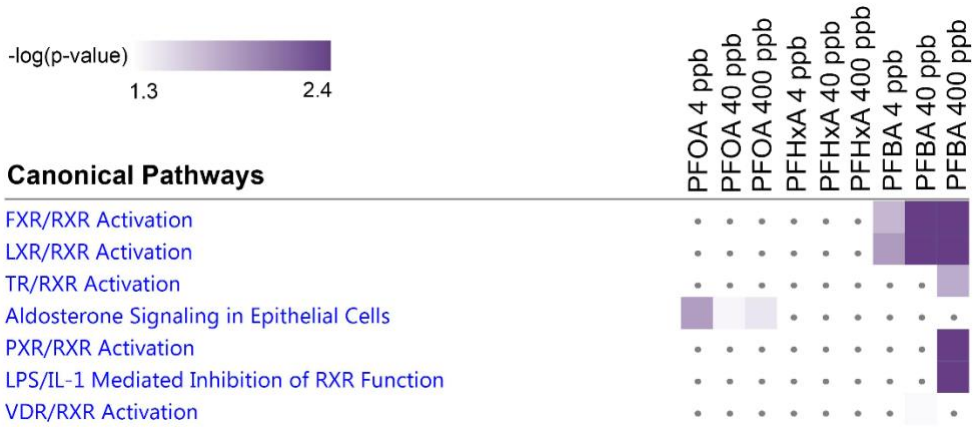

Figure 3-9: Comparison analysis showing canonical pathways related to nuclear receptor signaling following embryonic exposure to PFOA, PFHxA, or PFBA. Purple color indicates statistical significance (-log (p-value) = 1.3). Intensity of color correspond to – log (p-value).

Furthermore, exposure to PFOA or PFBA affected many cellular immune response canonical pathways, but there was no overlap between the affected pathways for the two chemicals (Figure 3.10). PFOA treatment groups showed a non-monotonic concentration response in the number of canonical pathways that were associated with cellular immune response between the 4, 40, and 400 ppb treatment groups. Additional unique pathways were identified in the 40 ppb PFBA treatment group and only 1 pathway was noted in the 400 ppb PFHxA treatment group (Figure 3.10).

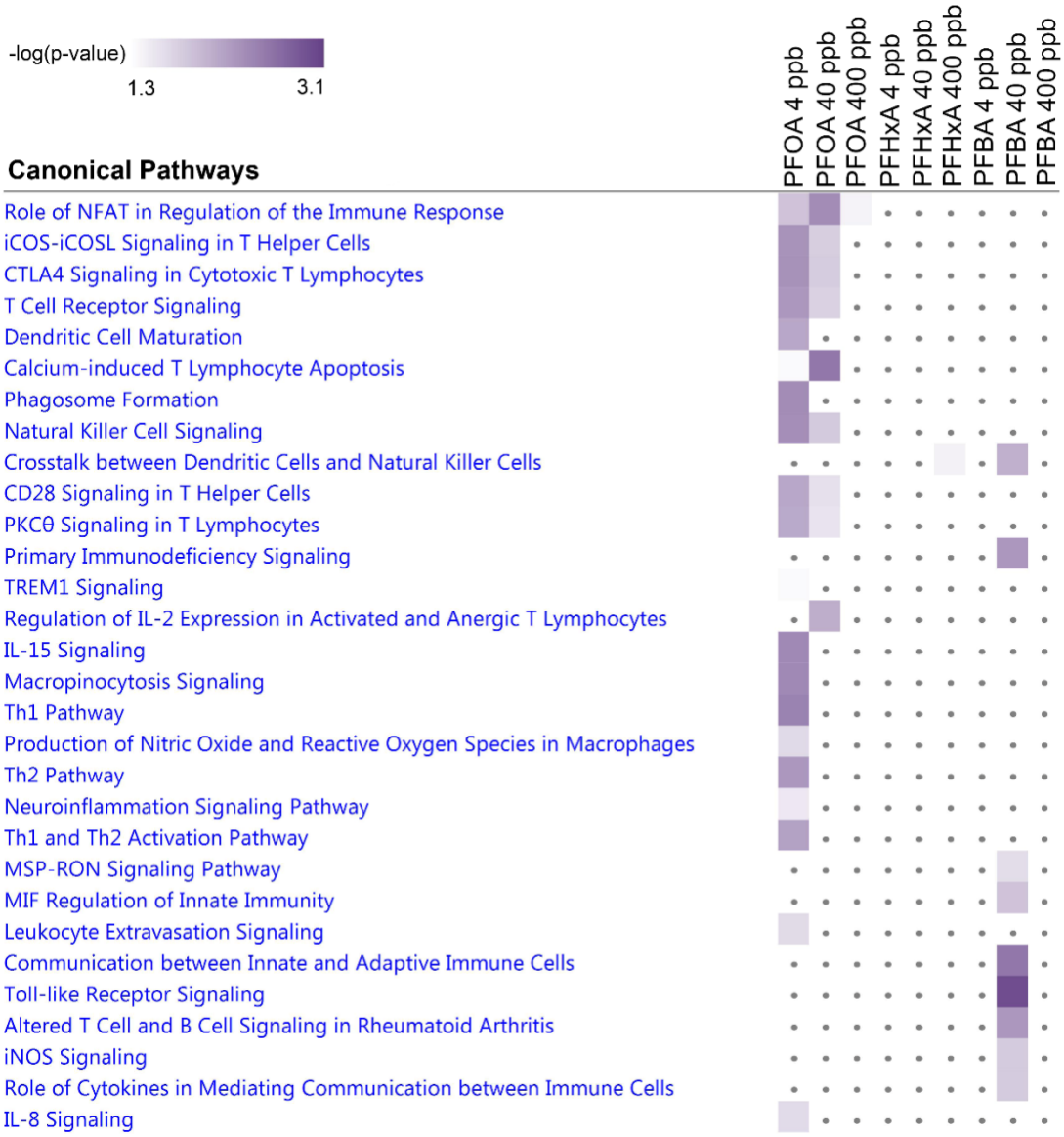

Figure 3-10: Comparison analysis showing canonical pathways related to cellular immune response following embryonic exposure to PFOA, PFHxA, or PFBA. Purple color indicates statistical significance (-log (p-value) = 1.3). Intensity of color correspond to – log (p-value).

3.5 Discussion

PFAS are a class of chemicals that are widely used in consumer and industrial products. Legacy PFAS (e.g., PFOA) being replaced by other shorter chain PFAS (e.g., PFHxA and PFBA) to increase their degradability in the environment and decrease their bioaccumulation in humans and other organisms, but limited toxicological data are available for these next generation PFAS. The goal of our study was to compare the developmental toxicity and developmental neurotoxicity

of shorter chain PFAS to legacy compounds. Our results revealed that the 120 hpf-LC50 for PFOA, PFHxA, and PFBA were 561.05, 8394.5, and >10,000 ppm, respectively. These results are in agreement with previously published data for these chemicals in the developing zebrafish, where LC50 decreases with increasing chain length (Ding et al. 2013; Godfrey et al. 2017; Hagenaars et al. 2011; Ulhaq et al. 2013a; Wasel et al. 2021).These results are also in agreement with those of the previous chapter where the exposure started at 5 hpf instead of 1 hpf. On the other hand, other zebrafish studies reported the 120 hpf-LC50 of PFOA to be around 72 ppm (continuous exposure started at <4 hpf) (Gebreab et al. 2020) and the 120 hpf-LC50 of PFHxA to be 290 µM (91 ppm) (exposure initiated at 3 hpf) (Annunziato et al. 2019). These values are much lower than our results. PFOA, PFHxA, and PFBA are acids, and highly concentrated solutions will overcome the buffering capacity of most solutions that zebrafish researchers use in their toxicity studies (Wasel et al. 2021). As such, if the solutions are not neutralized, the low pH of the dosing solution will greatly affect the survival of zebrafish embryos and larvae, which is hypothesized to be responsible for the difference observed in these two studies compared to the current findings.

LC50 is a quick measure for acute toxicity and it is used for environmental risk assessment, but it is less relevant to the environmental exposures and general human population exposure. It is very important to assess the effect of sublethal exposure. Our research is showing that although the acute toxicity of PFOA is much higher than shorter chain PFAS, the short chain PFAS can cause adverse effects at sublethal exposures.

Behavioral changes in developing zebrafish with PFAS exposure has been investigated in multiple studies, but the majority of these studies used relatively high concentrations (summarized in Table 3.6). In our study, sublethal concentrations (0.4, 4, 40, 400, and 4000 ppb) were used to assess effects of exposure on neurobehavior by assessing locomotor activity alterations. Evaluation of locomotor activity of zebrafish has been used to identify neuroactive drugs and assessment of neurobehavior of environmental toxicants (Basnet et al. 2019). In the visual motor response assay, zebrafish larvae are exposed to alternating periods of dark and light with locomotor activity increased in dark periods and decreased in light periods. Our goal was to assess the effect of an embryonic exposure to PFOA, PFHxA, or PFBA. Thus, the chemical treatments were ceased at the end of embryogenesis (at 72 hpf) and behavioral endpoints measured at 120 hpf, when the larvae have an inflated swim bladder and reliable movement (Padilla et al. 2011). The results showed that exposure to 400 or 4000 ppb PFOA increased the total distance moved and mean

velocity, which agrees with a past observation of hyperactivity in 120 hpf larvae exposed to 414 ppb PFOA (Rericha et al. 2021). Two additional studies also observed that PFOA exposure, albeit at higher concentrations, caused hyperactivity in 144 hpf larvae (Menger et al. 2020; Ulhaq et al. 2013b). Alternatively, a study using similar concentrations showed that a 10, 100, or 1000 ppb PFOA exposure decreased locomotor activity in zebrafish larvae aged 168 hpf and linked behavior changes to alterations in development and function of dopaminergic neurons (Yu et al. 2021). On the other hand, Khezri et al. (2017) at 96 hpf and Gaballah et al. (2020) at 144 hpf did not find that a developmental PFOA exposure altered behavior.

In our study, the embryonic PFHxA exposure did not induce any behavioral changes, which agrees with two other studies (Annunziato et al. 2019; Menger et al. 2020). Alternatively, hyperactivity was observed at 144 hpf but was at concentrations greater than were tested in the current study (Gaballah et al. 2020). Furthermore, another study found that exposure to 480 ppb PFHxA caused an increase in swimming activity in 120 hpf zebrafish larvae, but the effect wasn't observed in the higher exposure groups at 2,400 or 12,000 ppb (Guo et al. 2021).

PFBA exposure caused hypoactivity in all dark phases with effects in the highest treatment group (4000 ppb) being the most persistent in all outcomes measured. In addition, in the first light phase, hypoactivity was observed only in the 0.4 ppb exposure group in all outcomes. Furthermore, hyperactivity was also observed in the 4 ppb PFBA treatment group in only the first dark phase. As a result, PFBA caused behavioral changes at lower concentrations compared to PFOA. These biphasic effects on behavior have also been observed for other toxicants including ethanol where 1% and 2% ethanol increased activity of 144 hpf larvae and 4% ethanol caused a severe decrease in activity (MacPhail et al. 2009). Our findings are in contrast to two other studies reporting hyperactivity at 120 or 144 hpf in dark and light or only dark phases (Ulhaq et al. 2013b; Rericha et al. 2021).

Overall, the behavioral results showed that PFOA (mainly hyperactivity) and PFBA (mainly hypoactivity) induced behavioral alterations but were absent for PFHxA. In addition, these behavioral alterations were more prominent at lower exposure concentrations for PFBA compared to PFOA leading to the conclusion that the behavioral perturbations do not increase with chain length as is observed in acute toxicity studies. Furthermore, the overall trend of hyperactivity for PFOA and hypoactivity for PFBA suggest different toxicity mechanisms. The concentration-dependent and biphasic effects may also help to explain contradicting epidemiological results

regarding developmental neurotoxicity. For example, some studies observed an increase in risk of attention deficient/hyperactivity disorder (ADHD) and reduced executive functioning (Oulhote 2016) with PFAS exposure, while other studies showed no association between PFAS exposure and behavioral outcomes and motor coordination (Liew et al. 2018).

As noted above and as summarized in Table 3.6, our behavioral results agree with some studies but that there are differences in outcomes reported in the literature among the zebrafish larvae PFAS studies. These discrepancies may be attributed to differences in length of exposure (e.g., through 72 hpf compared to through 120 hpf) and/or the use of DMSO as a solvent. Although, DMSO at concentrations of 0.5% or less are reported not to alter behavior of zebrafish larvae (Padilla et al. 2011), DMSO can affect the uptake of the chemicals (Kais et al. 2013). It is important to note that multiple factors can affect behavior of zebrafish larvae including concentration of chemicals, age of larvae, duration of exposure, lighting conditions, dosing regime (static or daily renewal of dosing solutions), and rearing temperature (Fraser et al. 2017; Padilla et al. 2011) (Table 3.6). Another important factor that can affect results is the number of biological replicates and subsamples used in the experiments to influence statistical power. In our study, we used more than 100 subsamples per treatment from multiple biological replicates to account for intra-larvae variability to increase reliability of results (Ingebretson and Masino 2013).

Table 3-6. Summary of behavioral data from studies exposed developing zebrafish to PFOA, PFHxA, or PFBA

PFAA	Strain	Concentrations, Exposure Period	Age at assessment	Temperature	Well Plate	Light/dark protocol	Outcomes	Reference
PFOA	AB	100 X higher concentraion than human serum, 6-96 hpf	96 hpf	28 ± 1°C		10 min of light for acclimation; 10 min of light; 10 min of dark; 10 min of light	No effect on average swimming speed	Khezri et al. 2017
PFOA	Mixed wild type	1,822-33,125 ppb (4.4-80 µM), 1-144 hpf	144 hpf	26°C	96-well plate	20 min of dark for acclimation followed by 20 min light (L1: 10min then L2: 10 min) and 20 min dark (D1: 10 min then D2: 10 min)	No effect on locomotor activity	Gaballah et al. 2020
PFOA	AB	3,000-1,000,000 ppb, 0.5-144 hpf	144 hpf	26 ± 1°C	48-well plate	10 min of light for acclimation followed by two series of 10 min dark and 10 min light	Increased in activity in dark phases	Ulhaq et al. 2013b
PFOA	Wild type	0.1-100,000 ppb, 1-144 hpf	144 hpf	26 ± 1°C	96-well plate	10 min of light for acclimation followed with 4 series of 5 min dark and 5 min light	4,968 and 62,110 ppb (12 and 150 µM) increased swimming distance in dark phase	Menger et al. 2020

Table 3-6: Continued								
PFOA	5D	83-484 ppb (0.2−1.17 μM), 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	414 ppb (1 μM) caused hyperactivity in dark phase	Richa et al. 2021
PFOA	AB	25 ppb ppb, 2-30 hpf	120 hpf	28.5± 0.5°C	96-well plate	10 min of dark for acclimation, 10 min of alternating periods of dark and light for 60 min	Decreased total distance moved and average velocity	(Yu et al. 2022)
PFOA	AB Strain	10-1000 ppb at 2 - 168 hpf, solutions renewed daily	168 hpf	28.5± 0.5 °C	96-well plate	10 min of dark for acclimation, 10 min of alternative periods of dark and light for 60 min	Decreased total distance and average velocity	(Yu et al. 2021)

Table 3-6: Continued								
PFHxA	Wild type (strain not specified)	0.1-100,000 ppb, 1-144 hpf	144 hpf	26 ± 1°C	96-well plate	10 min of light for acclimation followed with 4 series of 5 min dark and 5 min light	No significant effects observed	Menger et al. 2020
PFHxA	5D	314-31,400 (1−100 µM), 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	785 and 5,150 ppb (2.5 and 16.4 µM) caused hyperactivity	Rericha et al. 2021
PFHxA	AB	62.8-6,280 ppb (0.2-20 µM), 3-120 hpf	14 dpf	26-27°C	24-well plate	30 min of dark	No changes in total distance moved and mean velocity	Annunzia to et al. 2019
PFHxA	AB	480, 2,400, 12,000 ppb, 2-120 hpf	120 hpf	28°C	24-well plate	10 min acclimation, the 4 cycles of 5 light, 5 min dark, and 5 min light	Only the 480 ppb caused increase in swimming activity	Guo et al. 2021

Table 3-6: Continued								
PFBA	5D	214-21,400 ppb (1-100 µM, 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	535, 1,391, 7491, and 15,839, and 21,404 ppb (2.5, 16.5, 35, 74, and 100 µM) caused hyperactivity in light and dark phases	Rericha et al. 2021
PFBA	AB	10,000-3,000,000 ppb, 0.5-144 hpf	144 hpf	26 ± 1 °C	48-well plate	10 min of light for acclimation followed by two series of 10 min dark and 10 min light	Increased activity in dark phases	Ulhaq et al. 2013b

Morphological measurements are reliable indicators of proper development of zebrafish (Kimmel et al.1995). Most studies evaluate gross malformation, but few studies have measured fine morphological changes after PFAA exposure. Our study showed that exposure to 4 ppb PFOA decreased the total body length and head width; however, there was no significant change in the ratio of head width to total body indicating that 4 ppb PFOA exposure resulted in overall smaller fish. Exposure to 400 ppb PFOA resulted in a decreased head length, head width, and ratio of the head length to body length suggesting that the 400 ppb PFOA exposure caused an unproportionate decrease in the head length. These findings align with a report that only exposure to 838 ppb PFOA from 3-120 hpf, but not 8.3 or 83 ppb increased total body length and yolk sac area and decreased interocular distance (similar to the head width) (Jantzen et al. 2016). For PFHxA, exposure to 40 ppb decreased total body length, head width, and head length with no changes in the morphological ratios. In addition, 400 ppb PFHxA exposure decreased head length and the head length to body length ratio. These findings are similar to a decreased body length and decreased yolk sac size at 120 hpf following 83 ppb PFHxA exposure (Annunziato et al. 2019). In addition, in rats an oral maternal exposure to PFHxA decreased body weight in offspring (Loveless et al. 2009). PFBA

exposure also caused concentration-dependent morphological alterations with a decrease in body length in the 4 and 400 ppb PFBA exposure groups. There was also an increase in head width in the 400 ppb PFBA-treated fish and an increase in the head width to body length ratio suggesting that the decrease in head width wasn't proportional to the decrease in body length.

Lastly, transcriptome analysis was completed in whole eleuthero-embryos following the embryonic PFAA exposure to compare genes and molecular pathway alterations among the different chain lengths. In addition, PFOA molecular pathways were compared to the literature to determine if perturbations in the developing zebrafish were concordant to adverse health outcomes observed in human populations. Overall, chemical- and concentration-dependent genes and molecular pathway alterations were detected with only a small number of genes being the same among the treatment groups of each PFAA (i.e., 5 for PFBA and 9 for PFHxA and PFOA).

For PFOA, the higher treatment groups (40 and 400 ppb) had a larger number of genes altered that were associated with cancer including breast, liver, prostate, ovarian, pancreatic, and testicular cancer (probable link identified in C8 Science Panel). In addition, the ERRB pathway, which is associated with many human cancers was specifically identified. PFOA is classified as possibly carcinogenic to humans by IARC due to a positive association between PFOA exposure and testicular and kidney cancers (IARC 2007). A National Toxicological Program (NTP) study also indicated strong evidence of association between PFOA exposure and increased incidence of hepatocellular neoplasms and increased incidence of acinar cell neoplasms of the pancreas, based on a 2-year study in male rats (NTP 2020). While, both 40 and 400 ppb PFOA exposure groups had enrichment in endocrine system disease disorders, each treatment group had unique pathways with exposure to 400 ppb PFOA alterations associated with thyroid cancer and the 40 ppb PFOA exposure associated with multiple pathways related to the female reproductive system. A 2017 review assessed the effects of PFOA on the thyroid gland using in vitro studies, animal studies, and human data and concluded that PFOA exposure reduces the circulating levels of thyroid hormones in animals (Coperchini et al. 2017). While this review also concluded the risk of thyroid cancer from PFOA exposure was low, the epidemiological studies showed contradicting conclusions including data from the C8 Science Panel that indicated a probable link with thyroid disease (Coperchini et al. 2017). Furthermore, among the 9 common altered genes in the three PFOA treatment groups and in multiple molecular pathways, genes associated with the immune system were identified. For example, *BATF* and *PLCG1* are associated with cell movement of

$CD4^+$ T-lymphocytes, homing of $CD4^+$ T-lymphocytes, homing of TREG cells, and migration of $CD4^+$ T-lymphocytes. These results align with other epidemiological studies that link PFOA to immunological diseases and the consideration that PFOA is an immune hazard due to its roles in suppression of the antibody response and increased hypersensitivity (NTP 2016). Interestingly, exposure of zebrafish embryos to PFOA lead to changes in genes involved in immune cell function and immune and trafficking in the 7 day-old F2 generation (Haimbaugh et al. 2022).

There are limited available studies on the toxicological effects of PFHxA and even less studies focused on its developmental toxicity. It was reported in a toxicological review for PFHxA released by the US EPA that PFHxA exposure is likely to cause developmental, hematopoietic, and hepatic effects in humans (US EPA, 2022), which aligns well with the observed perturbed molecular pathways in our study. For example, our results showed that exposure to 4, 40, or 400 ppb PFHxA caused alterations in genes associated with embryonic development. Other studies in rats have shown that 200 mg/kg/day of PFHxA decreased hemoglobin and red blood cells (RBCs) and in chronic and subchronic studies decreased reticulocytes (Chengelis et al. 2009; Klaunig et al. 2015; Loveless et al. 2009; NTP, 2018). Hematological disease was the top enriched disease in the 40 ppb PFHxA treatment group and hematological system development and function was a common physiological system development and function pathway in all PFHxA treatment groups. We also observed alterations in genes involved in the immune system in all the PFHxA exposure groups. Animal studies have shown an unclear pattern of the potential effects of PFHxA on the immune system including decreased spleen weights in male rats receiving 500 mg/kg/day PFHxA sodium salt for 90 days and alterations in immune cell counts in PFHxA exposure ranges between 20 to 500 mg/kg/day (Loveless et al. 2009). On the hand, no changes in immune cell counts or weight of spleen or thymus was observed after exposure to 200 mg/kg/day for 90 days in rats (Chengelis et al. 2009). In addition, a case-control study in children in Taiwan showed no association between PFHxA exposure and immunological markers (Dong et al. 2013). Metabolic disease was also among the top enriched disease pathways in the 4 and 400 ppb PFHxA exposure groups with expression alterations in uncoupled protein 3 (UCP3) and glyecerol-3 phosphate dehydrogenase (GPD2). UCP3 has a role in fatty acid metabolism, energy homeostasis, and modulates insulin sensitivity (Liu et al. 2013). UCP3 alterations are associated with familial non-insulin-dependent diabetes mellitus, severe obesity, and type 2 diabetes pathways, which is

concordant with a study in pregnant women reporting links with PFHxA exposure and gestational diabetes mellitus and impaired glucose homeostasis (Liu et al. 2019).

The PFBA exposures resulted in the highest number of gene alterations compared to PFOA and PFHxA. With PFBA as one the top replacements for PFOA, there is currently a lot of focus on furthering the understanding of health risks associated with PFBA. In the most recent toxicological review of PFBA by the US EPA, it was concluded that PFBA exposure may cause developmental, thyroid, and liver effects in humans in utero or during adulthood (US EPA 2021). PFBA treatment groups in our study resulted in distinct molecular pathways including neurodegenerative diseases in the 40 ppb PFBA exposure group and cancer in the 400 ppb PFBA exposure group. Additionally, the endocrine system development and function pathway was enriched in the 4 ppb PFBA exposure group and was associated with proliferation of pancreatic cells, proliferation of endocrine cells, proliferation of islet cells, and proliferation of beta islets cells. Endocrine disease was also one of the top disease pathways for the 40 ppb PFBA treatment group, which was associated with benign thyroid nodule, diabetes mellitus, hyperparathyroidism, and carcinoma of the pancreas. Moreover, canonical pathways were similar among the three PFBA treatment groups for their association with lipid metabolism, molecular transport, and small molecular biochemistry. Activation of the FXR/RXR pathway was of the most significant findings present within all three PFBA treatment groups in a concentration-response manner (i.e., 22% overlap at 400 ppb PFBA, 9.3% overlap at 40 ppb PFBA, and 5.6% overlap at 4 ppb PFBA for this pathway). In addition, comparative analysis revealed that the 4 and 40 ppb PFBA treatment groups also had LXR/RXR activation among the most enriched canonical pathways. The farnesoid X receptor (FXR), liver X receptor (LXR), and retinoid X receptor (RXR) are nuclear receptors that control metabolic pathways such as bile acid regulation, lipid and glucose homeostasis, and metabolism (Ding et al. 2014; Zheng et al. 2018). This finding is in agreement with others that report activation of FXR and LXR by PFAS (reviewed in Andersen et al. 2021; Haimbaugh et al. 2022) but are discordant with findings in human HepG2 hepatocarcinoma cells that identified PFOA, PFBA, and PFOS exposure activated the peroxisome proliferator-activated receptor alpha (PPARa) but did not affect FXR, LXR, or RXR (Behr et al. 2020).

Additionally, when comparing the same exposure concentration across the three different chemicals, enrichment in canonical pathways associated with neurological signaling were observed in the 4 ppb treatment groups of PFOA, PFHxA, and PFBA. The absence of significant

changes in 40 ppb and 400 ppb exposures can be due to non-monotonic dose-response of PFAS as endocrine disruptor chemicals (Vandenberg et al. 2012).

3.6 Conclusions

The results of this study were concordant with past studies showing that acute developmental toxicity of PFAAs is driven by chain length. Alternatively, embryonic exposure to PFOA and PFBA caused behavioral changes in zebrafish larvae, while PFHxA did not. These results support that the neurobehavioral changes are not dependent on the chain length and that the observed behavioral alterations are likely to function through different mechanisms given differences among PFOA and PFBA responses. Morphological alterations were observed among treatment groups of all three PFAAs in distinct patterns. The transcriptome analysis validated concordance of several of the reported human adverse health outcomes associated with PFOA exposure based on similarity of molecular pathways and networks and canonical pathways observed following the embryonic exposure in the developing zebrafish. These findings support the predictive value of this approach as we have observed in similar studies with other environmental toxicants (e.g., Weber et al. 2013) and can be used to build hypotheses for future studies addressing mechanisms of specific adverse health outcomes based on these identified molecular pathways. Overall, unique alterations were seen for each of the three PFAAs demonstrating the need to assess the toxicity risks of the replacement PFAS.

3.7 References

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CHAPTER 4 : DIFFERENTIAL DEVELOPMENTAL NEUROTOXICITY AND TISSUE UPTAKE OF THE PFAS ALTERNATIVES, GENX AND PFBS

4.1 Abstract

Perfluoroalkyl substances (PFAS) are a class of synthetic chemicals that are persistent in the environment. Due to the association of longer chain PFAS with multiple adverse health effects, shorter chain chemicals such as hexafluoropropylene oxide dimer acid (HFPO-DA; GenX) and perfluorobutane sulfonic acid (PFBS) and its potassium salt were used as replacements. Developmental neurotoxicity data on the shorter chain PFAS are lacking. The aim of this study was to evaluate the developmental neurotoxicity and tissue uptake of GenX and PFBS, the alternatives of PFOA and PFOS, respectively using the zebrafish (*Danio* rerio) model. LC50s were determined at 120 hours post fertilization (hpf) to assess acute toxicity of GenX and PFBS by exposing developing zebrafish to a range of concentrations of each chemical starting at 1 hpf. Tissue uptake was determined at 72 and 120 hpf after embryonic exposure (1-72 hpf) after exposure to 0, 4, 40, 400, or 4000 parts per billion (ppb; µg/L) of GenX and PFBS and then anchored to measured water concentrations by LC-MS/MS. Locomotor activities was assessed using visual motor response assay at 120 hpf after embryonic exposure (1-72 hpf) to the same nominal concentrations of GenX or PFBS. Dopamine levels and expression of genes involved in dopaminergic neuron signaling were assessed at 72 hpf after embryonic exposure to 0, 0.4, 4, 40, or 400 ppb. PFBS was more acutely toxic and more bioaccumulative than GenX with measured concentrations of dosing solutions near the nominal concentrations, but both chemicals had limited excretion 48 hours after exposure ceased. Both GenX and PFBS caused hyperactivity at 120 hpf, but the behavioral alterations were more persistent for PFBS among phases and occurred even at the lowest tested exposure concentration of 4 ppb. At 72 hpf, an increase in dopamine level was seen at 40 ppb GenX, while a decrease was observed at 400 ppb PFBS. No significant changes were observed in expression of monoamine oxidase (*mao*), dopamine transporter *(dat*), or tyrosine hydroxylase (*th*). Sulfonate functional group agreed with previous literature for higher acute toxicity and bioaccumulation. In addition, behavioral alterations were more persistent and observed at lower concentrations for PFBS. Coupled to the differences detected for dopamine

levels among the two PFAS, indicates differential mechanisms of developmental neurotoxicity. The lack of expression changes for the chosen genes calls for additional molecular and mechanistic assessments to determine this unique pattern.

4.2 Introduction

Perfluoroalkyl substances (PFAS) are used in aqueous film fighting foams (AFFF), non-stick cookware, waterproof clothing, carpets, food packaging, and for stain and water resistance in additional consumer products. PFAS have high chemical and thermal stability due to the strength of the carbon-fluoride bond. Therefore, these chemicals persist in the environment and bioaccumulate in humans and wildlife (Giesy and Kannan 2001). The long chain PFAS [(perfluoroalkyl carboxylic acids (PFCA)] with more than 7 carbons, or perfluoroalkyl sulfonic acids (PFSA) with more than 6 carbons) were phased out in the United States, but they are still used in other part of the world. In addition, products that contain long chain PFAS can be shipped to the United States. The longer chain PFAS such as perfluorooctane sulfonic acids (PFOS) and perfluoroocatonic acid (PFOA), have long half-lives in humans and are associated with many adverse health effects including reproductive and developmental toxicity, liver disease, thyroid disruption, and suppression of immune response (Olsen et al. 2007) (ATSDR, 2021.). In order to overcome the concerns regarding the longer chain PFAS, chemicals with shorter alkyl chain [e.g., perfluorobutane sulfonic acid and its potassium salt (PFBS)] or shorter chain with one or more ether group [e.g., hexafluoropropylene oxide dimer acid (HFPO-DA), known as GenX] were introduced into industry. GenX and PFBS replaced PFOA and PFOS, respectively, in their applications.

Although GenX and PFBS have shorter biological half-lives compared to PFOA and PFOS, there is evidence that they are persistent in the environment and their higher solubility can lead to higher mobility in the environment. The European Chemicals Agency (ECHA) listed GenX and PFBS under the substances of very high concerns due to their mobility and persistence in the environment (ECHA, 2019). GenX was detected in environmental water samples and in treated drinking water (Sun et al. 2016; Strynar et al. 2015; Brandsma et al. 2019). In addition, GenX was detected in grass and leaves in higher levels compared to PFOA. It has been shown that short chain PFAS (C4-C6) accumulate in leafy vegetables and fruits, while PFOA and PFOS accumulate in

roots of plants (Ghisi et al. 2019). GenX also was detected in Striped Bass in the Cape Fear River in North Carolina, US (Guillette et al. 2020). This study showed that the serum level of GenX was 136 times higher than the concentration found in surface water, indicating that GenX can accumulate in fish.

Multiple studies investigated toxicity of GenX and PFBS due to concerns of their persistence in the environment. GenX adverse health effects include hepatic, reproductive, developmental (Conley et al. 2019; Blake et al. 2020), thyroid (Conley et al. 2021), hematological, and immune effects (Rushing et al. 2017). PFBS caused multiple adverse effects in animals such as decreased thyroid hormones, decreased sperm health, delayed eye opening and decreased body weight of offspring, and increased liver weight (US Environmental Protection Agency 2021). Most recently, the United States Environmental Protection Agency (US EPA) released the human health toxicity values of GenX (US EPA 2021) and PFBS (US EPA 2021) with the chronic reference dose (RfD) estimated to be 0.000003 mg/kg/day and 0.00003 mg/kg/day, respectively. Currently, there is no federal maximum contaminant level (MCL) for PFAS in drinking water in the US. However, some US states have adopted more strict policies due to high concerns of PFAS. For example, Michigan has set regulatory limits for seven PFAS, including GenX [i.e., 370 parts per trillion (ppt; ng/L) and PFBS (420 ppt)] (EGLE, 2020).

In utero and early developmental exposure to PFAS is of concern as PFAS have been detected in the serum of pregnant women, in fetal cord blood serum, and in breast milk (Kim et al. 2011; Barbarossa et al. 2013). A study showed that prenatal PFOS exposure was associated with measures related to hyperactive-impulsive type ADHD (Vuong et al. 2021). On the other hand, other studies have shown no association between prenatal PFAS exposure and increase risk of ADHD in children or in abnormal IQ scores at 5 years of age (Liew et al. 2018; Liew et al. 2015). However, these studies were not conclusive, and they highlight the importance of further assessing neurodevelopmental effects of PFAS. Furthermore, few studies have investigated the developmental neurotoxicity of GenX and PFBS compared to other PFAS.

The zebrafish is continuing to grow in popularity as a model for developmental neurotoxicity studies after decades of use in developmental and neurobiology research. For example, assessing behavior of zebrafish is widely used in neuropharmacology for evaluating neurotoxicity of drugs (Basnet et al. 2019; Irons et al. 2010; de Esch et al. 2012; Kiper and Freeman 2019; Padilla et al. 2022). In addition, zebrafish have orthologs to 70% of human genes with this

number increasing to 82% when considering disease-related genes (Howe et al. 2013). Furthermore, the easy husbandry, high fecundity, and ex vivo development are advantages over other models that make toxicity testing easier during the earliest developmental stages. To this end, the zebrafish has been applied to investigate the developmental toxicity of some of the PFAS (Gaballah et al. 2020; Hagenaars et al. 2011; Spulber et al. 2014; Rericha et al. 2022; 2021b).

Given the current evidence of potential developmental neurotoxicity of PFOA and PFOS and their suggested effects on dopaminergic neuron development, it is important to investigate the developmental neurotoxicity of GenX and PFBS (Foguth et al. 2019; Yu et al. 2021; Wu et al. 2022). Moreover, assessing internal dose after PFAS exposure is highly important to anchor the observed effects to an internal dose given remaining questions on toxicokinetics of each PFAS in different organisms. This need for internal dose measurements was recently emphasized to assist regulatory agencies in their interpretations of toxicity data (Tal and Vogs 2021).

In this study, we hypothesized that GenX and PFBS causes developmental neurotoxicity by altering neurobehavior outcomes in developing zebrafish larvae and altering dopaminergic neuron development. In order to test this hypothesis, developing zebrafish were exposed throughout embryogenesis (1-72 hours post fertilization, hpf) to GenX or PFBS and then determined locomotor activity of larvae at 120 hpf, dopamine levels in whole eleuthero-embryos at 72 hpf, and gene expression alterations of targets associated with dopaminergic neurons at 72 hpf. In addition, internal doses were assessed at 72 hpf (at cessation of exposure and assessment time of dopamine levels and gene expression) and at 120 hpf (at time of locomotor activity assessment).

4.3 Materials and Methods

4.3.1 Zebrafish Husbandry

Zebrafish (*Danio rerio*) of the wild-type AB strain were housed in a Z-Mod system (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle. Water was maintained at 28±1°C, pH at 7.0–7.2, and conductivity at 550 µS. Water quality was assessed twice a day. Fish were fed a mixture of brine shrimp (Artemia International LLC., Fairview, Texas), Golden Pearls 500–800 µm (Artemia International LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros

Inc., Gardners, PA). Adult fish were bred in spawning tanks according to established protocols (Peterson et al. 2011, 2013). Embryos were collected within 1 hour post fertilization (hpf). The embryos were rinsed with embryo water (filtered reverse osmosis water of pH 7.2 and conductivity at 550 µS) and randomly distributed in groups of 50 embryos into 100 mm X 20 mm polystyrene Petri dishes for each treatment group within each biological replicate. All embryos used in experiments were incubated at 28°C through 72 or 120 hpf. Protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely with regard to prevention and alleviation of suffering.

4.3.2 Chemical Treatments for Zebrafish Assays

GenX (CAS# 13252-13-6, 97% purity, Matrix Scientific, Columbia, SC) and PFBS potassium salt (PFBS) (CAS# 29420-49-3, 98% purity, Sigma, St. Louis, MO) were used in the study (Table 4.1). The chemicals were solubilized in reverse osmosis water all within solubility limits. Stock solutions of GenX were neutralized to pH 7 with 5 M sodium hydroxide (Wasel et al. 2021).

Table 4-1. Physico-chemical properties of PFBS and GenX

Chemical	Perfluoro-2-propoxypropanoic acid	Potassium perfluorobutane sulfonate
Acronym	GenX, C6	PFBS, C4
CAS Registry No.	13252-13-6	29420-49-3
Chemical Formula	$C_6HF_{11}O_3$	$CF_3(CF_2)_3SO_3K$
Molecular Weight (g/mol)	330.05	338.19
Solubility in Water	>751 g/L at 20°C	46.2 mg/L at 20°C
pKa	2.8	Not Applicable

4.3.3 Acute Developmental Toxicity Assay

To assess the lethal concentration at which 50% mortality was observed (LC50) of the test chemicals, three biological replicates (embryos from 3 different clutches) of 50 embryos per

treatment were placed into a Petri dish and dosed with 20 mL of a range of concentrations of each PFAS within one hour after spawning (Weber et al., 2013; Peterson et al. 2011a). Selected concentrations were 0, 4000, 6000, 8000, and 10000 parts per million (ppm, mg/L) (13,331-33,328 μM) of GenX and 0, 1000, 1200, 1300, 1500, and 2000 ppm (3343.5-6687 μM) of PFBS. All solutions were adjusted to a neutral pH (Wasel et al. 2021). The developing zebrafish were exposed to the test chemicals until 120 hpf. The negative control was filtered water only. Mortality rates were monitored every 24 hours. Mortality rates of treatment groups were normalized to the control treatment group.

4.3.4 PFAS Tissue Dose and Water Concentration Determination

4.3.4.1 Collection of tissues for PFAS dose analysis at 72 and 120 hpf

Tissue dose was assessed at two time points: at 72 and 120 hpf. These tissue collection time points align with the exposure duration and conditions for the subsequent behavioral analysis at 120 hpf (i.e., 1-72 hpf PFAS exposure, cessation of exposure at 72 hpf, and development in water only until 120 hpf) and assessment of dopamine levels and gene expression by qPCR at 72 hpf (i.e., 1-72 hpf PFAS exposure with immediate analysis). In addition, concentration ranges aligned with exposure concentrations of these two time point assessments (i.e., 0, 4, 40, 400, and 4000 ppb at 120 hpf to align with behavior analysis exposure concentrations and 0, 0.4, 4, 40, and 400 ppb at 72 hpf to align with dopamine level and gene expression analyses exposure concentrations).

4.3.4.1a Collection of tissues for PFAS dose at 72 hpf and confirmation of dosing solution concentration

Embryos were rinsed with filtered water (reverse osmosis water of pH 7.2 and conductivity at 550 μS/cm) and randomly distributed into groups of 50 for each treatment group at 1 hpf. Fish were exposed to 0, 0.4, 4, 40, or 400 parts per billion (ppb, μg/L) of GenX or PFBS. Samples from the solutions were stored at 4°C for chemical analysis to measure concentrations of GenX or PFBS (at 1 hpf). Concentrations were chosen to encompass a log scale of sublethal concentrations with inclusion of and concentrations above an earlier US EPA provisional health advisory limit for

PFOA at 0.4 ppb (EPA 2021). In addition, these concentrations align with another PFAS study by our laboratory with PFOA, PFHxA, and PFBA with similar exposure conditions (Chapter 3). Each treatment group of each biological replicate had two Petri dishes with 50 fish to total 100 fish exposed. Fish were incubated in the treatment solutions at 28°C from 1 to 72 hpf. At 72 hpf, eleuthero-embryos were rinsed twice with filtered water and euthanized by cooling for ~2-7 hrs in 4°C. At 72 hpf (immediately following PFAS exposure), 70 fish were pooled (35 from each plate) into a microcentrifuge tube for each treatment group of each biological replicate. The weight of the tubes before adding the fish was measured. Samples were placed at -20°C until freezing drying. Samples were freeze-dried, the weight of tubes (containing samples) measured, and stored at -20°C until further preparation for chemical analysis.

4.3.4.1b Collection of tissue for PFAS dose at 120 hpf and dosing solutions at 1, 72, and 120 hpf

Zebrafish embryos were exposed to 0, 4, 40, 400 or 4000 ppb GenX or PFBS through 72 hpf. In order to assess tissue dose at 120 hpf, instead of collecting the tissue samples at 72 hpf after ending the exposure, larvae were allowed to develop until 120 hpf with no additional PFAS exposure and incubated in filtered fish water only for an additional two days. This time point aims to determine the concentrations in the tissues at the same timepoint of behavioral assessment using the same exposure regime. At 120 hpf, 70 larvae were collected as mentioned above. Water samples from the dosing solutions were collected 1) at 1 hpf (at initiation of PFAS exposure) and then from the Petri dishes of each treatment group 2) at 72 hpf (end of PFAS exposure) and 3) at 120 hpf (48 hours following cessation of PFAS exposure). These collection time points were chosen to 1) confirm expected PFAS concentration of dosing solution at exposure initiation, 2) PFAS concentration at cessation of exposure period, and 3) to determine if PFAS were eliminated into dosing solution during the 48 hours following the exposure period. Following collection, water samples were stored in the dark at 4°C until further processing for analysis.

4.3.4.2 Preparation and analysis of PFAS in tissue samples

Tissue samples were spiked with 25 µL of an internal standard solution in methanol (about 200 ng/mL of each mass-labeled PFAS). Extraction was performed by adding 600 µL of tetrahydrofuran and 200 µL of water. Samples were then vortexed for 10 minutes, sonicated for

30 minutes, and centrifuged at 13,000 rpm for 20 minutes. Supernatant was transferred to a 1.5 mL glass injection vial, gently blown down with nitrogen, and solvent exchanged to a 500 µL 50:50 v/v methanol: water. The extract was vortexed, transferred to 1.5 mL microcentrifuge tubes, and centrifuged for 10 minutes at 13,000 rpm. 200 µL of the supernatant was transferred back to the 1.5 mL glass injection vials and stored at 4°C until analysis. Laboratory blanks (ultrapure water) and spiked control samples (ultrapure water spiked with 20 µL of a 100 ng/mL stock solution containing the chemical of interest dissolved in methanol) were prepared with each batch of samples. Analysis was performed using reverse-phase chromatography with two automated liquid chromatography mass spectrometry (LC-MS/MS) as previously described by (Hoover et al. 2017). The limit of quantification (LOQ) varied between the different runs and it ranged between (0.04-0.06 ppb). Four biological replicates were completed for each treatment group at each time point.

4.3.4.3 Preparation of dosing solutions for PFAS concentration analysis

Prior to analysis, the 400 and 4000 ppb dosing solutions collected at initiation of exposure (1 hpf) and at cessation of exposure (72 hpf) were diluted by adding 50 µL of the PFAS dosing solution with 450 µL of ultrapure water for the 400 ppb solutions (1:10) or by adding 10 µL of the PFAS dosing solution with 490 µL of ultrapure water for the 4000 ppb solutions (1:50). 500 µL of the dosing solution (i.e., either at full concentration or at diluted concentration) was transferred into a 1.5 mL plastic injection vial. 475 µL of methanol and 25 µL of an internal standard solution in methanol were added (about 200 ng/mL of each mass-labeled PFAS). Samples were vortexed and stored at 4°C until analysis. Similar to the tissue dose assessment, laboratory blanks (ultrapure water) and spiked control samples (ultrapure water spiked with 20 µL of a 100 ng/mL stock solution containing the chemical of interest dissolved in methanol) were prepared with each batch of samples. Analysis was performed using reverse-phase chromatography with two automated liquid chromatography mass spectrometry (LC-MS/MS) (Hoover et al. 2017). Four biological replicates were completed for each treatment group at each time point.

4.3.4.4 Calculation of bioconcentration factor

Bioconcentration factors (BCF) were calculated using the tissue dose at 72 hpf and the measured concentrations of corresponding dosing solutions at 1 hpf as follows:

$$BCF = \frac{C\text{zebrafish}}{C\ media}$$

where Czebrafish is the dry weight concentration of GenX or PFBS in zebrafish larvae (ng/g), and Cmedia represents the concentrations of PFAS exposure media at 1 hpf (ppb), respectively.

4.3.5 Visual Motor Response Behavior Assay

Larval locomotion was assessed to determine if the embryonic exposure to sublethal concentrations of GenX or PFBS resulted in behavioral alterations. A visual motor response test was performed using Noldus DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands). In each biological replicate, 50 zebrafish embryos were exposed to 0, 4, 40, 400, or 4000 ppb of GenX, (0.012-12.12 μM) or PFBS (0.013-13.33 μM) within 1 hpf. 0 ppb represents the negative control treatment consisting of filtered water (0 ppb).

At 72 hpf, exposure to the chemicals was terminated by rinsing the fish with filtered water twice. Fish were then incubated in water only at 28°C until 120 hpf when behavioral analysis was completed similar to our past studies (Horzmann et al. 2018a). Subsamples from each replicate were placed in separate wells in a 96-well plate. Grossly malformed or dead larvae were excluded. The wells were filled with 500 μl filtered water, the plate placed in the DanioVision observation chamber, and temperature maintained at 28°C throughout the experiment using the Noldus temperature control unit. After a 10-minute dark acclimation period, the test was started by exposing larvae to 10 minutes of alternating periods of dark and light for a total of 50 minutes (dark-light-dark-light-dark) (Horzmann et al. 2018). Infrared light that is not visible to zebrafish larvae was used for tracking movement. During the light phase, a 5000 lux light was activated under the DanioVision observation chamber. The infrared movement traces were recorded at a rate of 25 frames per second with a Basler GenICam acA 1300-60gm camera. Tracks were smoothed via a minimum distanced moved profile set to >0.2 mm. The exposure to dark or light was controlled by EthoVision 12 software. All behavioral experiments were performed at 11 am-2 pm

to minimize circadian variability in movement. Total distance moved, mean velocity, and cumulative time spent moving were calculated using EthoVision 12 software. Seven biological replicates were completed for GenX with 15-19 subsamples per treatment per replicate to total 129-132 total fish per treatment group. Six biological replicates were performed for PFBS with 18-19 subsamples per treatment in each replicate to total 111-113 total fish per treatment group.

4.3.6 Assessment of Dopamine Levels

A total of 50 zebrafish embryos were randomly sorted into a Petri dish and dosed with 0, 0.4, 4, 40, or 400 ppb GenX or PFBS from 1-72 hpf at 28°C. One or two plates were collected for each treatment group. At 72 hpf, fish were rinsed twice with filtered water and then pooled in a 1.5 mL tube (35 or 70 total). Eleuthero-embryos were homogenized with a pestle in 500 µL 1X phosphate buffered saline (PBS) and stored at -20°C. After two freeze-thaw cycles, the homogenates were centrifuged for 5 minutes at 5000 x g at 2-8°C. The supernatant was removed and assayed immediately or stored -20°C. Dopamine levels were measured using a competitive inhibition enzyme-linked-immunoassay (ELISA) (Cusabio, CSB-EQ027496FI) following manufacturer instructions. The plate design included a standard curve supplied with the kit. Each standard curve and experimental sample were run in duplicate (technical replicates). The optical density (OD) of each well was assessed using a microplate reader set to 450 nm. Optical density at 630 nm was set to account for background. The duplicate readings for each standard and sample were averaged. Average OD of the blank wells was subtracted from the OD of the standards and samples. The standard curve was created using GraphPad Prism (8.4.1) using the four-parameter logistic (4-PL) curve fit. Dopamine concentrations of the samples were calculated from the standard curve. Five (PFBS) or ten (GenX) biological replicates were assessed for each treatment group.

4.3.7 Quantitative Polymerase Chain Reaction (qPCR)

Gene expression of monoamine oxidase (*mao*), dopamine transporter (*dat*), and tyrosine hydroxylase (*th*) were assessed using qPCR. 50 zebrafish embryos were exposed to a 0, 0.4, 4, 40, or 400 ppb GenX or PFBS from 1-72 hpf in Petri dishes. At 72 hpf, eleuthero-embryos in each treatment group of each biological replicate were pooled, homogenized in TRIzol (Life

Technologies, Carlsbad, CA), flash frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and cDNA synthesized with the SuperScript IV first-strand synthesis system (Invitrogen, Carlsbad, CA) as previously described (Peterson et al., 2009). Primers specific to the target genes (Integrated DNA Technologies, Coralville, IA; Table 4.2) were designed using the Primer3 website (http://primer3.ut.ee/) and checked for genomic alignment using NCBI Primer-BLAST. Melt and standard dilution curves and no template controls were included in the plate design to ensure efficiency (100 ± 10%) and specificity. Samples were run in triplicate as technical replicates. Target genes were normalized to β-actin (target gene/ β-actin), which showed the most consistent gene expression among different treatments of GenX and PFBS. qPCR was performed following similar methods as described previously (Peterson, Zhang, and Freeman 2013b; Peterson et al. 2011b; Horzmann et al. 2021; Wirbisky et al. 2014) following MIQE guidelines. The Bio-Rad CFX Connect Real-Time PCR Detection System was used with the SSO Advanced Universal SYBR Green Supermix according to manufacturer recommendations (Bio-Rad, Hercules, CA). Six biological replicates were completed for each target.

Table 4-2. Primers for qPCR analysis

Gene Name	Sequence ID	Gene Symbol	Primer sequence
Monoamine oxidase	NM_212827.3	*Mao*	Forward: cccaaactgcataacatggtcg Reverse: acaaaagggtctgaacgtagct
Dopamine transporter (slc6a3)	NM_131755.1	*dat*	Forward: catcatttacccagaagccatt Reverse: tcgattcctaaagtcagcaaca
Tyrosine hydroxylase	NM_131149.1	*Th*	Forward: tttggcatctcttggagcttct Reverse: aatcacccctccctgtttacac
β-actin	NM_181601	*actb2*	Forward: ctaaaaactggaacggtgaagg Reverse: aggcaaataagtttcggaacaa

4.3.8 Statistical Analysis

For the acute toxicity assessment, the LC50 and associated 95% confidence limits at 120 hpf in zebrafish larvae were determined using non-linear regression with a hill slope curve fitting using GraphPad Prism 8.4.1. In the visual motor response assay, total distance moved, mean velocity, and time spent moving were analyzed. A Grubb's outlier test was used to detect outliers

within a treatment group of each outcome and then a repeated measures analysis of variance (ANOVA) using SAS 94 software (α=0.05) was completed. The phase (dark or light) and chemical treatments were the independent variables and locomotor activity was the dependent variable. A one-way ANOVA with SAS 94 software was used to analyze survival rates, dopamine content, and relative gene expression with a least significant difference post hoc test when a significant ANOVA was observed (α=0.05). All data are presented as mean ± standard deviation.

4.4 Results

4.4.1. Acute Toxicity at 120 hpf of GenX and PFBS

The 120-hpf LC50 of GenX was 8617 ppm (95% CI: 8280-8974) ppm. There was a significant decrease in survival in the 2000 ppm PFBS treatment group at 120 hpf (p=0.002); however, the average percent survival was 84.4%. Thus, the 120 hpf-LC50 for PFBS is greater than 2,000 ppm (Figure 4.1). The 120-hpf LC50 for PFBS was greater than 2000 ppm.

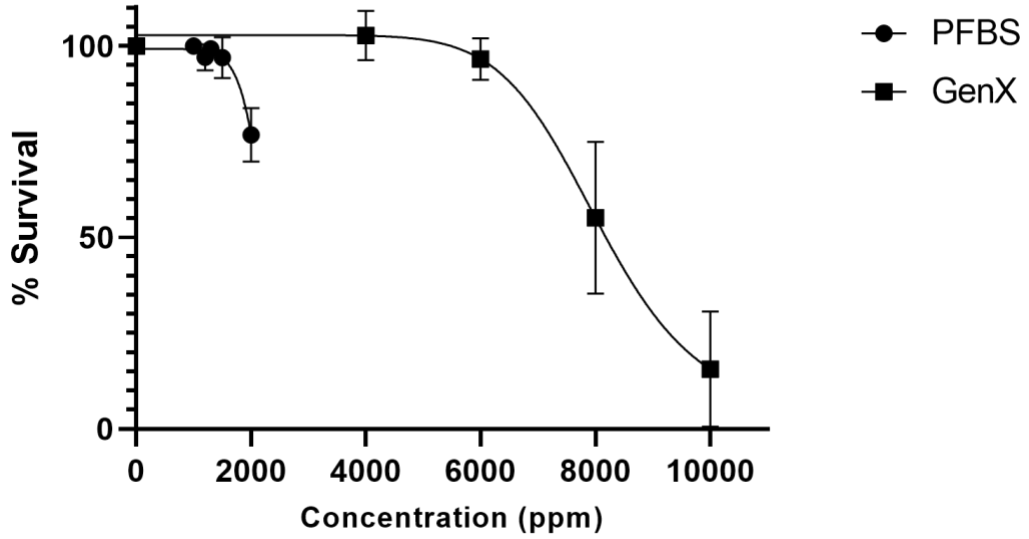

Figure 4-1: 120 hpf-LC50 curves of zebrafish exposed to GenX or PFBS from 1-120 hpf. N=3 with 50 fish per treatment per replicate. Error bars are standard deviation.

4.3.2 GenX and PFBS Tissue Dose Assessment

4.3.2.1 Tissue dose at 72 hpf

There was a dose-dependent accumulation of GenX and PFBS in zebrafish eleuthero-embryos immediately following the exposure period at 72 hpf (Figure 4.2A). For GenX, only the 40 and 400 ppb GenX treatment groups had a measurable concentration at 38.5 and 639.2 ng/g, respectively. The control (0 ppb), 0.4, and 4 ppb treatment groups were below the limit of quantification (LOQ). PFBS was more bioaccumulative with higher tissue concentrations observed immediately following the exposure period at 72 hpf (Figure 4.2A). PFBS concentrations in fish in 40 ppb treatment group averaged 166.1 ng/g, and the 400 ppb treatment group measured at 1921.8 ng/g. The PFBS control (0 ppb), 0.4 ppb and 4 ppb treatment groups were below the LOQ.

Concentrations of GenX and PFBS in test solutions were close to the expected concentrations at the beginning of the exposure period (1 hpf) (Figure 4.2B). The negative control treatment (0 ppb GenX or PFBS) and the 0.4 ppb treatment groups for each GenX and PFBS were below the LOQ (Figure 4.2B). A concentration-dependent increase in the treatment groups was observed with GenX measured concentrations of 4.3, 41.4, and 374.6 ppb close to the 4, 40, and 400 ppb expected concentrations, respectively (Figure 4.2A). A similar result was detected in the PFBS dosing solutions with the observed concentrations at 4.0, 50.1 and 510.1 ppb for the 4, 40, and 400 ppb expected concentrations, respectively (Figure 4.2B)

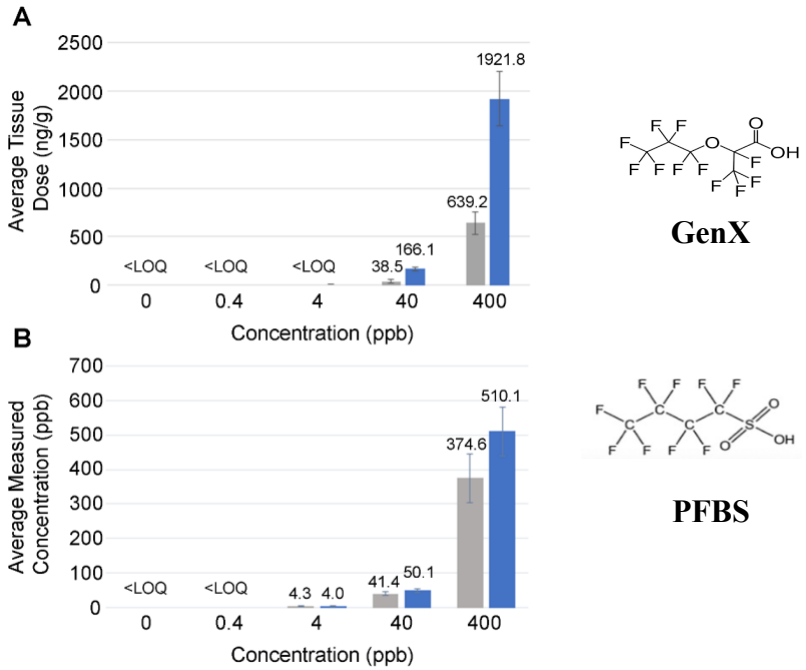

Figure 4-2: Tissue dose at 72 hpf and concentration of dosing solution at exposure initiation for GenX and PFBS. (A) Tissue dose of whole pooled eleuthero-embryos at 72 hpf at cessation of chemical exposure (N=4 with 70 subsamples pooled per treatment per replicate). (B) Concentration of dosing solutions at initiation of chemical exposure (at 1 hpf, N=4). Error bars are standard deviation.

4.3.2.2 Determination of bioconcentration factor (BCF)

Because the tissue dose for 0.4 ppb and 4 ppb treatment groups were below the LOQ, only measurements of tissue dose for 40 and 400 ppb were used to determine BCF (Table 4.3). As expected, the BCF for PFBS is higher than GenX. Interestingly, the BCF for PFBS at 40 ppb and 400 ppb were relatively close to each other while the BCF for GenX at 400 ppb is almost double the BCF at 40 ppb (Table 4.3).

Table 4-3: Calculated BCFs of GenX and PFBS[a]

Chemical	Nominal Concentration (µg/L)	Measured water (media) Concentration (µg/L)	Measured Tissue Dose (ng/g)	Average BCF (L/kg)
GenX	40	41.42 ± 4.63	38.51 ± 16.85	0.93 ± 0.38
	400	374.63 ± 70.68	639.22 ± 112.61	1.72 ± 0.25
PFBS	40	50.07 ± 3.92	166.14 ± 17.16	3.35 ± 0.55
	400	510.00 ± 28.79	1921.79 ± 282.98	3.80 ± 0.72

[a]All measurements are expressed as average± standard deviation.

4.3.2.3 Tissue dose at 120 hpf

A dose-dependent accumulation of GenX and PFBS was observed in zebrafish larvae immediately following the exposure period at 120 hpf (Figure 4.3A). For GenX, only the 40, 400, and 4000 ppb GenX treatment groups had a measurable concentration at 26.4, 546.9, and 7396.83 ng/g, respectively. The control (0 ppb) and 4 ppb treatment groups were below the LOQ. 4, 40, and 400 ppb PFBS exposures were detected in 120 hpf larval tissues at 99.42, 1806.85, and 15011.56 ppb, respectively. (Figure 4.3A). The PFBS negative control (0 ppb) and 4 ppb treatment groups were below the LOQ.

The concentrations of dosing media were measured at the beginning of exposure (1 hpf), at 72 hpf, and at 120 hpf (Figure 4.3B). The average measured concentrations during the exposure period (at 1 hpf and 72 hpf) of GenX and PFBS were close to the nominal concentrations (Figure 4.3B and 4.3C). Water samples from the depuration period (120 hpf) showed much lower concentrations (Figure 4.3B and 4.3C). Only 400 and 4000 ppb GenX and 400 ppb PFBS exposures showed measurable concentrations in water at 120 hpf (0.16, 1.51, and 1.74 ppb, respectively).

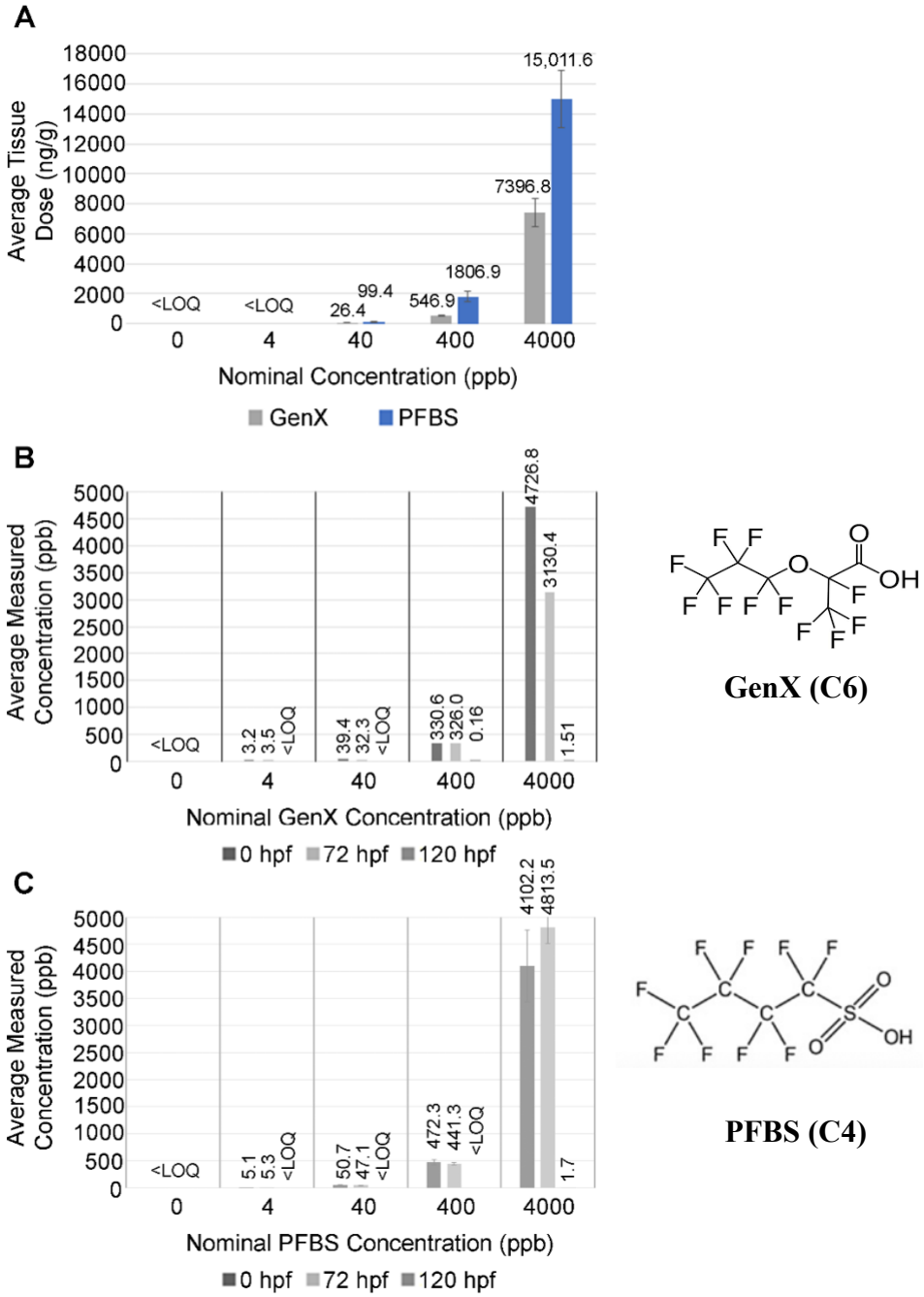

Figure 4-3: Tissue dose at 120 hpf and concentrations of dosing solutions at initiation of exposure, cessation of exposure, and time of behavior analysis. (A) Tissue dose of whole pooled larvae at 120 hpf aligning with time of behavior analysis 48 hours after chemical exposure ceased (N=3-4 with 70 larvae pooled per treatment per replicate). (B) Concentration of GenX dosing solution at initiation of exposure (1 hpf), at end of chemical exposure (72 hpf), and 48 hours after the chemical exposure ceased (at 120 hpf when behavior analysis was completed, N=2-4). (C) Concentration of PFBS dosing solution at initiation of exposure (1 hpf), at end of chemical exposure (72 hpf), and 48 hours after the chemical exposure ceased (at 120 hpf when behavior analysis was completed, N=3-4). Error bars are standard deviation.

4.4.3 Locomotor Activity of Zebrafish Larvae at 120 hpf with Embryonic GenX or PFBS Exposure

Overall, zebrafish larvae moved more in dark periods compared to light periods as expected with different trends in behavioral alterations observed for GenX and PFBS (Figure 4.4). The statistical analysis was done using a repeated measure ANOVA. In all data, the F statistic is expressed as F((df of variable), df (error))= F value. At 120 hpf, the GenX embryonic exposure resulted in a significant effect on the interaction between treatment group and phase (dark or light condition) in all tested outcomes including total distance moved and velocity [(F16, 2552) =3.36, $p<0.0001$ for both outcomes] and time spent moving [(F16, 2552 =3.29, $p<0.0001$] (Figure 4.4A, 4.4C, 4.4E). There was no significant difference in overall treatment group effect for each outcome signifying overall alterations were dependent on phase [total distance moved and velocity [(F4, 638)=1.14, p=0.331] and time spent moving [(F4, 638) =0.83), p=0.50]]. Phase specific effects in the treatment groups included an increase in the total distance moved, mean velocity, and time spent moving during the first dark phase of larvae exposed to 40, 400, or 4000 ppb GenX. This effect only persisted in the 400 ppb treatment group for total distance moved and velocity in the second dark phase (Figure 4.4A, 4.4C). In addition, an increased time spent moving was also seen in the larvae exposed to 4000 ppb (Figure 4.4E).

The embryonic PFBS exposure resulted in a significant overall effect of treatment group [(F4, 549) = 21.01, $p<0.0001$] for the total distance moved and mean velocity (same values for each outcome) (Figure 4.4B, 4.4D). In addition, PFBS exposure caused a significant overall effect of treatment [(F4, 549) = 18.55, $p<0.0001$] for time spent moving (Figure 4.4F). In the three dark phases, all treatment groups (4, 40, 400, and 4000 ppb) showed hyperactivity with an increase in total distance moved and mean velocity (Figure 4.4B, 4.4D). A similar effect was observed for time spent moving, except only 40, 400, and 4000 ppb were hyperactive in the first dark phase (Figure 4.4F). In the light phases, all treatment groups except the 4 ppb exposure group were hyperactive for all three outcomes. No significant interaction of treatment and phase (dark or light) was observed for total distance moved [(F16, 2196) = 1.6, $p<0.06$], mean velocity [(F16, 2196) = 1.6, $p<0.06$], or time spent moving [(F16, 2196) = 1.5, $p<0.09$] indicating similar effects were observed among the phases.

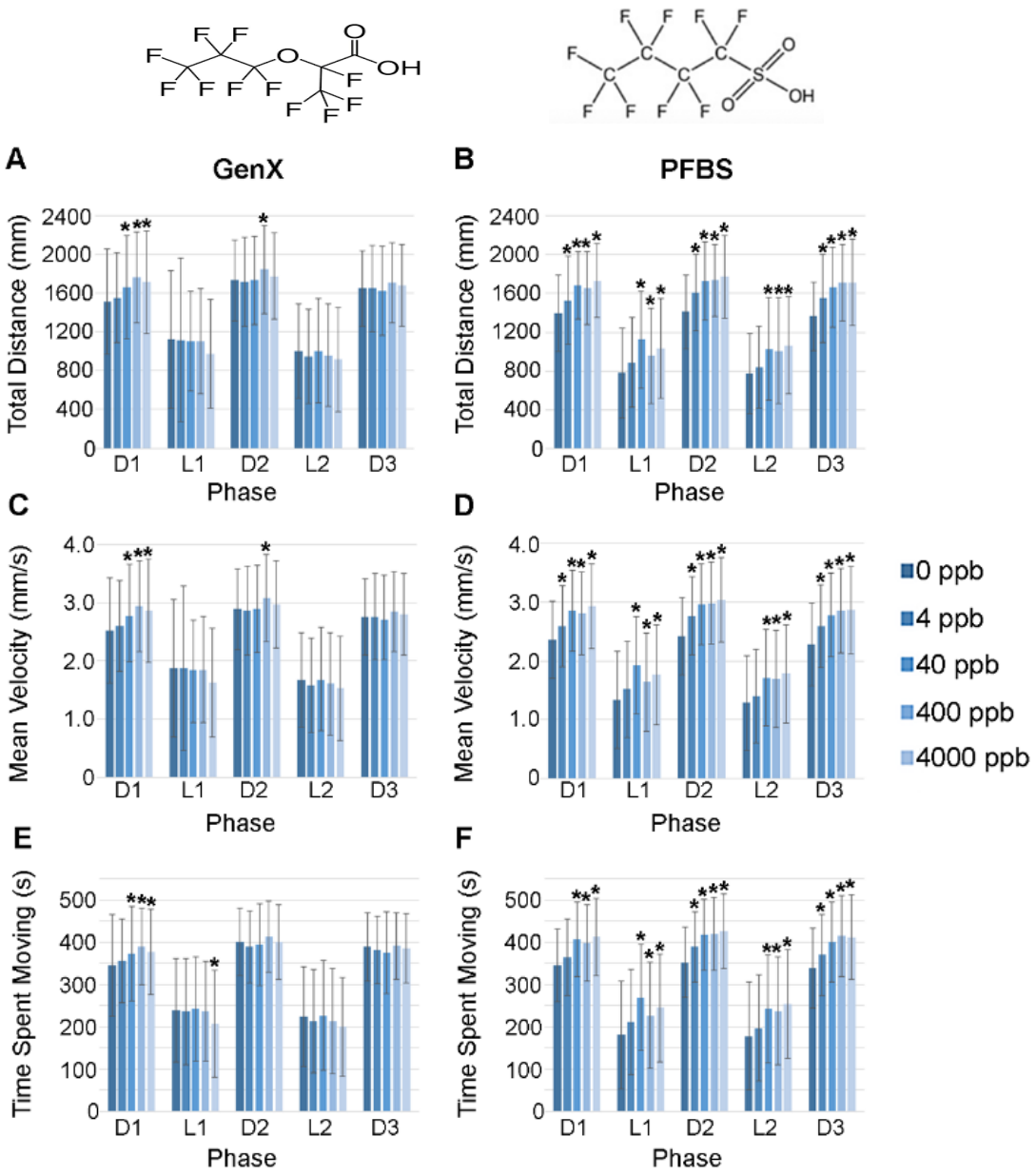

Figure 4-4: Visual motor response behavior assay in zebrafish larvae at 120 hpf following an embryonic exposure (1-72 hpf) to GenX (A, C, E) or PFBS (B, D, F). N=7 biological replicates for GenX with 15-19 subsamples per treatment per replicate to total 129-132 fish per treatment group. N= 6 for PFBS exposure with 18-19 subsamples per treatment per replicate to total 111-113 fish per treatment group. Error bars represent standard deviation. *p<0.05 compared to the control treatment group within each phase. (D1: first dark phase; L1: first light phase; D2: second dark phase; L2: second light phase; D3: third dark).

4.4.4 Dopamine Levels in Eleuthero-Embryos

A significant effect of GenX treatment was observed on dopamine level in zebrafish eleuthero-embryos (p=0.04). The LSD test showed that a significant increase in dopamine content was observed in zebrafish eleuthero-embryos exposed to 40 ppb GenX during embyrogenesis (p<0.05, Figure 4.5A). While an increasing trend was observed in the 400 ppb treatment group, this difference failed to reach a significant difference from the negative control treatment (0 ppb). Also, a significant effect of PFBS treatment was observed on dopamine level in zebrafish eleuthero-embryos (p=0.007). Alternatively, a decreasing trend was observed for the PFBS exposure groups with only the 400 ppb treatment group reaching a significant difference from the negative control treatment (0 ppb) (p<0.05, Figure 4.5B).

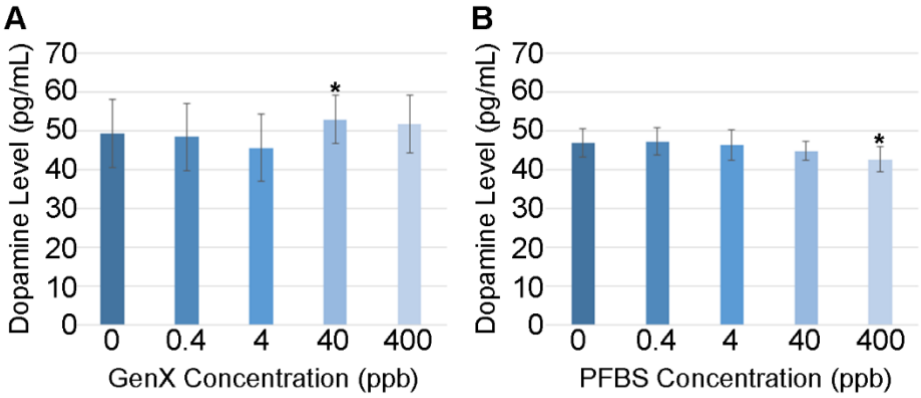

Figure 4-5: Average whole-body dopamine content in eleuthero-embryos exposed to (A) GenX or (B) PFBS from 1-72 hpf. N=5 (for PFBS) and N=10 (for GenX) with pools of 35-70 fish per treatment group per biological replicate. Error bars are standard deviation. *p<0.05.

4.4.5 Gene Expression of *mao, dat,* and *th* in Eleuthero-Embryos Following Embryonic Exposure to GenX or PFBS

To investigate gene expression alterations in dopaminergic neuronal development that may be caused by an embryonic exposure to GenX or PFBS, qPCR targeting *mao*, *dat*, and *th* was performed. No significant changes in the expression of *mao* (p=0.62), *dat* (p=0.16), or *th* (p= 0.14) was seen in the GenX treatment groups compared to unexposed treatment group (0 ppb) (Figure 4.6A, 4.6C, 4.6E). Also, there was no significant changes in the expression of *mao* (p=0.46), *dat* (p=0.62), or *th* (p= 0.32) in PFBS exposed groups (Figure 4.6B, 4.6D, 4.6F)

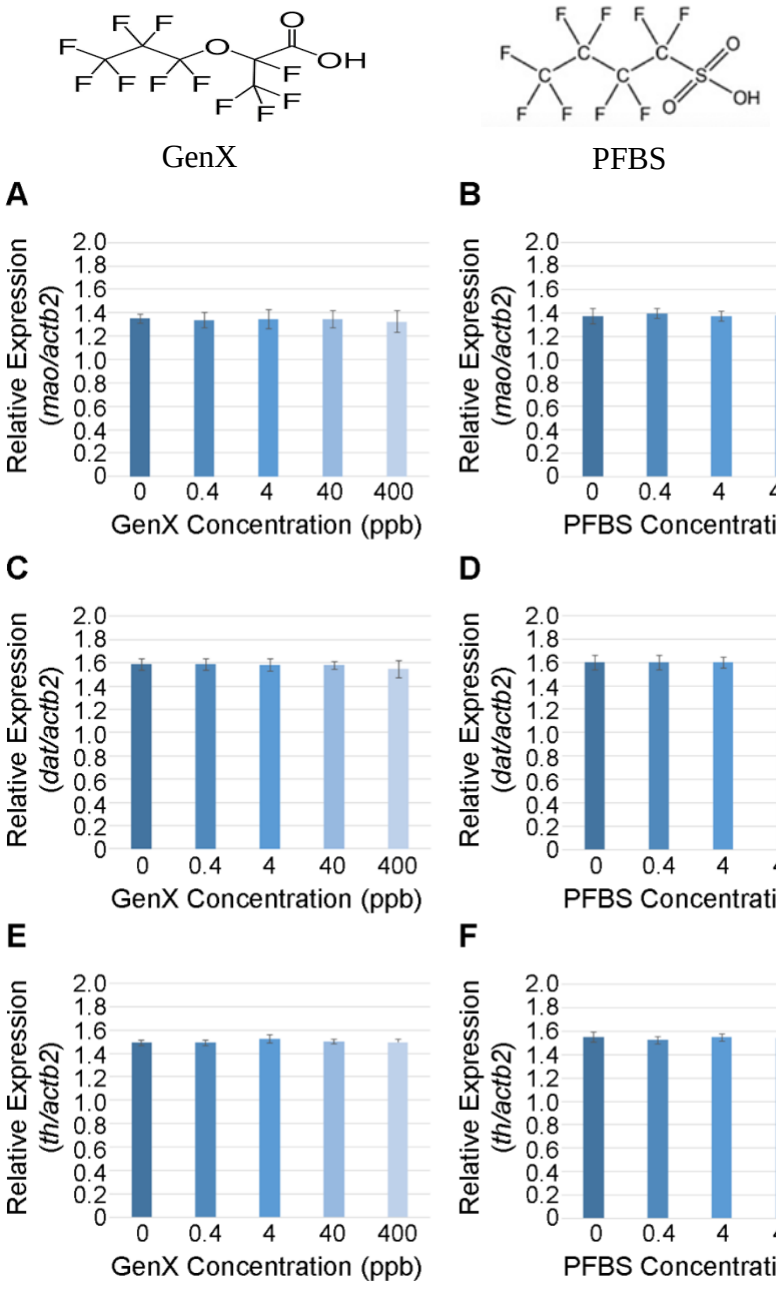

Figure 4-6: Relative gene expression at 72 hpf for mao, dat, and th in eleuthero-embryos exposed to GenX (A, C, E, respectively) or PFBS (B, D, F, respectively. N = 6 pools. Error bars represent standard deviation. $p>0.05$.

4.5 Discussion

There is relatively limited information on the developmental toxicity, especially the developmental neurotoxicity, of the emerging PFAS, GenX and PFBS, which are the shorter chain replacements of PFOA and PFOS, respectively. In this study, we first assessed the acute toxicity of GenX and PFBS by exposing zebrafish to a range of each chemical beginning at 1 hpf and monitoring the survival rates through 120 hpf. The 120 hpf-LC50 for GenX was determined as 8617 ppm (95% CI: 8280-8974). Our previous study found the 120 hpf-LC50 of PFOA to be 560 ppm (Chapter 3). These results indicate that the acute toxicity of GenX is less than PFOA in zebrafish larvae. The 120 hpf-LC50 of PFBS was > 2000 ppm. No higher exposure concentrations were tested since this concentration is magnitudes above environmental concentrations, agreed with our previous study, and is similar to previously published data (Hagenaars et al. 2011; Ulhaq et al. 2013a). This 120 hpf-LC50 of PFBS is substantially different from another study reported at 105 ppm (Gebreab et al. 2020), but our previous study showed that this discrepancy in reported LC50s in the literature is due to a lack of neutralizing the acidic dosing solution that results when working with higher concentrations of these perfluoroalkyl acids (Wasel et al. 2021a). As such, some studies inaccurately report much more toxic LC50s and can vary depending on the buffering capacity of the dosing solution being used by the group if not accounting for the acidic conditions created, which in itself is toxic to the developing zebrafish. Overall, these results are in agreement with previous studies that show the acute toxicity of PFAS is dependent on the chain length, where longer chain chemicals have higher acute toxicity compared to shorter chain chemicals (Ulhaq, Carlsson, et al. 2013b; Mahapatra et al. 2017; Hagenaars et al. 2011). In addition, these results are in agreement with the 120 hpf-LC50 values reported in developing zebrafish in studies with similar exposure paradigms and anchor our further evaluations at sublethal concentrations.

Second, we aimed to determine tissue dose of GenX and PFBS in our exposure scenario from 1-72 hpf without replenishing dosing solutions. We assessed tissue dose immediately after exposure at 72 hpf and then again 48 hours after cessation of the chemical exposure (120 hpf). These tissue doses were integrated with the measured concentrations of the dosing solutions at the beginning of the exposure period, at the end of the chemical exposure period (72 hpf), and 48 hours after the chemical exposure (120 hpf). These time points reflect the chemical exposure period (1-72 hpf) and time points and conditions at which analyses in this study were completed (i.e., at 120 hpf for behavioral analysis following embryonic exposure and at 72 hpf for dopamine levels and

gene expression immediately after embryonic exposure). Dose-dependent accumulation of both chemicals was observed immediately following the PFAS exposure, but concentrations at and below 4 ppb GenX and 0.4 ppb PFBS were below the limit of detection (LOD). In addition, the internal dose of PFBS was higher than GenX at the same nominal concentrations with not much difference in measured concentration of dosing solution. This result aligns with other sulfonate group-containing PFAS having a higher potential to accumulate compared to carboxylic group-containing PFAS in multiple organisms. For example, Gomis et al. (2018) reported the lowest-observed effect level (LOEL) of GenX was lower than the LOEL of PFOA for liver enlargement in rat. They showed that different toxicokinetic properties among the PFAS may explain these differences when at the same administered dose (Gomis et al. 2018). Similarly, zebrafish larvae at 144 hpf had higher levels of PFHxS compared to PFOA or PFHxA when exposed to the same nominal concentrations (Gaballah et al. 2020). Menger et al. (2020) showed that bioconcentration factor for PFSA was higher than PFCA. Furthermore, Vogs et al. (2020) found that the bioconcentration factors of different PFAS in 120 hpf zebrafish larvae vary and the difference in potency was reduced by three orders of magnitude when considering internal doses.

Bioconcentration factor for PFBS was higher than GenX at 40 and 400 ppb exposures. The BCF of 400 ppb GenX (BCF=1.72 L/kg) was much higher than 40 ppb exposures (BCF=0.93 L/Kg). On the other hand, the difference between BCF of 40 and 400 ppb PFBS was relatively small (3.35 and 3.8, respectively). Gaballah et al. (2020) revealed that 1 (500.1 ppb) and 1.8 µM (900.2 ppb) of PFOS had almost same BCF of 1374 L/Kg in zebrafish larvae; however, those exposed to 3.2 µM (1600.4 ppb) had a lower BCF of 684.03 L/Kg. In addition, other studies reported an inverse relationship between water concentration of PFAS and BCF, which has been hypothesized to be due to saturation of the active transporter at high doses that lead to a decreased BCF (reviewed by Tal and Vogs 2021). This effect may explain the small difference between the BCF at 40 and 400 ppb PFBS and the larger difference for 40 and 400 ppb GenX.

Researchers are starting to explore mechanisms that may explain the different toxicokinetic properties among PFAS. Some studies report variable abilities to bind to tissue proteins. Luebker et al. (2002) showed that PFOS and PFOA can bind to the secondary binding sites in rat liver fatty acid binding protein (L-FABP). In a competitive binding assay, PFOS showed a higher inhibition level of ligand binding to L-FABP compared to PFOA (Luebker et al. 2002). In addition, using molecular docking analysis it was determined that PFOS bound to L-FABP by creating three

hydrogen bonds compared to two hydrogen bonds by PFOA, indicating stronger binding interactions for the sulfonated PFAS (Jones et al. 2003). These results may explain the higher accumulation potential of sulfonate group-containing PFAS. Overall, these studies highlight the importance of assessing the internal dose with PFAS exposure to account for differences in toxicokinetics and for a more informed comparison of the toxicity of the different chemicals. Also, determination of internal dose accounts for differences in exposure regimes in different studies, where some studies use static exposure (e.g., what was done in the current study) and others use daily renewal of dosing solutions, which will likely change the internal dose.

In addition, to measuring tissue dose immediately after the PFAS exposure at 72 hpf, we also measured tissue at 120 hpf, two days after cessation of exposure, to anchor these doses to the behavior outcomes. Overall, tissue doses only slightly decreased for GenX and PFBS indicating minimal elimination during the 48 hour period and trends remained the same for concentration-dependency, LOQ, and higher doses for PFBS. Moreover, the concentrations of the dosing solutions at the beginning of the exposure and concentrations at the end of the exposure (72 hpf) revealed that concentrations of GenX remained close to the nominal concentrations through the whole duration of exposure with slight decrease at the end of exposure. Similar results were observed in PFBS dosing solutions where all dosing solutions at 1 hpf and 72 hpf remained close to nominal concentrations.

The developing central nervous system is especially sensitive to toxicant exposure. Locomotor activity of zebrafish larvae can be used to determine neuroactive and neurotoxic chemicals (Basnet et al. 2019; Irons et al. 2010) with the neuronal pathways and neurotransmission systems conserved throughout vertebrate evolution (Horzmann and Freeman 2016; Basnet et al. 2019). A recent study concluded that a chemical can be suggested as a developmental neurotoxicant if it induces a significant effect compared to the negative control in multiple lighting phases of the visual motor response assay or if multiple concentrations of a chemical caused a significant effect within one lighting phase (Gaballah et al. 2020). Locomotor activity has been included after developmental PFAS exposure in multiple studies but are limited in specifically investigating GenX and PFBS at lower concentration ranges (Ulhaq et al. 2013b; Gaballah et al. 2020; Khezri et al. 2017; Menger et al. 2020; Truong et al. 2022; Rericha et al. 2021). In our study, we exposed zebrafish embryos to relatively low concentrations compared to other studies throughout embryogenesis (from 1 through 72 hpf) and then assessed locomotor activity of larvae

at 120 hpf. Exposure to 40, 400, or 4000 ppb GenX resulted in hyperactivity as an increase in total distance moved, mean velocity, and time spent moving during the first dark phase. This increase in activity was persistent in the second dark phase but only in the 400 ppb treatment group for total distance moved and velocity. Our results are different to what is reported by two other groups for GenX. Gaballah et al. (2020) did not observe locomotor changes in zebrafish larvae at 144 hpf when exposed to 4.4 to 80 μM GenX (1,527 to 27,766 ppb) for 6 days. Only the highest treatment group in the current study overlapped with this previous study and there were differences in exposure period, age of assessment, and replenishment of solutions every 24 hours (Table 4.4). Alternatively, Rericha et al. (2021) found that exposure to 0.76 μM (250 ppb) GenX from 6-120 hpf caused hypoactivity in the larval photomotor response test in the light phase, which has different light/dark phases compared to the visual motor response assay (Table 4.4).

Compared to GenX, the locomotor alterations induced by the embryonic PFBS exposure were more significant with changes observed in all treatment groups (even the 4 ppb exposure) in all outcomes during all dark phases. These results highlight that PFBS exposure is affecting the ability for the larvae to habituate to the stimulus (dark phase). Hyperactivity was also observed in the 40, 400, and 4000 ppb treatment groups in all light phases further indicating persistent neurotoxicity. Rericha et al. (2021) also observed hyperactivity in light phases in the photomotor response assay, but in contrast to our results, other studies showed that developmental PFBS exposure in zebrafish did not induce behavioral changes (Gaballah et al. 2020; Menger et al. 2020). In addition, Ulhaq et al. (2013) observed a biphasic alteration in activity with PFBS exposure but was at much higher concentrations than used in the present study. Similar to the discrepancies for GenX, the different behavioral outcomes observed in this study compared to others can be explained by variations in exposure concentrations, duration of exposure, age of the larvae at time of assessment, removal of chorion after 24 hours, and lighting regime during the experiments (Table 4.4). In addition, some studies maintained zebrafish at 26°C during development, which would have resulted in a slower developmental time course compared to the majority of studies that maintain zebrafish at 28°C [as recommend in The Zebrafish Book (Westerfield 2000) and for which the developmental stages are standardized (Kimmel et al. 1995)].

Table 4-4. Comparative findings for behavioral analysis of larval zebrafish exposed to GenX or PFBS during development

PFAS	Strain	Concentrations/ Exposure Period	Age at assessment	Temperature	Well Plate	Behavior Test Protocol	Outcomes	Reference
GenX	Mixed wild type	4.4-80 µM (1452.2-26,440ppb) from 1-120 hpf	144 hpf	26°C	96-well plate	20 min of dark for acclimation followed by 20 min light (L1: 10min then L2: 10 min)and 20 min dark (D1: 10 min then D2: 10 min)	No effect on locomotor activity	(Gaballah et al. 2020)
GenX	5D	0.76 µM (250.84 ppb) from 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	0.76 µM (250.84 ppb) caused hypoactivity in light phase	(Rericha et al., 2021)

Table 4-4: Continued								
PFBS	AB	10,000-3,000,000 ppb from 0.5-144 hpf	144 hpf	26 ± 1 °C	48-well plate	10 min of light for acclimation followed by two series of 10 min dark and 10 min light	-1,000,000 ppb (1000 ppm): decrease in overall activiy during dark phases	(Ulhaq et al. 2013b)
PFBS	Mixed wild type	5.5-100 µM (1650-3000 ppb) from 1-144 hpf	144 hpf	26°C	96-well plate	20 min of dark for acclimation followed by 20 min light (L1: 10min then L2: 10 min) and 20 min dark (D1: 10 min then D2: 10 min)	No effect on locomotor activity	(Gaballah et al. 2020)
PFBS-salt	Wild type	0.02-72 µM (6-21,607 ppb) from 0.75-144 hpf	144 hpf	26 ± 1 °C	96-well plate	10 min of light for acclimation followed with 4 series of 5 min dark and 5 min light	PFBS caused no effects	(Menger et al. 2020)

Table 4-4: Continued								
PFBS	5D	0.74 µM (222,07 ppb) from 6-120 hpf	120 hpf	28°C	96 well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	0.74 µM exposure caused hyperactivity in light phase	(Rericha et al., 2021)

Previous studies report that behavioral abnormalities in zebrafish larvae are associated with dopamine deficits or alterations in dopaminergic neurons development (Spulber et al. 2014; Yu et al. 2021; Wu et al. 2022). Here, we observed that exposure to 40 ppb GenX during the embryonic period caused increased in dopamine levels at 72 hpf (13% increase), while exposure to 400 ppb PFBS resulted in decreased dopamine (9% decrease). This result is interesting in showing a potential for two different mechanisms of action for each chemical on the dopaminergic pathway, which agrees with an earlier study that modeled developmental neurotoxicity of a few PFAS in differentiating PC12 cells (Slotkin et al. 2008). The study in PC12 cells concluded that PFAS will not have one shared mechanism of developmental neurotoxicity and observed variations in promotion and/or suppression of dopamine and acetylcholine neurotransmitter phenotypes. Specifically, they observed PFBS to suppress dopamine neuron differentiation (around 35% decrease), which aligns with the findings of our study reporting decreased dopamine for PFBS. While studies are limited that have included GenX or PFBS, others have observed dopamine-related alterations with exposure to other PFAS in multiple organisms. Reports include decreased dopamine and dopamine turnover in Northern leopard frogs with developmental exposure to PFOA (1000 ppb) or PFOS (100 and 1000 ppb) (Foguth et al. 2019); dopaminergic neuropathology in C. elegans with a 72 hour PFOS exposure (Sammi et al. 2019); alterations in dopamine in planarians with PFOS exposure (Yuan et al. 2018); perturbations to dopaminergic gene transcription in the mouse cerebral cortex and hippocampus with a postnatal PFOS exposure in mice (Hallgren and Viberg 2016); multiple modifications in the dopaminergic system in the adult

male rat amygdala, prefrontal cortex, and hippocampus with PFOS exposure for 28 days (Salgado et al. 2016); impairment of human dopaminergic neurons with PFOA exposure for 24 hours at 10 ppb (di Nisio et al. 2022); among others (reviewed in (Foguth et al. 2020)).

To further investigate impacts of GenX and PFBS on the dopaminergic system, gene expression analysis was completed for *mao*, *th*, and *dat*. Monoamine oxidase (MAO) is responsible for metabolizing dopamine in the cytosol into 3,4-dihydroxyphenylacetic acid (DOPAC). Tyrosine hydroxylase (TH) is the rate limiting step enzyme in dopamine synthesis that converts tyrosine to dihydroxyphenylalanine. Dopamine transporter (DAT) is presynaptic transporter that is responsible for dopamine uptake from the synapse (Wasel and Freeman, 2020). Generally, *th* and *dat* are used as markers for dopaminergic neurons. Although other studies report transcription alterations in these genes for other PFAS (Yu et al. 2021, Wu et al. 2022), our results showed that there were no significant changes in the expression of these three genes. Future work should assess other targets such as the dopamine receptors (i.e., D1- and D2-like receptors), the vesicular monoamine transporter (VMAT) that transports dopamine from the cytoplasm to secretory vesicles, and catechol-o-methyltransferase (COMT) that degrades dopamine in the synaptic cleft into methyltyramine. Alterations in expression of these targets may explain the alterations that were observed in dopamine levels. Furthermore, other studies showed that expression of *th* and *dat* were increased in larvae exposed to PFOA from 48 to 168 hpf (Yu et al. 2021). In that study, only the 100 ppb PFOA exposure, and not 10 or 1000 ppb, induced a significant increase in *th* expression. In addition, a similar observation was detected for *dat*, where only the 100 ppb PFOA exposure show a significant increase in expression. Furthermore, another study assessing the effects of PFOS on dopamine pathways reported exposure from 2-120 hpf caused an increase in dopamine (33% increase) and upregulation of *th* expression in the 3.2 ppm treatment group but no significant change in *dat* expression (Wu et al. 2022). This study reported an increase of swimming speed in 0.32 ppm and 3.2 ppm treated larvae. study also showed increased protein expression of TH and DAT in the 0.32 and 3.2 ppm treatment groups. Thus, it is likely that impacts on gene expression may be non-monotonic and that changes in protein levels may occur in absence of alterations in gene expression at the same assessment time point. Similarly, another study showed increased dopamine level in the prefrontal cortex of the adult rat after exposure to 1 mg/kg/day PFOS but no changes were seen in the 3 or 6 mg/kg/day treatments again supporting a non-monotonic dose response (Saldago et al. 2016).

4.6 Conclusions

The results of this study confirm that PFBS is more acutely toxic compared to GenX. Assessment of tissue dose showed variation in accumulation between GenX and PFBS with dosing solutions at the same nominal exposure concentration, which were then confirmed to be near the expected treatment concentration and emphasizes importance of assessing internal dose based on different toxicokinetic properties among the PFAS. In addition, an embryonic exposure to GenX or PFBS induced behavioral changes in zebrafish larvae. PFBS had more persistent behavioral alterations at lower nominal exposure concentrations compared to GenX, highlighting that sulfonate-containing PFAS are triggering stronger behavioral alterations compared to carboxylic-containing PFAS and confirming that the functional group is playing an important role in induction of PFAS toxicity. Furthermore, different mechanisms of toxicity are hypothesized for these two PFAS based on opposite effects observed for dopamine levels for each. Future work is needed to further investigate the molecular mechanisms driving developmental neurotoxicity on the dopaminergic system.

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CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation aimed to investigate the developmental toxicity of short chain perfluoroalkyl substances (PFAS): perfluorobutanoic acid (PFBA), perfluorobutane sulfonate (PFBS), perfluorohexanoic acid (PFHxA), and perfluoro-2-propoxypropanoic acid (GenX) using the zebrafish model. These chemicals are used as replacements to longer alkyl chain PFAS such as perfluorooctanoic acid (PFOA). There is relatively limited information regarding toxicity of these chemicals. Comparison between developmental toxicity including LC50, morphological alterations, neurobehavior, and transcriptomic profile of the short chain chemicals to effect induced by PFOA exposure was performed. In addition, effects of embryonic exposure of the two emerging PFAS, GenX and PFBS, on development of dopaminergic neurons were assessed.

The first study revealed that chain length and functional group contribute to the toxicity of the tested PFAS where the toxicity ranking was PFOA > PFHxA > PFBA and PFBS > PFBA. There was an agreement between results obtained from zebrafish larvae or cells when compared to other aquatic models and in vitro models. This study highlighted the importance of neutralizing perfluoroalkyl acids solutions when assessing acute toxicity with high concentrations to avoid getting results influenced by the acidity, and not the actual chemical.

The second study was focused more on the effects of embryonic exposure to sublethal concentrations of PFHxA and PFBA in comparison to PFOA. PFOA and PFBA caused alterations in neurobehavior of 120 hpf larvae, while PFHxA exposure caused no change in behavior of larvae. These results indicated potential neurotoxic effect of PFOA and PFBA. PFOA, PFHxA, and PFBA exposures induced alterations in morphology of exposed larvae compared to control. In addition, exposure to PFOA, PFHxA, and PFBS caused alterations in expression of multiple genes associated with diseases. The transcriptome analysis showed concordance of several of the reported human adverse health outcomes associated with PFOA exposure, such as cancer, altered immune response, and endocrine disruption, based on similarity of molecular pathways and networks and canonical pathways observed following the embryonic exposure in the developing zebrafish. This result validates the use of early life stage of zebrafish in predicting effects of toxicants and identifying molecular targets to guide future research. The transcriptome profile for each chemical was unique. These differential effects of the three chemicals can be explained by

differences in mechanisms of action or the differences in internal doses corresponding to same nominal concentration, which wasn't measured in this study. Overall, this study showed that shorter chain chemicals, PFHxA and PFBA, caused developmental toxicity in developing larvae.

The third study was focused on assessing developmental neurotoxicity of GenX and PFBS. PFBS induced hyperactivity in exposed larvae with all tested exposures (4, 40, 400, 4000 ppb). GenX also induced hyperactivity but only with high exposures levels (40, 400, and 4000 ppb). Gene expression analysis of tyrosine hydroxylase, monoamine oxidase, and dopamine transporter showed no changes with either GenX or PFBS exposures; however, dopamine levels were altered in larvae exposed to GenX or PFBS, but the alterations were in two opposite directions. The study showed that accumulation of PFBS in zebrafish tissue was higher than GenX using the same nominal exposure. This result agrees with previous studies that showed sulfonate-containing PFAS have longer half-lives and accumulate at higher levels compared to carboxylate-containing PFAS. However, the differences in tissue dose may explain differences in responses after exposure GenX and PFBS. Future studies should investigate the molecular mechanisms behind the observed changes in dopamine.

An important finding of our research is that the acute toxicity of PFOA is much higher than shorter chain PFAS (PFHxA, PFBA, PFBS, and GenX); however, these short chain PFAS can cause adverse effects at sublethal exposures. This result highlights the importance of not only relying on the LC50 when comparing toxicity of chemicals, especially during introducing safer alternatives. Overall, in this research embryonic exposure to the short chain PFAS caused developmental toxicity and developmental neurotoxicity in zebrafish. Our results showed that the functional group and chain length are a driver of toxicity, but the functional group has a higher influence on toxicity. Assessment of internal dose is essential to help understand the hazard associated with PFAS exposure. Understanding toxicokinetics of PFAS in zebrafish will enhance the use of data from this animal model to inform human risk assessment regarding PFAS, which is an important step towards regulating these chemicals. Here, we showed that transcriptome analysis was able to capture many of the known adverse health effects associated with PFOA. Data is currently limited for PFHxA and PFBA to be able to make a similar comparison but given findings in this research with PFOA and similar studies with other environmental chemical contaminants there is support for future studies to use transcriptome analysis using zebrafish to predict toxicity of other environmental chemical contaminants and environment-relevant mixtures.

These analyses will enhance our understanding of the risk associated with exposure to these chemicals. Also, the use of behavioral analysis is an important tool to predict PFAS developmental neurotoxicity. Our results showed that embryonic exposure to PFOA, PFBA, GenX, and PFBS caused alterations in locomotor activity with alterations in dopamine in PFBS and GenX exposures. Future studies should investigate the mechanism behind these neurotoxic effects.

VITA

EDUCATION

PURDUE UNIVERSITY West Lafayette, Indiana

Doctor of Philosophy – Toxicology August 2022
Master of Science- Toxicology August 2018
Master of Public Health -- August 2017

ALEXANDRIA UNIVERSITY Alexandria, Egypt

Master of Science- Biotechnology August 2010
Bachelor of Science- Biochemistry/*chemistry* *May 2006*

RESEARCH FUNDING

-Neurotoxicity of Polyfluoroalkyl Substances (PFAS) Mixtures, Pilot Project Research Training Grant, CDC-NIOSH Illinois ERC, NIOSH #T42/OH008672 (PI, $20,000) Jul'19-Jun'20

-Neurotoxicity of Polyfluoroalkyl Substances (PFAS) Mixtures in Firefighting Materials, Pilot Research Project Grant, CDC-NIOSH U. of Cincinnati ERC (UC-ERC), NIOSH #T42/OH008432 Jul'19-Jun'20

WORK EXPERIENCE

-Research Assistant, Freeman Laboratory- Purdue University Aug'16-Aug'22
-Safer Materials & Biocompatibility Intern, Environmental Technologies- Apple Inc.
July'20- Dec'20
-Teaching Assistant, School of Health Sciences- Purdue University Aug'17- May'19
-Intern, Radiological and Environmental Management- Purdue University May'17- July'17
-Research Assistant, Tissue Engineering Laboratories- Alexandria University Jan'07-Aug'17

AWARDS

◊ Purdue Graduate School Global Ambassador *Aug'18 - present*
◊ Best Graduate Student Abstract Award, Neurotoxicology Specialty, Society of Toxicology *Mar'22*
◊ Wayne V. Kessler Graduate Student Award, School of Health Sciences, Purdue University (**$2,250**) *Mar'22*
◊ Toshio Narahashi Trainee Conference Endowment, Neurotoxicology Specialty, Society of Toxicology (**$500**) *Mar'22*
◊ Purdue Graduate Student Government Top Tier Travel Award to attend SOT'22 (**$750**) *Mar'22*

◊ Best Graduate Student Abstract, Arab Toxicologist Association, Society of Toxicology, SOT'22 *Mar'22*
◊ Designated alternate Graduate Student Representative, Arab Toxicologist Association, SOT'22 (**$700**) *Mar'22*
◊ 2nd place Oral Poster Presentation award, Ohio Valley Society of toxicology (**$400**) *Nov'21*
◊ Purdue Graduate Student Government Top Tier Professional Development Award (**$750**) *Sep'21*
◊ Society of Toxicology Supplemental Training for Education Program to attend TERA's Boot camp (**$1000**) *Jul'21*
◊ 1st place, School of Health Sciences' annual research retreat poster session, Purdue University, IN (**$250**) *Apr'21*
◊ 2nd place, Purdue's 2020 Health and Disease Poster Session Competition, Purdue University, IN (**$300**) *Mar'20*
◊ Society of Toxicology Graduate Student Travel Support, SOT annual meeting, Anaheim, CA (**$800**) *Mar'20*
◊ Frederick N. Andrews Environmental Travel Grant, The Graduate School, Purdue University (**$1500**) *Oct'19*
◊ Distinguished Poster Presentation, 20th Annual Pilot Research Project Symposium NIOSH UC-ERC, OH *Oct'19*
◊ Society of Toxicology SIG-TAO Graduate Student Travel Award, Baltimore, MD (**$200**) *Mar'19*
◊ Purdue Graduate Student Government 2nd Tier Travel Award (**$500**) *Jan'19*
◊ Purdue Graduate Student Government Top Tier Travel Award (≈ 2% of applicants) (**$750**) *Jan'18*
◊ Egyptian Academy of Scientific Research and Technology: Grant to pursue masters studies *Jan'07*
◊ Full-Ride scholarship for undergraduate studies in chemistry and biochemistry *Aug'02 - May'06*

PUBLICATIONS

- C. J. Ley, O. Wasel, K. P. Isaacson, C. R. Proctor, M. Tariq, A. D. Sha, J. Freeman, and A. Whelton, Crosslinked Polyethylene (PEX) Pipe Leaching: Toxicological Impacts on Zebrafish, Carbon Release, and Microbial Growth (submitted)
- O. Wasel, K. M. Thompson, and J. L. Freeman, Evaluation of developmental toxicity and transcriptomic alterations in zebrafish (Danio rerio) exposed to PFOA, PFHxA and PFBA, (in preparation)
- O. Wasel, K. M. Thompson, Y. Gao, A. E. Godfrey, J. Gao, C. T. Mahapatra, L. S. Lee, M. S. Sepúlveda, and J. L. Freeman, *Comparison of zebrafish in vitro and in vivo developmental toxicity assessments of perfluoroalkyl acids (PFAAs)*, Journal of Toxicology and Environmental Health, Part A, 84 (2021), pp. 125–136. PMID: 33143551
- O. Wasel and J. L. Freeman, *An overview on tungsten toxicity*, in Metal Toxicology Handbook, D. Bagchi and M. Bagchi, eds., CRC Press, Boca Raton, Florida, 2020
- O. Wasel and J. L. Freeman, *Chemical and genetic zebrafish models to define mechanisms of and treatments for dopaminergic neurodegeneration*, International Journal of Molecular Sciences, 21 (2020), p. 5981

- R. Zhang, M. R. Silic, A. Schaber, O. Wasel, J. L. Freeman, and M. S. Sepúlveda, *Exposure route affects the distribution and toxicity of polystyrene nanoplastics in zebrafish*, Science of The Total Environment, (2020), p. 138065
- O. Wasel and J. Freeman, *Comparative assessment of tungsten toxicity in the absence or presence of other metals*, Toxics, 6 (2018), p. 66
- O. Wasel, *Investigating the effects of cobalt and nickel mixtures on tungsten toxicity in zebrafish (danio rerio)*, Master's thesis, Purdue University, 2018
- O. Wasel, *Differentiation of bone marrow-derived mesenchymal stem cells into hepatocyte-like cells*, Master's thesis, Alexandria University, 2010

TEACHING EXPERIENCE **Aug'17-May'19**

-HSCI 13100 – Introduction to Medical Terminology Fall 2015

-HSCI 34500 – Introduction to Occupational and Environmental Health Sciences Spring'18,Fall'18,Spring'19

-HSCI 57500 – Introduction to Environmental Health Spring 2018, 2019

-HSCI 6900 – Seminar in Health Sciences Spring 2018

PRESENTATIONS

- Developmental Neurotoxicity of GenX and PFBS using Zebrafish

- Society of Toxicology Annual Meeting, SanDiego, CA Mar'22

- Health and Human Sciences Spring Research Day, Purdue University Mar'22

- Comparative Toxicity Assessment of Legacy and Emerging Perfluoroalkyl Substances Using Zebrafish Model Nov'21
  - Oral Poster Presentation, Ohio Valley Society of Toxicology annual meeting (Virtual)

- Developmental Toxicity of Perfluoroalkyl Substances Using Zebrafish

- (PhD Platform Presentation) Ohio Valley Society of Toxicology Summer meeting (Virtual) July'21

- Ecological and Environmental Engineering Research Seminar, Purdue University Mar'21

-Purdue Center for the Environment, Environmental Research Expo (Virtual) Oct'20

- Society of Toxicology Annual Meeting, Anaheim, CA (Virtual) Mar'20

- Purdue's 2020 Health and Disease Poster Session, Neuroscience and Neurodegenerative Diseases, IN Mar'20

- Neurotoxicity of Perfluoroalkyl Substances (PFAS) Mixtures in Firefighting Material

- (Podium talk) 2020 Pilot Research Project Symposium, U. of Cincinnati NIOSH ERC (Virtual) Oct'20 · 2019

- Pilot Research Project Symposium, U. of Cincinnati NIOSH Education and Research Center, OH Oct'19

- *Investigating Effects of Cobalt and Nickel on Tungsten Toxicity in a Zebrafish model*

- Society of Toxicology Annual Meeting, Baltimore, MD Mar'19

- 5th Annual Purdue Environmental Community Mixer, Center for Environment (C4E), Purdue, IN Oct'18

- Society of Toxicology Annual Meeting, San Antonio, TX Mar'18
- Health and Human Sciences Research day, Purdue, IN Nov'17
- 4th Annual Purdue Environmental Community Mixer, C4E, Purdue, IN Sep'17

PROFESSIONAL MEMBERSHIPS

- The Association for the Advancement of Alternatives Assessment (A4) Indiana Environmental Health Association
- Purdue Graduate Student Public Health Association
- Society of Toxicology
    - Arab Toxicologists Association (founding member)
    - Toxicologist of African Origin
    - Risk Assessment Specialty Section
    - Regulatory and Safety Evaluation Specialty Section
    - Neurotoxicology Specialty Section
    - Medical Device and Combination Product Specialty Section
    - Metals specialty group
    - Occupational and Public Health Specialty Section