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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ASSESSMENT OF THE TOXICITY OF LEGACY AND NEXT-GENERATION PERFLUOROALKYL SUBSTANCES (PFAS) IN EARLY-LIFE STAGES OF FRESHWATER AND MARINE FISH

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Kiflom Y. Gebreab

2022

To: Dean Michael R. Heithaus
Collage of Arts, Sciences and Education

This dissertation, written by Kiflom Y. Gebreab, and entitled Assessment of the Toxicity of Legacy and Next-Generation Perfluoroalkyl Substances (PFAS) in Early-Life Stages of Freshwater and Marine Fish, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Kiflom Y. Gebreab is approved.

Dean Michael R. Heithaus
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Andrés G. Gil
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and Dean of the University Graduate School

Florida International University, 2022

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DEDICATION

I dedicated this dissertation to my wonderful wife and my children. Without your love, patience and support, the completion of this work would not have been possible.

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I would like to thank Dr. John P. Berry for his excellent mentorship, welcomed me in his lab and introduced me to the aquatic toxicology science. I would like to express my greatest gratitude to his boundless support, supervision, guidance and believing on me.

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Finally, I would like to thank my wife for her love, encouragement, and endless support. Without her relentless and unconditional support, this work would not be accomplished.

ABSTRACT OF THE DISSERTATION

ASSESSMENT OF THE TOXICITY OF LEGACY AND NEXT-GENERATION PERFLUOROALKYL SUBSTANCES (PFAS) IN EARLY-LIFE STAGES OF FRESHWATER AND MARINE FISH

by

Kiflom Y. Gebreab

Florida International University, 2022

Miami, Florida

Professor John P. Berry, Major Professor

Per- and polyfluoroalkyl substances (PFASs) are a group of synthetic surfactants that have utilized for several decades in a wide range of industrial and manufacturing applications. Owing to their high chemical stability, PFAS are widespread and persistent in the environment, and have been shown to be bioaccumulative in diverse organisms including plants, wildlife, and humans. Exposure to PFASs has been linked to a range of documented toxic and/or adverse effects in relation to human and animal health. The potential environmental health and ecotoxicity of next-generation PFAS remain unclear. In the present study, toxicity, and bioconcentration potential, of several perfluoroether carboxylic acids (PFECA), as emerging pollutants of concern, in early life stages of marine (Mahi-Mahi and Olive Flounder) and freshwater fish (zebrafish) was

assessed. Toxicity including lethality, behavioral and developmental effects of PFECA, and the legacy PFAS, perfluorooctanoic acid (PFOA), was assessed in embryonic stages of zebrafish, alongside high-resolution magic angle spin nuclear magnetic resonance (HRMAS NMR) metabolomics techniques to elucidate metabolic pathways affected by PFAS. These studies identified acute embryotoxicity (i.e., lethality), as well behavioral and developmental effects, in the nominal micromolar range for all PFAS tested, which was correlated with fluoroalkyl chain length (and relative lipophilicity, i.e., log P), and suggested quantitatively comparable toxicity for next-generation (i.e, PFECA) and legacy (i.e., PFOA) variants. Metabolomic studies indicated targeting of liver, and specifically mitochondria, and associated metabolic pathways. Subsequently, toxicity assays were developed and applied to early life stages of mahi-mahi to assess toxicity of PFECA and PFOA. Mahi-mahi embryos were significantly more sensitive to PFAS with lethal concentrations extending into the environmentally relevant (e.g., parts-per-billion) concentration range. Finally, bioconcentration of PFECA and PFOA were comparatively assessed in embryos of zebrafish and flounder. These studies measured bioconcentration factors (BCF) ranging from 83 to 226-fold and 22 to 329-fold bioconcentration (relative to measured concentration in water) in embryos of zebrafish and flounder, respectively, with BCF values significantly correlated with fluoroalkyl chain length and log P values, in both cases. These findings raise concerns regarding PFECA as environmental toxicants, and specifically, as next generation replacements to legacy PFAS.

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ABBREVIATIONS AND ACRONYMS

Lethal concentration for 50 % of the population	LC50
High-resolution magic angle spin Nuclear magnetic resonance	HRMAS- NMR
Perfluoroalkyl substance	PFAS
Perfluoroethercarboxylic acid	PFECA
Perfluoro(4-oxapentanoic) acid	PFMOBA
Perfluoro(5-oxa-6-dimethylhexanoic) acid	PFDMMOBA
Perfluoro(2-methyl-3-oxahexanoic) acid	PFPrOPrA (GenX)
Perfluoro(3,6-dioxheptanoic) acid	PFO2HpA
Perfluoro(3,6-dioxadecanoic)	PFO2DA
Perfluoro(3,6,9-trioxadecanoic) acid	PFO3DA
Perfluoro(3,6,9-trioxatridecanoic) acid	PFO3TDA
Perfluorooctanoic acid	PFOA
Observed-adverse-effect level	NOAEL
Lowest-observed-adverse-effect level	LOA

CHAPTER 1

Introduction

1.1 Perfluoroalkyl Substances (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are a broad class of synthetic compounds characterized by fluorocarbon "tail," consisting of partial or full substitution of the hydrogen atoms of a hydrocarbon backbones by fluorine atom, typically coupled to a polar headgroups including, most frequently, carboxylic acid and sulfonic acid functional groups (Figure 1.1; Buck et al., 2011) which impart amphiphilicity, and corresponding surfactant properties. The remarkably strong C–F bonds of the fluorocarbon tail, in particular, imparts extreme resistance to hydrolysis, photolysis, and biodegradation (Buck et al., 2011; Prevedouros et al., 2006; Wang et al., 2015). Owing to this chemical and thermal stability, and their amphiphilic nature, PFAS have been utilized widely as surfactants (Kissa, 2001), and these distinctive properties have led to widespread industrial, household, and commercial applications including cosmetics, firefighting foams, food contact materials, medical devices, pesticide formulations, household products (e.g., Teflon), water repellents, textile coatings, paper products, food packaging, and aqueous film-forming foams (Armitage, 2009; Brendel et al., 2018; Buck et al., 2011; Kemi, 2015; Prevedouros et al., 2006; Wang et al., 2013). Due to their extensive use, and remarkably high stability, they are both widespread and highly persistent in the environments, and have been shown to be bioaccumulative in a range of organisms including plants, wildlife and humans (Giesy & Kannan, 2001; Kemi, 2015; Prevedouros et al., 2006; Wang et al., 2017).

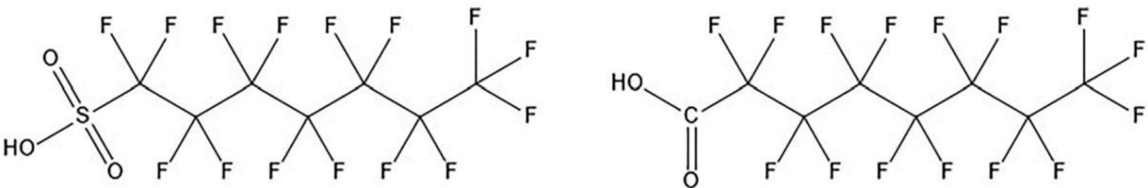

Figure 1. 1 Chemical structure of Perfluoro sulfonic acid (PFOS) perfluorooctanoic acid (left) and PFOA (right)

Perfluoroalkyl substances have been in use and released into the environment over the past 60 years (Lindstrom et al., 2011). As a result of the widespread occurrence of PFASs in the environment, numerous studies in the past two decades have investigated their presence in a range of environmentally relevant matrices including surface water, soils, sediments, groundwater and landfill effluents (Bai & Son, 2021; Banzhaf et al., 2017; Brusseau et al., 2020; Dalahmeh et al., 2018; Houtz et al., 2013; Zhao et al., 2016). High concentrations of PFASs were detected, for example, in the Las Vegas Wash water (2234.3 ng/L) and Truckee River water (441.7 ng/L) including, in particular, so-called "legacy" PFAS, such as perfluorooctanoic acid (PFOA; 65.5 ng/L; (Bai & Son, 2021). Their release into the environment affects, in particular, aquatic environments (Ahrens, 2011; Ahrens & Bundschuh, 2014; Clara et al., 2009; Groffen et al., 2018), as well as humans and wildlife associated with these systems (Alkhalawi et al., 2016; De Silva et al., 2021; Giesy & Kannan, 2001; Hall et al., 2022; Shi et al., 2015). Although the exposure mechanism of PFASs to humans and the environment is uncertain, it may occur through different pathways, including direct exposure from the commercial product (Dinglasan-Panlilio & Mabury, 2006; Kim et al., 2012; Prevedouros et al., 2006; Sinclair et al., 2007), abiotic breakdown of PFASs during production (D'eon et al., 2006; Martin et al., 2006), and metabolic transformation of precursors (Martin et al., 2005; Tomy, Tittlemier, et al., 2004).

An indoor source of exposure was reported in a study conducted by Shoeib et al. (2011): in 152 households in Vancouver, Canada, PFOA was detected in indoor air with a geometric mean of 28 pg/m^3. Human exposure may, thus, occur through dietary routes, drinking water, air and dust inhalation, and even perhaps direct skin absorption (Ostertag et al., 2009; Vestergren & Cousins, 2009; Vestergren et al., 2008). The most widely used PFAS including PFOA and perfluorooctane sulfonic acid (PFOS) have even been discovered in remote polar locations (Giesy & Kannan, 2001). And, being water soluble, PFASs may spread globally through ocean circulation.

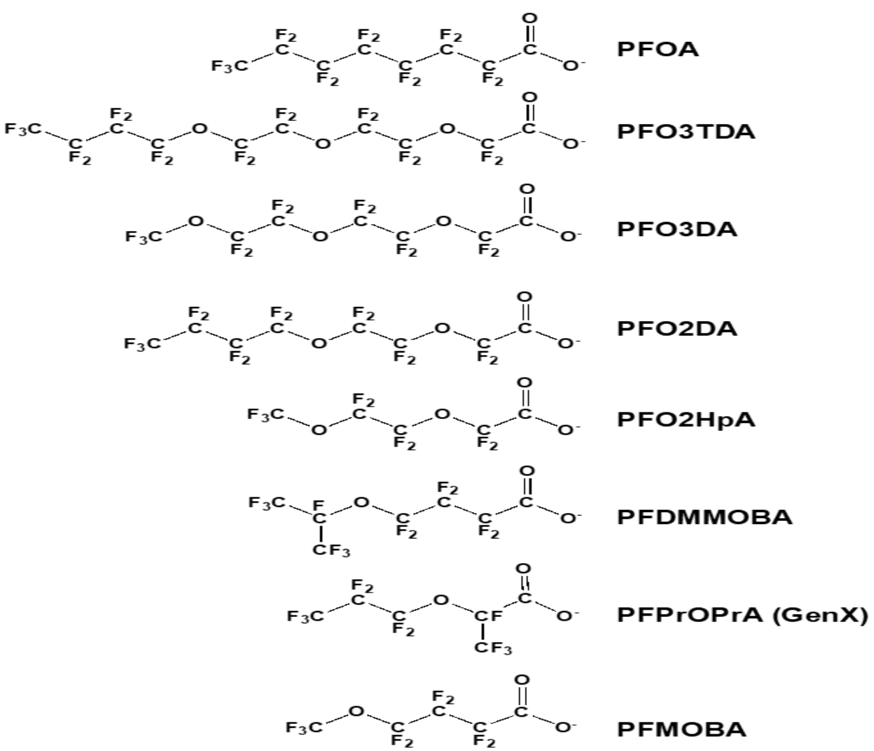

Figure 1. 2 Perfluoralkyl substances investigated in the current study, including PFOA and seven different PFECA. Abbreviations: PFOA = perfluorooctanoic acid, PFO3TDA = perfluoro-3,6,9-trioxatridecanoic acid, PFO3DA = perfluoro-3,6,9-trioxadecanoic acid,

PFO2DA = perfluoro-3,6-dioxadecanoic acid, PFO2HpA = perfluoro-3,6-dioxaheptanoic acid, PFDMMOBA = 4-(heptafluoroisopropoxy)hexafluorobutanoic acid, PFPrOPrA or "GenX" = perfluoro (2-methyl-3-oxahexanoic) acid, PFMOBA = perfluoro (4-methoxy butanoic) acid.

In view of their persistent and widespread distribution in the environment, and potential for bioaccumulation, as well as toxicity (discussed further below), the production and use of several of the most common PFASs – including long-chain ($\geq$ 7 C) perfluorocarboxylic acids, such as PFOA (Figure 1.2), and perfluoroalkyl sulfonic acids, such PFOS – have been phased out from production in the United States and Europe (Cadore et al., 2009; Pérez et al., 2013; Sheriff et al., 2020; Wang et al., 2017). That said, these long-chain "legacy" PFASs have been largely replaced by a variety of "next-generation" compounds, and the occurrence of these alternative PFASs – especially short-chain PFASs – is increasing (Ahrens & Bundschuh, 2014). Among these PFAS of *emerging* concern are numerous perfluoroether carboxylic acids (PFECA; Figure 1.2) in which one or more fluorocarbon (i.e., $-CF_2-$) is replaced by an ether oxygen which largely retains chemical properties of legacy PFAS (Cadore et al., 2009; Schuchat & Breysse, 2018; Wang et al., 2015). Emerging chemical variants including PFECA – as alternatives for legacy PFAS– are, however, also highly stable, and recent studies suggest that they are equally persistent, and may similarly bioaccumulate in the environment, as well as sharing similar toxicity (Gebreab et al., 2020; Parsons et al., 2008; Sun et al., 2016; Wang et al., 2015; Wang et al., 2013; Young & Mabury, 2010).

1.2. Exposure and Toxicological Risks of PFAS

Alongside potential for bioaccumulation, PFAS including PFECA and other alternative chemical variants are problematic environmental pollutants that are associated

with multiple adverse health effects. A number of studies have reported the detection of PFASs in living organisms (Apelberg et al., 2007; Butenhoff et al., 2006; Langberg et al., 2019). Humans can consume these compounds through drinking contaminated water, ingesting seafood (e.g., fish), inhaling indoor air, and contact with other consumer products (Tomy, Budakowski, et al., 2004; Vestergren et al., 2008). Drinking water is known as one of the main sources of PFAS exposure for humans, particularly those living near polluted areas ((Domingo & Nadal, 2019). A few US states have their own PFAS guidelines, while others have adopted the US Environmental Protection Agency (EPA) lifetime health advisory level for PFOS and PFOA of 70 ng/L in drinking water (Boone et al., 2019; Post, 2021). Some US states additionally have their own guidelines for short-chain PFAS such as perfluorohexanoic acid (PFHxA), perfluorobutane sulfonate (PFBS), and GenX. For example, in North Carolina the guideline for GenX in drinking water is 140ng/L, in Michigan 370 ng/L, and in Ohio 700ng/L (Post, 2021). Animal studies have revealed that PFAS are completely absorbed orally and by inhalation (Flynn et al., 2021; Poothong et al., 2020).

Short-chain PFASs are generally less bioaccumulative in animals and humans than their long-chain counterpart (Conder et al., 2008; Wang et al., 2013). There is, however, limited experimental data on the bioaccumulation of PFECAs in animals and humans. In fact, only two of the most commonly used PFECAs – under the trade names GenX and ADONA (4,8-dioxa-3H-perfluorononanoate) – have been studied with respect to bioaccumulation. Earlier studies demonstrated the serum elimination half-lives of these PFECAs in rats and in humans (Gomis et al., 2018; Wang et al., 2015). Although these

compounds were bioaccumulative in rats and humans, the findings of these studies are not conclusive enough to make any recommendation. Large-scale bioaccumulation studies on PFECAs in animal and human models are needed in order to arrive at any conclusions about serum elimination half-lives of PFECAs.

This class of PFAS is, therefore, emerging as a potential concern as environmental toxicants, having been detected in humans (Nicole, 2020; Yao et al., 2020), in the air (Fang et al., 2018), in fish, and in water and other environmental samples (Li et al., 2020; Sun et al., 2016; Sunderland et al., 2019). Moreover, their presence (and bioaccumulation) in the environment has been linked to adverse effects in animal models, including hepatotoxicity, embryotoxicity (Gebreab et al., 2020), cytotoxicity (Gorrochategui et al., 2014), immunosuppression, thyroid malfunction, and reproductive and developmental toxicity (Fenton et al., 2021).

A number of PFAS accumulate in organs, and high concentrations have been measured in human blood samples, including maternal blood and fetal cord blood, where they bind strongly to human serum albumin (HSA) – increasing concern about potential *in utero* exposure and developmental toxicity (Pérez et al., 2013; Poothong et al., 2020; Weiss-Errico et al., 2018). They are easily absorbed and bind to proteins in blood serum, and accumulate in the target organs, including the liver, kidneys, and spleen (Jensen & Leffers, 2008). Long-chain PFASs (e.g., PFOA and PFOS) are associated with various adverse effects on human health, such as reproductive toxicity, carcinogenicity (e.g., liver, kidney and bladder cancer), thyroid malfunction, and immunotoxicity (ATSDR, 2018; Prevedouros et al., 2006; Shrestha et al., 2017; Tarapore & Ouyang, 2021).

Although the carcinogenic mechanism of PFASs has not been fully evaluated in humans, the US EPA classified PFOA as an animal carcinogen and the International Agency for Research on Cancer regards it as a likely carcinogen in humans (IARC, 2017; USEPA, 2009).

For emerging PFASs, such as PFECA, there is limited data on the toxicity, health impacts and possible environmental health concerns (Wang et al., 2013; Wang et al., 2017). An assessment of toxicity of short-chain PFAS by the U.S. EPA(EPA,2018) has, however, reported toxicity for GenX and PFBS. Furthermore, recent studies in early life stages of the zebrafish - as a vertebrate toxicological model - have confirmed that next-generation PFASs including PFECA appear to be comparable in potency to their homologous long-chain PFASs (Gaballah et al., 2020; Gebreab et al., 2020). In a 2020 study conducted by Gebreab et al. (2020; see Chapter 2), acute embryotoxicity, together with impaired development and variable effects on locomotory behavior, were observed for PFAS in the zebrafish embryo model. Notably, both toxicokinetics and toxicity of PFASs have been demonstrated as highly correlated with fluoroalkyl chain length (Buhrke et al., 2013; Gebreab et al., 2020; Vogs et al., 2019), and in studies in the zebrafish embryo model, median lethal concentration (LC_{50}) was, for example, significantly correlated with alkyl chain length. Metabolic profiling of zebrafish embryos exposed to PFAS including PFOA and representative PFECA further revealed an alteration of metabolic profiles in zebrafish embryos, suggesting targeting of hepatocytes (i.e., hepatotoxicity), as well as apparent alteration of neural metabolites.

1.3 Animal Models in Toxicity Studies

Due to close phylogenetic proximity to humans, animal models have frequently been used in various environmental and toxicological studies. Toxicity of environmental pollutants in animal models can be extrapolated to adverse effects in humans. Multiple studies on the toxicity of PFAS have, thus, been conducted in a wide range of relevant vertebrate models ranging from rodents to fish. With respect to the latter, species of finfish as both established laboratory models including, in particular, the zebrafish (*Danio rerio*), as well as ecologically and commercially relevant species have been employed to assess environmental toxicants including PFAS (Nelson et al., 2016; Weiss-Errico et al., 2017; Fair et al., 2019; Fenton et al., 2021; Gebreab et al., 2020).

1.3.1 Zebrafish Embryos as a Toxicological Model

Zebrafish were first introduced to laboratories, as an animal model, in the 1980s, and on a large scale starting in the early 1990s (Yang et al., 2009). Due to transparent embryos and rapid organogenesis, zebrafish are widely used as a model for various toxicological and environmental studies. They have many characteristics that make them a convincing tool for toxicology studies. They are small in size and have rapid embryogenesis and high fecundity, which means that many embryos (200–250 eggs) can be produced per mating. This enables inexpensive husbandry, and larger sample sizes within a shorter period (Berry et al., 2007; Horzmann & Freeman, 2018). Zebrafish embryos are transparent – an advantage that makes them suitable for *in vivo* studies (Fishman, 1999). The embryos develop outside the body (*ex vivo*), making them easy to deal with in relation to chemical exposure and monitoring. Moreover, the zebrafish genome is approximately 75%

homologous with the human genome. This high degree of similarity has resulted in zebrafish being used in a wide range of studies on human genetic disease (Howe et al., 2013). Due to the above characteristics and advantages, zebrafish have emerged as one of the most important research tool in many toxicological studies.

Zebrafish (*Danio rerio*) larvae and embryos have become established as a vertebrate toxicological model, and indeed, most PFASs toxicity studies in fish have been conducted with zebrafish (Hagenaars et al., 2011; Jantzen et al., 2016; Weiss-Errico et al., 2017; Gaballah et al., 2020; Gebreab et al., 2020). In a 2017 study by Weiss-Errico et al., sub-lethal mortality (LC_{50}) relevant to developmental and acute toxicity was analyzed in 1–7 dpf zebrafish (*Danio rerio*)

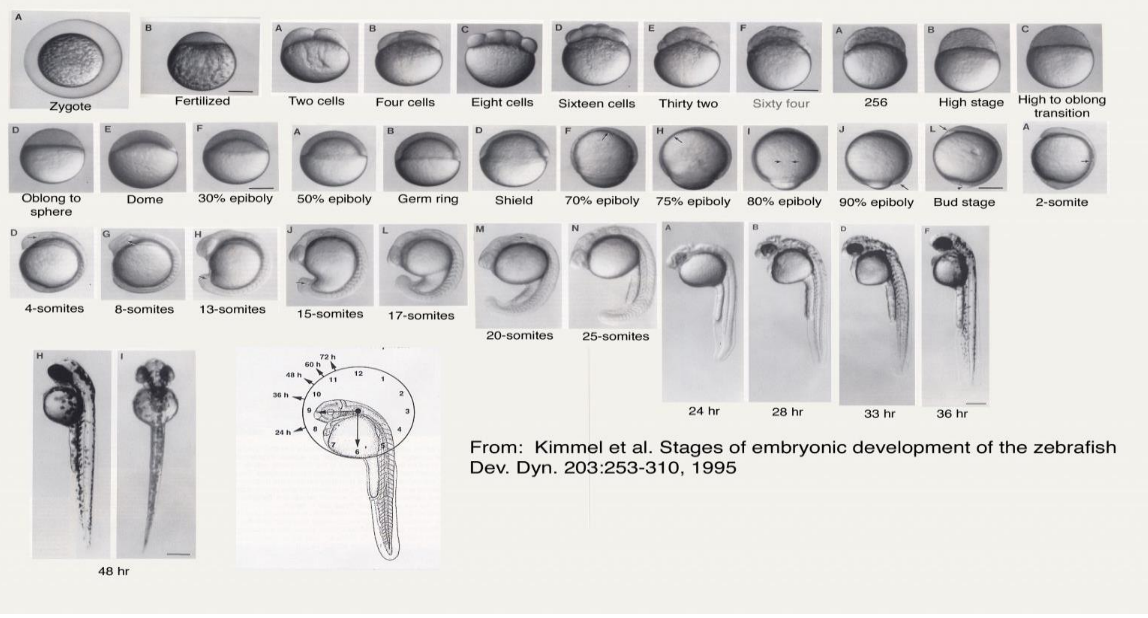

Figure 1. 3 Stages of embryonic development of the zebrafish

exposed to a range of PFOA and PFECA concentrations. In this study, acute embryotoxicity, along with neurobehavioral effects, of PFOA was observed at LC_{50} value of 50 ppm. In similar studies, zebrafish exposed to PFASs demonstrated morphological changes such as deformities, edemas, and effects on the swim bladder (Hagenaars et al., 2011). Short- and long-term (i.e., 5 days post-fertilization [dpf] to 6 months) morphometric and behavioral effects of PFASs were evaluated in zebrafish, and the results revealed substantial biochemical and behavioral alterations (Jantzen et al., 2016). Acute embryogenesis, developmental toxicity, and impaired embryonic development have also been widely observed in numerous other studies in zebrafish (e.g., Hagenaars et al., 2011; Jantzen et al., 2016; Gaballah et al., 2020; Gebreab et al., 2020). Exposure to PFASs has negative effects on the metabolism, reproductive system, neurological system, and endocrine system in zebrafish (Gaballah et al., 2020; Jantzen et al., 2017; Vogs et al., 2019). Comparable to long-chain PFAS, emerging PFECAs (e.g., GenX and ADONA) affected developmental toxicity and developmental neurotoxicity in zebrafish (Gaballah et al., 2020; Gebreab et al., 2020; Han et al., 2021).

Several zebrafish studies have demonstrated that PFASs with longer chain lengths are more toxic than PFASs with shorter chain lengths (Gaballah et al., 2020; Hagenaars et al., 2011). Levels of toxicity were associated with increasing carbon chain length for developmental neurotoxicity, developmental toxicity, and acute toxicity. That said, although insufficient research has been carried out on the potency of short-term PFASs, they have generally been found to be equally potent as their long-chain counterparts (Gaballah et al., 2020; Gebreab et al., 2020; Hagenaars et al., 2011). Due to its many

advantages, the zebrafish embryo holds considerable promise for toxicology studies of PFAS including these emerging chemical variants such as PFECA.

1.3.2 Ecologically and Commercially Relevant Fish Species as Toxicological Models

Although the zebrafish including, in particular, early life stages (i.e., embryos, larvae) of the species has shown considerable value as a model system for toxicological studies including, in particular, assessments of PFAS, with potential relevance - as vertebrate model - to human health, the translational value with respect to *ecotoxicology* is arguably limited. Despite being a representative aquatic vertebrate, laboratory lines of zebrafish have limited relevance as "ecological receptors" both as a species, and moreover, as genetically non-wild type (i.e., laboratory bred) representatives of the species. At the same time, relatively few studies have assessed toxicity in marine species, compared to studies of freshwater species, and even fewer have focused on marine vertebrates. Accordingly, there is a need to similarly develop and establish ecologically relevant fish species – including early life stages - as toxicological model for environmental pollutants such as PFAS. In partnership with hatcheries, recent studies have begun to demonstrate the potential of environmentally and commercially relevant finfish species for such toxicants.

1.3.2.1 Early Life Stages of Mahi-Mahi as a Toxicological Model

Mahi-mahi, or "Dolphin Fish," (Coryphaena hippurus) is a common pelagic, mid-trophic level, highly migratory species dispersed throughout the world's tropical and subtropical seas and oceans (Maggio et al., 2019; Palko et al., 1982; Perrichon et al., 2019). Like many other pelagic marine fish, the species are characterized by both frequent spawning, and high fecundity (Beardsley Jr, 1967; Maggio et al., 2019). They are in high demand in both sport fishing and commercial

fishing, and are, therefore, continually sought out as food fish (Kraul et al., 1993; Schlenker et al., 2021). Early life stages of mahi-mahi are likely vulnerable to environmental pollutants (Alloy et al., 2016; Esbaugh et al., 2016), and the high spawning frequency and reproductive capacity of the species makes it a good model for environmental toxicology studies, and in particular, assessment of possible ecotoxicological impacts at the population level, e.g., juvenile recruitment (Perrichon et al., 2019).

Source: Perrichon, P., Stieglitz, J. D., Xu, E. G., Magnuson, J. T., Pasparakis, C., Mager, E. M., ... & Burggren, W. W. (2019). Mahi-mahi (Coryphaena hippurus) life development: morphological, physiological, behavioral and molecular phenotypes. Developmental Dynamics, 248(5), 337-350.

Figure 1. 4 Embryo-larval development of mahi at 26°C. A-C, Cell cleavage. D-I, Morula/blastula. J-P, Gastrula/segmentation. Q–S, Pharyngula/hatching period. T–V, Post-hatching period/yolk sac larvae. W–Y, Juvenile period. Z, Adult phase.

Embryos and larvae of mahi-mahi have, in fact, been recently used as a model species for toxicological and physiological studies in relation to crude oil associated with oil spills (Esbaugh et al., 2016; Nelson et al., 2016; Perrichon et al., 2017; Stieglitz et al., 2016; Wang et al., 2019). These previous studies in mahi-mahi have demonstrated that this species is very sensitive to

components of crude oil with effects on heart, kidney, craniofacial, and central nervous system, as well as adverse effects caused by physiological abnormalities (e.g. reduced swimming performance) (Brette et al., 2014; Esbaugh et al., 2016; Incardona et al., 2004; Stieglitz et al., 2016). Mahi-mahi has also been widely studied as a model in other environmental pollutants (Adema-Hannes & Shenker, 2008; Sweet et al., 2017), as well as population genetics (Tripp-Valdez et al., 2010) and developmental physiology (Perrichon et al., 2017).

Prior to the current study, there has, however, been no available literature on the toxicity of PFAS in mahi-mahi. Knowing the value of these species as a tractable model for experimental studies and the potential bioaccumulation of PFASs in the aquatic environment, this research endeavored to develop this species as a toxicological model to assess both legacy and emerging PFAS.

1.3.3.2 Early Life Stages of Olive Flounder as a Toxicological Model

Olive Flounder, or "Japanese Halibut," *(Paralichthys olivaceus)* is a threatened benthic species of marine fish. In view of their high market value as a food fish in the commercial and sporting spheres, the species has been protected through cryopreservation (Chen & Tian, 2005; Lanes et al., 2008; Zhang et al., 2003), and the aquaculture of Olive Flounder has been actively pursued in many parts of the world, including the Far East and Europe (Washio et al., 2015). They can survive in a broad range of temperatures and saline waters (Iwata et al., 1994; Sampaio et al., 2001).

Alongside their commercial importance, flounder species are ecologically relevant as a benthic species, and have been used as a model in toxicological studies of various environmental toxicants (Mimura et al., 1998; Washio et al., 2015), and given their unique morphology, as a model for studies of genetics (Coimbra et al., 2003; Kang et al., 2008),

transcriptomic responses (George et al., 2004; Lewis et al., 2006; Williams et al., 2008), and morphological and developmental studies (Schreiber & Specker, 1998; Jee et al., 2001). Findings from toxicological studies have revealed that environmental pollutants such as ozone, trace metals (e.g., silver, nickel, and cobalt) and other toxicants (e.g., bis-oxide and aluminum hydroxide) induce cytochrome p450, and negatively affect the genetic and morphological development of flounders. On the other hand, temperature and fish size are apparently not significant factors with regard to the effects of environmental pollutants on flounder (Furuta et al., 2008).

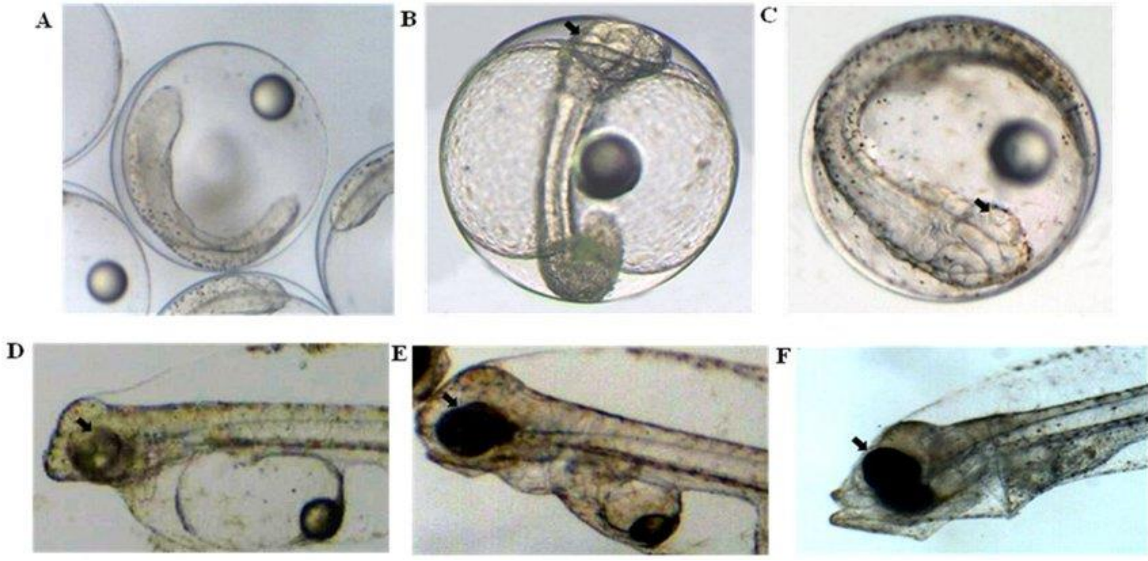

Figure 1. 5 Developmental stages of olive flounder (Paralichythys olivaceus). Larva for 0 day to 6 days. (a: Myotome, b:, d: eye-sac formation, c: before Hatching, d: 1day after hatching, e: 3 days after hatching, f: 5 days after hatching).

Source: Yang, H., Lee, Y. M., Noh, J. K., Kim, H. C., Park, C. J., Park, J. W., ... & Lee, J. H. (2012). Differential Expression Patterns of Crystallin Genes during Ocular Development of Olive Flounder (Paralichthys olivaceus). *Development & Reproduction*, *16*(4), 301.

A few studies have also been conducted on the accumulation of PFASs in flounder (Järv et al., 2017; Kallenborn, 2004; Marchand et al., 2004). Significant levels of PFAS (75% of total PFAS) were detected in southern flounders in Cooper River, South Carolina (Marchand et al., 2004). In addition, PFASs were measured in flounders in the Nordic environment – in the coastal waters of Denmark and Sweden (Kallenborn, 2004). These studies revealed that legacy PFAS potentially bioaccumulate in flounder. Accumulation of next-generation PFASs, including PFECAs, in flounders has not yet been studied.

1.4 Research Objectives

The aim of this dissertation research is to investigate the effect of PFECA, as emerging pollutants of concern, alongside legacy PFAS (i.e., PFOA), in early life stages of both zebrafish, as an established laboratory model, as well as ecologically and commercially relevant fish species. The comparative toxicity of legacy (i.e., PFOA) and emerging (i.e., PFECA) PFAS was investigated in the zebrafish embryo model through assay-based approaches, alongside the use of high-resolution magic-angle-spin (HRMAS) nuclear magnetic resonance (NMR) metabolomic techniques to elucidate metabolic pathways affected by these toxicants (Chapter 2). Subsequently, assays were developed to assess toxicity in early life stages (i.e., embryos) of mahi-mahi as a representative marine species, and in turn, used for a comparative toxicological of both PFOA and PFECA between this ecologically relevant species, and the zebrafish as a laboratory model system (Chapter 3). Finally, the toxicity and bioconcentration of PFOA and PFECA were evaluated comparatively in the zebrafish and Olive Flounder (Chapter 4) using LC/MS measurement, and an assay-based approach developed for these species.

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CHAPTER 2

Comparative Toxicometabolomics of Perfluorooctanoic Acid (PFOA) and Next-Generation Perfluoroalkyl Substances

(adapted from Gebreab, K., Eeza, M.N.H, Bai, T., Zuberi, Z., Matysik, J., O'Shea, K., Alia, A. and Berry, J.P. (2020), *Environ. Pollut.,* 265: 114928)

2.1 Abstract

Owing to environmental health concerns, a number of per- and polyfluoroalkyl substances (PFAS) have been phased-out, and replaced by chemical analogs including, in particular, *perfluoroether carboxylic acids* (PFECA). Toxicity, and environmental health concerns associated with these next-generation PFAS, however, remains largely unstudied. The zebrafish embryo was employed, as a toxicological model system, to investigate toxicity of a representative sample of PFECA, alongside perfluorooctanoic acid (PFOA) as one of the most widely used, and best studied, of the "legacy" PFAS. In addition, *high-resolution magic angle spin* (HRMAS) NMR was utilized for metabolic profiling of intact zebrafish embryos in order to characterize metabolic pathways associated with toxicity of PFAS including PFOA and two representative PFECA. Acute embryotoxicity (i.e., lethality), along with impaired development, and variable effects on locomotory behavior, were observed for all PFAS in the zebrafish model. Acute toxicity was significantly correlated with alkyl chain-length, and toxic concentrations were quantitatively similar to those reported previously for PFAS. Metabolic profiling of zebrafish embryos exposed to selected PFAS, specifically including PFOA and two representative PFECA (i.e., GenX and PFO3TDA), enabled elaboration of an integrated model of the metabolic pathways associated with toxicity. This systems-level model, based on observed alteration of metabolic profiles, suggests targeting of hepatocytes (i.e., hepatotoxicity), as well as apparent modulation of neural metabolites, and an inferred role of mitochondrial disruption and peroxisome proliferator-activated receptor (PPAR) activation as reflected by dysfunctions of carbohydrate, lipid and amino acid metabolism, and consistent with a previously

proposed contribution of PFAS to *metabolic syndrome*. Taken together, it was generally concluded that toxicity of PFECA is quantitatively and qualitatively similar to PFOA, and these analogs, likewise, represent potential concerns as environmental toxicants.

2. 2 Introduction

Per- and polyfluoroalkyl substances (PFAS) are environmental pollutants of emerging concern. Chemically speaking, the PFAS are a class of highly fluorinated organic compounds in which all, or most, hydrogen atoms of "long" (≥ 6 carbon) or "short" aliphatic chains are substituted by fluorine, and to which terminal polar functionalities (e.g., carboxylic acid, sulfonate) are typically added (Appendix A1) in order to impart highly effective surfactant properties (Prevedouros et al., 2006; Wang et al., 2017). Owing to these properties, the PFAS have been extensively used since the late 1940s in a range of industrial and consumer applications including textiles, household products (e.g., Teflon), cosmetics, firefighting foams, medical devices, oil production, pesticide formulations, and in water-repellants (Kemi, 2015; Prevedouros et al., 2006; Wang et al., 2013).

Due to chemical recalcitrance bestowed by C-F bonds, PFAS are also highly persistent, and potentially bioaccumulative (Prevedouros et al., 2006; Wang et al., 2017), and have been widely detected in the environment, wildlife and humans (Giesy & Kannan, 2001; Kemi, 2015; Prevedouros et al., 2006; Wang et al., 2017). Concentrations of PFAS in drinking water, for example, typically range from ≤ 200 ng L^{-1} to a several parts-per-billion (ppb, i.e., µg L^{-1}) (Sunderland et al., 2019). Levels of PFAS in fish and seafood has, in turn, been generally reported in the range of a few to hundreds ppb (i.e., ng g^{-1}), and

has suggested, in fact, to be the primary non-occupational route of human exposure to PFAS (Domingo & Nadal, 2017; Fair et al., 2019). Moreover, studies by the Center Disease Control (CDC) reported detection of PFAS in as much as 98% of the U.S. population (Sunderland et al., 2019) with serum concentrations reaching levels of a few ppb, but up to parts-per-million (ppm, i.e., mg L-1) among occupationally exposed individuals (Anderson et al., 2019; Worley et al., 2017). And a recent study (Mamsen et al., 2019) found that PFAS can be transferred maternally via placenta to embryo and fetal tissues (reaching levels up to ng g^{-1}).

At the same time, PFAS have been demonstrated to have a range of adverse effects relevant to human health including reproductive toxicity, carcinogenicity, hormonal dysfunction, hepato/nephrotoxicity, developmental toxicity, neurotoxicity, immunotoxicity, and pulmonary effects (Lau et al., 2007; Prevedouros et al., 2006; Shrestha et al., 2017; Steenland et al., 2009). One of the notable links to human health – identified through both molecular and epidemiological studies – has been a possible association with *metabolic syndrome* including obesity and dyslipidemia, impairment of glucose tolerance and regulation, and increased blood pressure all of which are linked, in turn, to increased risks of heart disease, stroke and diabetes, although findings in this regard have been collectively inconclusive (Christensen et al., 2019; Matilla-Santander et al., 2017; Sunderland et al., 2019). Based on available data regarding adverse health effects, the U.S. Environmental Protection Agency (EPA) has, for example, previously proposed a lifetime health advisory level of 70 ng/L in drinking water, though this has recently been lowered (Sunderland et al., 2019).

Given their persistent and widespread distribution in the environment, and potential for bioaccumulation and toxicity (and consequent adverse health effects), the production and use of several of the most common PFAS including, in particular, long chain (≥ 7 C) representatives of perfluorocarboxylic acids, such as perfluorooctanoic acid (PFOA; Appendix A1), and perfluoroalkyl sulfonic acids, such as perfluorooctane sulfonate (PFOS), have been phased-out in the United States and Europe (Cadore et al., 2009; Pérez et al., 2013; Wang et al., 2017). These so-called "legacy" PFAS, however, been largely replaced by a diversity of "next-generation" compounds. Among these are numerous *perfluoroethercarboxylic acids* (PFECA; Appendix A1) in which one or more carbon (i.e., -CF_2-) is replaced by an oxygen (i.e., ether) while still enabling similar chemical properties (ATSDR, 2018; Cadore et al., 2009; Wang et al., 2019). Like legacy PFAS, however, this means PFECA are also highly stable, and recent studies suggest that they are equally persistent, and may similarly bioaccumulate, in the environment(Sun et al., 2016; Wang et al., 2015; Wang et al., 2013).This class of PFAS are, thus, emerging as a potential concern as environmental toxicants (Prevedouros et al., 2006; Wang et al., 2017). To date, however, there remains very limited information on the PFECA with respect to toxicity, and possible environmental health concerns (Wang et al., 2013; Wang et al., 2017). Among the PFECA, in fact, only two of the most commonly used – under the trade names, GenX (Appendix A1) and ADONA (4,8-dioxa-3H-perfluorononanoate) - have been studied with respect to toxicity, and specifically in mammalian (i.e., mice and rat) systems (DeWitt, 2015; Wang et al., 2015).

Zebrafish (*Danio rerio*) and, in particular, early life stages (i.e., embryos and larvae) of the species, have become well established as a vertebrate toxicological model (Berry et al., 2007; Berry et al., 2016; Jaja-Chimedza et al., 2017; Roy et al., 2017; Weiss-Errico et al., 2017; Zuberi et al., 2019). In addition to numerous practical advantages of this system including ease of husbandry and breeding, high fecundity, a small and nearly transparent embryo, and rapid (i.e., ~3-5 day) embryogenesis, the zebrafish embryo - as a toxicological model - is both potentially translational to human health, and moreover, represents access to a wide range of relevant and quantifiable toxiciological endpoints. As such, this model system has been employed to investigate a myriad of environmental toxicants (Berry et al., 2007; Ulhaq et al., 2013; Weiss-Errico et al., 2017; Zuberi et al., 2019). Indeed, zebrafish embryos and larvae - and even adult stages – have been previously used to investigate toxicity and toxicokinetics of PFAS including, in particular, PFOA, PFOS and related compounds (Ulhaq et al., 2013; Vogs et al., 2019; Weiss-Errico et al., 2017; Wen et al., 2019; Zheng et al., 2012). In the present study, we utilized the zebrafish embryo model to evaluate a representative sample of PFECA with respect to their toxicity including acute toxicity (i.e., lethality) and developmental impairment, as well as behavioral endpoints. Although the zebrafish embryo has been used previously to investigate several legacy PFAS (e.g., PFOA, PFOS), the current study is among the first to investigate the toxicity of the PFECA, in general, and in this model system specifically.

Given the considerable promise of the zebrafish embryo model, it is perhaps not surprising that a number of biotechnological advances have evolved alongside the toxicological model. Among these is a wide range of "omics" approaches. Very recently,

this has included adaptations of nuclear magnetic resonance (NMR)-based metabolomics techniques for metabolite profiling and imaging in the zebrafish model (Chatzopoulou et al., 2015; Kabli et al., 2009; van Amerongen et al., 2014). In particular, approaches based on *high-resolution magic angle spin* (HRMAS) NMR have shown remarkable potential for metabolomics of *intact* zebrafish (Berry et al., 2016; Chatzopoulou et al., 2015; Kabli et al., 2009; Roy et al., 2017; van Amerongen et al., 2014; Zuberi et al., 2019). Briefly stated, HRMAS NMR utilizes spinning of a sample at the so-called "magic angle" (of 54.74° relative to the magnetic field) to minimize major interactions (specifically dipolar and quadrupolar interactions, and chemical shift anisotropy) that, otherwise, cause broadening of signal (i.e., "chemical shift") peaks, and thus, enables resolution of the chemical shifts of multiple compounds (e.g., metabolites) even within complex biological samples including intact zebrafish embryos. This technique has been previously applied to understanding of metabolic contributions to disease state (Chatzopoulou et al., 2015; van Amerongen et al., 2014), and more recently, metabolic alterations associated with environmental toxicants (Berry et al., 2016; Roy et al., 2017; Zuberi et al., 2019). In the latter case, this approach was specifically applied to several naturally occurring biotoxins (e.g., cyanobacterial toxins, mycotoxins), and enabled facile characterization of integrated pathways of toxicity, and identification of potential metabolic biomarkers of toxin-specific effects (Berry et al., 2016; Roy et al., 2017; Zuberi et al., 2019).In the present study, this state-of-the-art technique was applied to a comparative toxicological investigation of PFECA and PFOA as a means of characterizing pathways and targets of PFAS toward an integrated systems-level model of toxicity in relation to possible health concerns.

2.3 Materials and Methods

2.3.1. Chemicals

Perfluoroethercarboxylic acids (PFECA) including perfluoro (4-methoxy butanoic) acid (PFMOBA), perfluoro-3,6,9-trioxatridecanoic acid (PFO3TDA), perfluoro-3,6,9-trioxadecanoic acid (PFO3DA), perfluoro (2-methyl-3-oxahexanoic) acid (trademarked commercially under the name "GenX"), perfluoro-3,6-dioxaheptanoic acid (PFO2HpA), 4-(heptafluoroisopropoxy)hexafluorobutanoic acid (PFDMMOBA) and Perfluoro-3,6-dioxadecanoic acid (PFO2DA) were purchased from SynQuest Laboratories (Dallas, TX U.S.A.). Perfluorooctanoic acid (PFOA), and all other chemicals (i.e., deuterated phosphate buffer and reference standard for NMR), were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock solutions of PFECAs and PFOA were prepared with deionized water in polypropropylene tubes (to prevent adsorption to glass;(Shafique et al., 2017), and were sonicated until complete dissolution of the compounds was achieved. All chemicals were utilized without further purification.

2.3.2 Zebrafish Rearing and Breeding

Zebrafish embryos (of the PSA line) for primary toxicity testing were obtained from the University of Miami Rosenstiel School of Marine and Atmospheric Science zebrafish facility. Zebrafish (*Danio rerio*) were reared and bred as previously described (Weiss-Errico et al., 2017). Briefly, adult zebrafish were maintained in 30-L tanks at 28 °C with 14 h:10 h light/dark cycle, and bred (from approximately 10–30 individuals) above 10-L tanks in mesh enclosures. Eggs were collected (from the bottom of tanks) within 1 h of the end of the dark cycle, and, following collection and washing (with water), transferred to

plates containing E3 medium (Brand et al., 2002). Eggs containing dead or obviously poor quality embryos were removed, and the remaining embryos were used, within 3 h post-fertilization (hpf), for toxicity assays. All rearing and breeding was conducted under protocols approved by the University of Miami's Institutional Animal Care and Use Committee (IACUC), and performed by trained investigators.

For additional, confirmatory toxicity assays (in order to establish concentration ranges, and appropriate developmental stage, for exposure of zebrafish embryos in NMR metabolomics studies; see below), embryos were obtained from Helmholtz Centre for Environmental Research (UFZ; Leipzig, Germany). Husbandry and experimental procedures were performed in accordance with the German animal protection standards, and were approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64). Adult wild-type zebrafish (*D. rerio* OBI/WIK line) were maintained in recirculating aquarium systems according to established rearing procedures (van Amerongen et al., 2014). The water temperature was maintained at 28ºC with a flow rate of 150 L/min, with day/night light cycles (12h dark versus 12h light). The fish were fed twice daily with commercial flake food according to the guidelines for the laboratory use of zebrafish (Westerfield, 1995). Breeding and embryo collection were performed as per standard procedure described previously (Ali et al., 2011). Briefly, eggs were obtained by random pairwise mating of zebrafish. Three adult males and four females were placed together in small (L 26 cm; H 12.5 cm; W 20 cm) breeding tanks containing mesh egg traps (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. Eggs were harvested the following morning, and transferred in to 92-mm plastic Petri dishes (50 eggs

per dish) containing 40 mL fresh embryo medium and washed four times to remove debris, and unfertilized, unhealthy and dead embryos were removed under a dissecting microscope. At 3 hpf, embryos were again screened, and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28°C, either in the incubator or climatized room. All incubations of embryos were carried out in an incubator with orbital shaking (50 rpm) under a light cycle of 14 h light: 10 h dark (lights on at 8 h).

2.3.3. Zebrafish embryo toxicity assays

Zebrafish embryo toxicity of PFAS was assessed as previously described (Berry et al., 2007; Jaja-Chimedza et al., 2017; Weiss-Errico et al., 2017). Evaluation of toxicity was primarily conducted in laboratories at Florida International University under protocols approved by the FIU Animal Care and Use Committee (IACUC). Additional details of rearing and breeding of zebrafish, and toxicity assays, are given in Appendix A. For comparison to a representative legacy PFAS, the toxicity of PFECA (and subsequent metabolic profiling experiments) was assessed alongside PFOA for which toxicity in the zebrafish embryo model has been previously evaluated(Hagenaars et al., 2011; Weiss-Errico et al., 2017). Preliminary studies enabled establishment of effective concentration ranges for PFAS, and accordingly, embryos (PSA line at ≤ 4 hours post-fertilization [hpf]) were exposed to a concentration series of 25, 50, 75, 100, 150, 200 ppm for PFMOBA, Gen X, PFO2HPA , PFO3DA and PFOA; 100, 150, 200, 250 and 300 ppm for PFDMMOBA; 5, 10, 50, 75 and 100 ppm for PFO2DA; and 1, 5, 10, 20, 30 and 40 ppm for PFO3TDA.

Embryo toxicity was observed, and recorded, at four developmentally relevant timepoints, i. e., 1, 2, 5 and 7 days post-fertilization (dpf), over 7-d continual exposure. Relevant toxicological endpoints included lethality/mortality, developmental inhibition and neurobehavioral endpoints. With respect to mortality, median lethal concentration (LC_{50}) was calculated for each test compound at each timepoint, based on percent mortality (N = 3 replicates of 5 embryos per well) fitted to sigmoidal concentration-response curves, specifically using Origin 2018b software (Origin Lab, Northampton, Massachusetts, USA) with LC_{50} values calculated by Probit Analysis in SPSS (version 23.0; IBM Corporation Armonk, NY, USA, 2015). Alongside mortality, morphological developmental deformities were recorded by light photomicrography using Olympus DP2-BSW imaging software (Olympus, Center Valley, PA, USA, 2009). Inhibition of development and behavioral dysfunction were assessed, respectively, based on interocular distance (IOD) and vestibular righting reflex (i.e., percent listing in 30 s interval, n = 6) for pooled surviving embryos (at 7 dpf), compared to untreated (E3 medium only) controls, as previously described (Weiss-Errico et al., 2017). One-way ANOVA was used to calculate the statistical significance of differences between LC_{50} values, calculated at each timepoint, and IOD and percent listing (at 7 dpf) relative to untreated (E3 only) controls, for all treatments.

2.3.4. Exposures for HRMAS-NMR metabolic profiling

Toward comparative characterization of targets and pathways of toxicity, metabolic profiling of zebrafish exposed to three representative PFAS - specifically PFOA, GenX and PFO3TDA – was performed by HRMAS NMR, as previously described (Berry et al., 2016; Roy et al., 2017; Zuberi et al., 2019). All exposures and subsequent NMR analyses

were conducted at the University of Leipzig with embryos (OBI/WIK line) provided by the the Helmholtz Centre for Environmental Research. Prior to NMR metabolomics studies, additional assessments of toxicity were subsequently conducted in laboratories to confirm toxicity in this line, in general, and to determine relevant exposure parameters including sublethal concentrations and optimal developmental stage, for PFAS (i.e., PFOA, GenX and PFO3TDA) selected for metabolomic studies. Details of rearing and breeding of zebrafish, and these toxicity assays, are given in the Appendix A. Exposures for NMR analyses were accordingly performed using 72 hpf embryos exposed (in 25 mL of ISO medium (Knöbel & Busser, 2012) in 75 cm^2 tissue culture flasks) to final concentrations of PFOA, GenX or PFO3TDA, below LC_{50} for these compounds (50, 100 and 10 ppm, respectively), for 24 h, i.e., collected at 96 hpf. Additional exposure replicates were made to account for any losses due to mortality, and in order to generate a sufficient number of embryos (N = 100) and replicates (n = 3) to achieve quantitative NMR analyses. After washing 3 times with MilliQ water to remove residual PFAS, embryos were transferred to 4-mm zirconium oxide rotors (Bruker BioSpin AG, Switzerland) to which 10 μL deuterated phosphate buffer (100 mM, pH 7.0) containing 0.1% (w/v) 3-trimetylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) was added as a reference (^1H chemical shift at 0 ppm).

2.3.5. HRMAS NMR analysis

All HRMAS NMR experiments were performed on a vertical Bruker magnet (DMX 600-MHz), which was equipped with a 4-mm HRMAS doubly tuned $^1H/^{13}C$ inverse probe with a magic angle gradient. Measurements were carried out at a spinning rate of 6 kHz

and a temperature of 277 K which was achieved by a Bruker BVT3000 control unit. Data acquisition and processing were carried out using Bruker TOPSPIN 2.1 software (Bruker Analytische Messtechnik, Germany).

One-dimensional ^{1}H HRMAS NMR spectra were obtained as described previously (Roy et al., 2017). A rotor synchronized Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with water suppression was used. Each one-dimensional spectrum was acquired applying a spectral width of 8000 Hz, domain data points of 16k, number of averages of 512 with 8 dummy scans, constant receiver gain of 2048, and acquisition time of 2 s and relaxation delay of 2 s. The relaxation delay was set to a small value to remove short T_2 components due to the presence of lipids in intact embryo samples. All spectra were processed by an exponential window function corresponding to a line broadening of 1 Hz and zero-filled before Fourier Transformation. NMR spectra were phased manually, and automatically baseline corrected using TOPSPIN 2.1 (Bruker Analytische Messtechnik, Germany). The total analysis time (including sample preparation, optimization of NMR parameters and data acquisition) of ^{1}H-HRMAS NMR spectroscopy for each sample was approximately 20 min.

All HRMAS NMR spectra were manually phased, baseline corrected and analyzed using TOPSPIN 4.0.6 (Bruker Analytische Messtechnik, Germany). Quantification of metabolites was performed by Chenomx NMR Suite 8.2 (Chenomx Inc., Edmonton, Alberta, Canada). Consequently, the concentrations of metabolites were calculated as a ratio relative to tCr; since the external reference may lead to misleading results, and Cr resonance has been previously shown to be a reliable internal reference in a wide range of

animal studies. Statistical analysis of metabolite quantification was done by one-way analysis of variance (ANOVA) using OriginPro v. 8 (OriginLab, Northampton, MA, USA), and calculated F-values larger than 2.8 ($p < 0.05$) were considered significant. Multivariate analysis of the one-dimensional NMR spectra were performed as described below.

2.3.6 Multivariate statistical analysis

Multivariate statistical analysis of primary metabolites in the ^{1}H NMR spectra was performed using SIMCA software package (Version 14.0, Umetrics, Umeå, Sweden). The spectra, collected from embryos, were subdivided in the range between 0.3 and 9 ppm into buckets of 0.04 ppm (total 218 buckets), using MestReNova v.12.0.4 (Mestrelab research S.L., Santiago de Compostela, Spain). The region of 4.80 – 6.00 ppm was excluded from the analysis to remove the water signal. To compensate for the differences in the overall metabolite concentration between individual samples, the data obtained were mean centered, scaled to unit variance and then normalized by dividing each integral of the segment by the total area of the spectrum. The resulting data matrix was exported into Microsoft office Excel (Microsoft Corporation, USA). This was then further imported into SIMCA software for multivariate statistical analysis. A probability of p-value of <0.05 was taken to indicate statistical significance. Multivariate analysis specifically was done by supervised partial least squares discriminant analysis (PLS-DA) as previously described (Roy et al., 2017). This method gives both a score matrix and a loading matrix, with score matrix showing the relation between observations, while the loading matrix gives the individual contribution of each parameter, which is a peak in the case of NMR spectra. The HRMAS spectra were investigated by multivariate analysis to probe if control and PFAS-

treated embryos can be discriminated and to determine the spectral regions and corresponding compounds mainly responsible for the separation. A clear clustering could be observed in the score plot of PLS-DA1 vs PLS-DA2 (Appendix A5 A-B). In order to determine the variables, i.e. metabolites assigned to the corresponding buckets that are mainly responsible for the separation of three groups, the load values of the PLS-DA1 and PLS-DA2 were determined (Appendix A5 C).

2.4. Results and discussion

2. 4.1. Toxicity of PFECA and PFOA in the zebrafish embryo model

Toxicity of the PFECA and PFOA was evaluated for zebrafish embryos continually exposed over 7 dpf at compound-specific ranges of exposure concentrations. Concentration-dependent toxicity of the PFECA was observed with respect to lethality (Appendix A2) which was accompanied by clear impairment of development and locomotory function. Generally speaking, the range of acutely toxic concentrations, based on median lethal concentration (i.e., LC_{50}; see Table 2.1), of PFECA were similar to those previously reported for other PFAS, i.e., 10 to 10^2 μM (Jantzen et al., 2016; Weiss-Errico et al., 2017; Zheng et al., 2012), and LC_{50} values for PFOA were, likewise, comparable to those previously reported (Ding et al., 2013; Zheng et al., 2012) for this compound (i.e., 261 and 271ppm, respectively).

Table 2. 1 Median lethal concentration (LC50) of PFECA and PFOA in the zebrafish embryo model. Calculated values (in µM), along with 95% confidence intervals, are specifically given for 24 h exposure of ≤ 3 hours post-fertilization (hpf) embryos. LC50 values were calculated by Probit analysis; dose-response curves are given in Appendix A. Shown, for comparison, are number of fluorocarbons (-CFx) and ether oxygens (-O-) in the alkyl chain of each PFAS.

	Alkyl Chain		
	-CF$_x$-	**-O-**	**LC$_{50}$ (µM) ± 95% CI**
PFECA			
PFMOBA	5	1	499±0.0
GenX	5	1	383±30
PFDMMOBA	6	1	248±81
PFO2HpA	4	2	441±66
PFO2DA	7	2	157±8
PFO3DA	6	3	202±8
PFO3TDA	9	2	38±6
PFOA	7	0	232±29

Although frequency of deformities was not dose-dependent, a range of developmental deformity including bent body axes and edema, as well as general moribundity accompanied by sloughing of dermal layers, was observed in approximately 7-10% of surviving embryos (Appendix A3).No significant effect on hatching rate, however, was observed with >90% hatching observed for both control and treated embryos by 4 dpf (data not shown). Embryo mortality, furthermore, increased for all PFECA and PFOA over the 7 days of continuous exposure (Figure.2.1): median lethal concentrations significantly decreased between 1 to 7 dpf for 6 of the 7 PFECA including PFMOBA ($p < 0.001$), GenX ($p < 0.0001$), PFO2HpA ($p < 0.001$), PFO2DA ($p < 0.0001$), PFO3DA ($p < 0.001$) and

PFO3TDA ($p < 0.0001$), as well as PFOA ($p < 0.001$), and increased most drastically post-hatch (approximately 72 hpf). (Figure. 2.1).

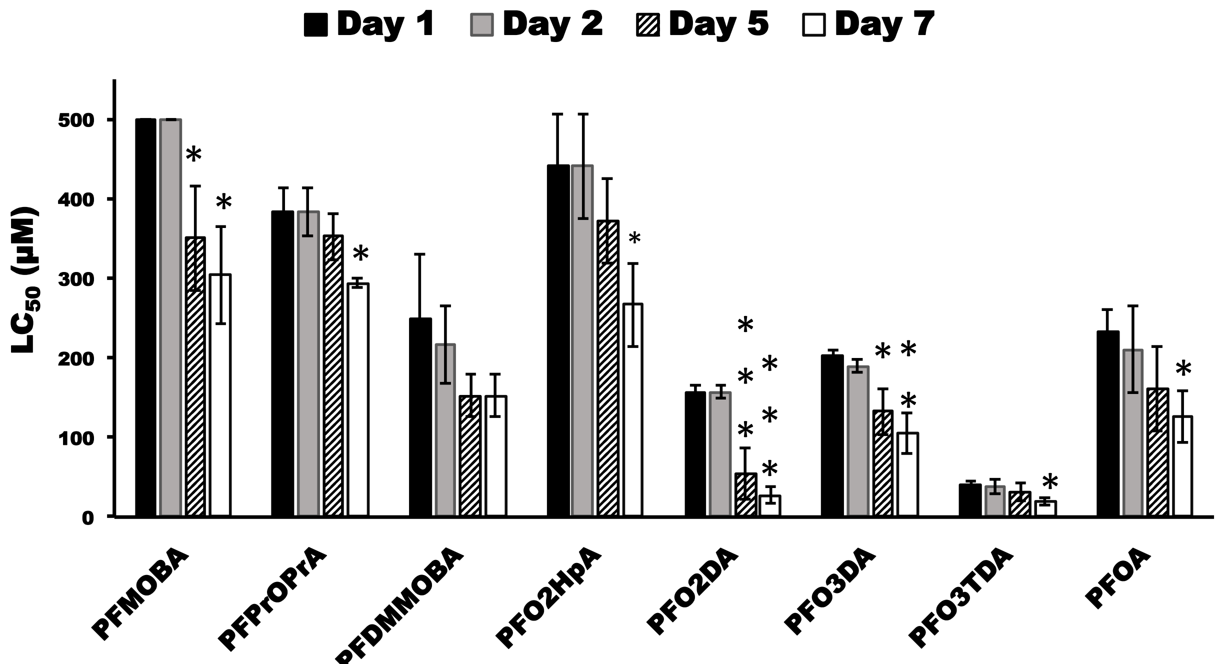

Figure 2. 1 Increased mortality, as measured by median lethal concentrations (LC_{50}), with continuous exposure of zebrafish embryos to PFECA and PFOA over 7 dpf. Asterisks (i.e., ** and ***) indicate that LC_{50} is significantly different ($p < 0.001$ and $p < 0.0001$) from 1 dpf values. Error bars represent 95% confidence interval.

Alongside cumulative toxicity (i.e., total duration of exposure), it is proposed that increased toxicity of PFAS at later embryonic stages may be related to two factors. The first is a development of the liver, and corresponding role hepatic enzyme. Previous studies have demonstrated targeting of hepatocytes by PFAS, and a role of the hepatic *phase I* (i.e., cytochrome P450 [CYP]) and *phase II* enzymes (e.g., glutathione-S-transferase [GST]) has been specifically implicated in a range of mammalian and teleost fish (e.g., carp, rare minnow) models (Cheng & Klaassen, 2008; Liu et al., 2007; Liu et al., 2009; Rotondo et al., 2018). Targeting of hepatocytes by PFECA and PFOA would be consistent with the

significant increase in toxicity at later stages of embryo development, as observed in our study (Figure.2.1), since it has been shown that differentiation of the liver, and significant expression of relevant enzymes (e.g., CYP), in zebrafish occurs at approximately 72 hpf. A similar increase in acute toxicity has, indeed, been previously demonstrated for recognized hepatotoxins (e.g., aflatoxin, acetaminophen) in the zebrafish embryo model (Pandya et al., 2015; Zuberi et al., 2019). Alternatively, stage-dependent toxicity of the PFECA may be simply related to hatching – which, likewise, occurs at approximately 72 hpf – and specifically, to a role of the chorion as a barrier to these compounds. Recent studies have investigated uptake and toxicokinetics of PFAS, and concluded a clear contribution of the chorion as a barrier for uptake until approximately 48 hpf after which uptake, as measured by PFAS concentration in post-hatch embryos, steadily increased (de Koning et al., 2015; Huang et al., 2010; Keiter et al., 2016; Vogs et al., 2019).

Embryotoxicity (i.e., LC_{50}) of PFECA was significantly correlated with alkyl-ether chain length of PFECA (Figure.2.2). A similar relationship between alkyl chain length and both toxicity, and uptake and bioconcentration, has been consistently reported for other PFAS in numerous previous studies including the zebrafish model (Buhrke et al., 2013; Mahapatra et al., 2017; Vogs et al., 2019; Wen et al., 2017; Zheng et al., 2012). Uptake and subsequent toxicity are presumptively due to the positive correlation between lipophilicity and alkyl-ether chain length which is supported by a generally positive correlation between lipophilicity and uptake previously documented for a diversity of compounds in the zebrafish embryo (Berghmans et al., 2008; de Koning et al., 2015; Jaja-

Chimedza et al., 2017). Most notably, the LC_{50} of PFOA generally falls within the same range, relative to chain length, as PFECA (Figure.2.2A), whereas the degree of

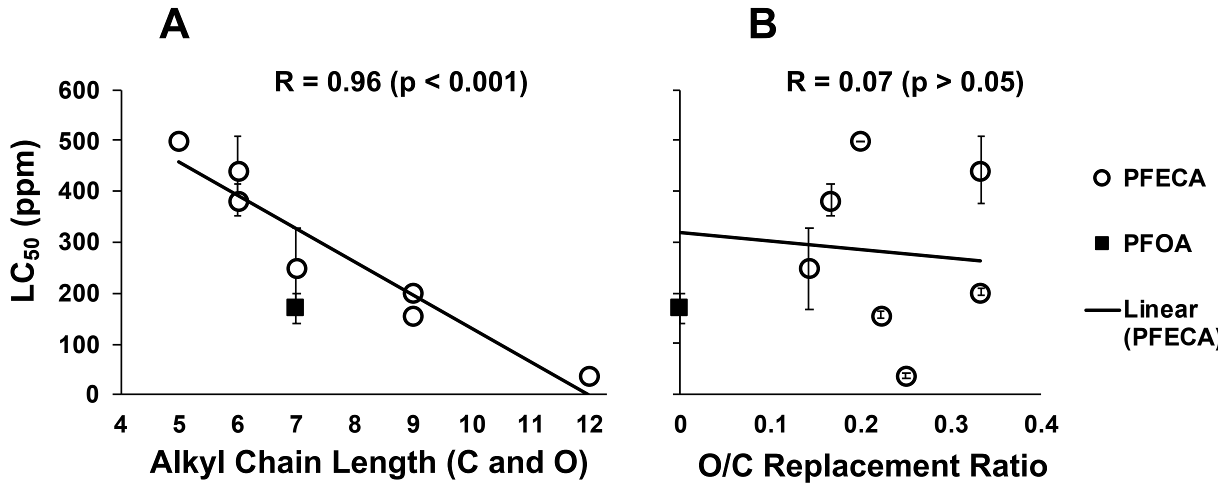

Figure 2. 2 Correlation between acute embryotoxicity, based on lethality (LC_{50}) at 24 hpf, relative to alkyl chain length (A) and O/C ratio, as a measure of replacement of $-CF_2-$ by ether oxygen, in alkyl chain (B) of PFECA and PFOA. Alkyl chain length is defined as number of carbon and oxygen atoms not including terminal carboxylic acid group. Significant correlation was observed between alkyl chain length and LC_{50}, whereas no significant correlation was observed between LC_{50} and ratio of O/C (i.e., number of ether groups) in the alkyl chain. Pearson correlation coefficient (R), and statistical significance (p-value) are given for correlations.

substitution of $-CF_2-$ by ether groups in PFECA (as measured by O/C ratio of PFECA) was not correlated with toxicity (Figure.2.2.B). Taken together, these findings suggest that while chain length of PFAS (including both PFOA and PFECA) is consistently correlated with embryo toxicity, the replacement of fluorocarbons by ether, as in the PFECA, has no effect on toxicity in the zebrafish embryo (as measured by lethality). This further suggests that acute toxicity (and presumably toxicokinetics, i.e., uptake) are, in fact, quantitatively similar for these next-generation chemical variants.

In addition to lethality, PFECAs and PFOA impaired embryo development as evidenced by morphometric measurements, and specifically IOD (Figure.2.3A) as a proxy of embryo size (Weiss-Errico et al., 2017). The effect, however, was independent (in the concentration range tested) of chain-length or any other apparent structural feature. Generally speaking, all PFECA and PFOA significantly ($p < 0.0001$) reduced IOD by nearly 50% (Figure.2.3A). A similar

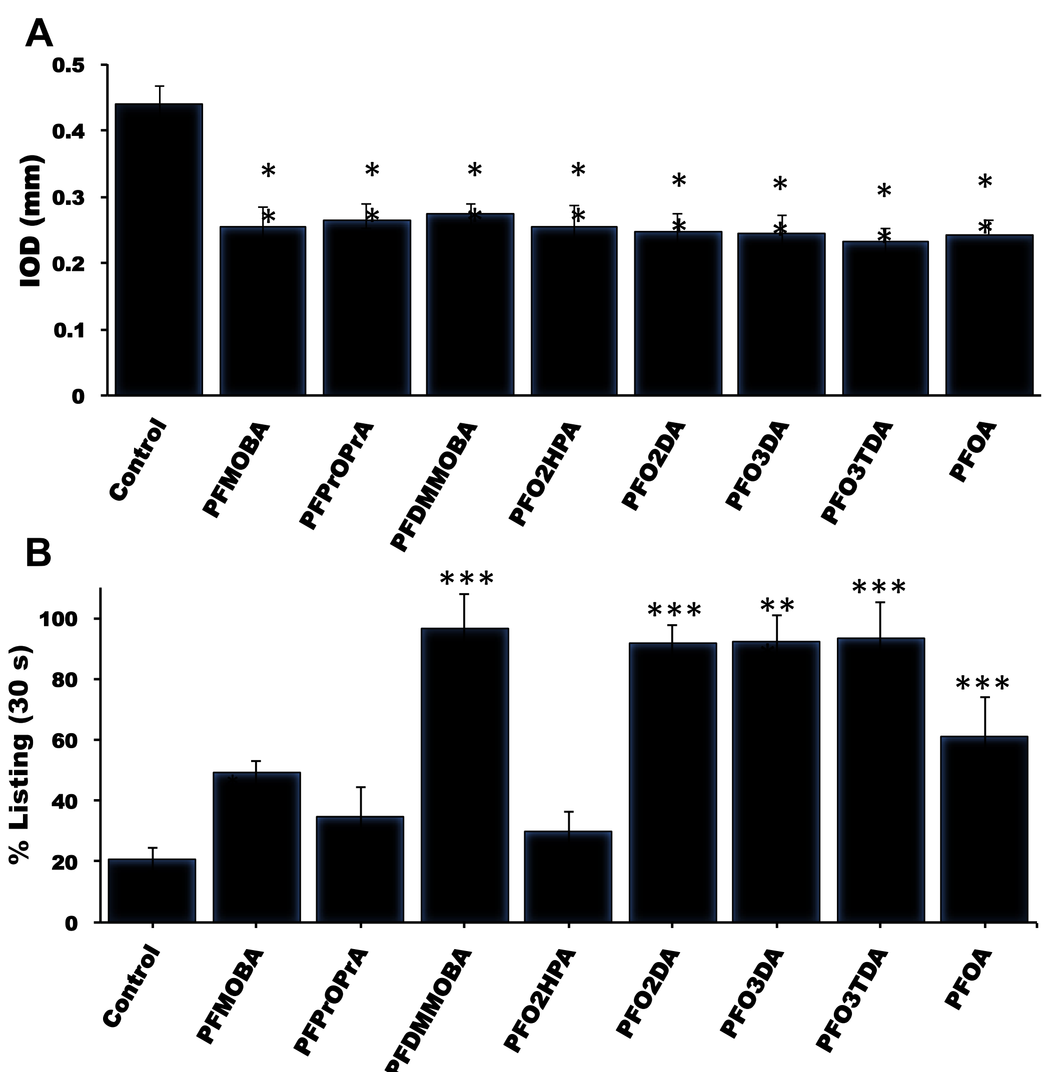

Figure 2. 3 Developmental impairment and behavioral dysfunction of zebrafish embryos (at 7 dpf) exposed to PFECA and PFOA. (A) Interocular distance (IOD), as a morphometric measure of embryo body size, for surviving embryos exposed to sublethal concentrations (< LC_{50}) of PFECA and PFOA at 7 dpf, compared to controls. The IOD was significantly reduced (*** p < 0.0001) for embryos exposed to all PFECA and PFOA, compared to controls. (B) Percent listing of surviving embryos exposed to sublethal concentrations (< LC_{50}) of PFECA and PFOA at 7 dpf, compared to controls. Percent listing was significantly higher for embryos exposed to PFDMMOBA, PFO2DA, PFO3DA, PFO3TDA and PFOA (*** = p < 0.0001), as well as PFMOBA (** p < 0.005). Error bars represent 95% CI.

decoupling of lethality and morphometrically assessed developmental toxicity was previously observed for PFOA in the zebrafish embryo model: specifically, in this case, while complexation with □-CD significantly reduced acute toxicity (i.e., lethality), no effect on IOD - which was equally (~50%) decreased by PFOA in this previous study - was observed (Weiss-Errico et al., 2017).

Finally, alongside lethality and developmental toxicity, several of the PFECA signficantly affected locomotory behavior of exposed zebrafish embryos, specifically measured by righting behavior – and, to be exact, rate of listing - of hatched eleutheroembryos at 7 dpf (Figure. 2.3B). A similar effect was, in fact, previously reported for PFOA in the zebrafish embryo model (Weiss-Errico et al., 2017). Although 5 of the 7 PFECA, as well as PFOA, significantly increased percent of embryo listing (compared to controls) in the present study, no quantitative relationship between chain length, or any other structural feature of the compounds was discernable. Whether the effect of PFECA and PFOA was due to neurotoxicity – that is modulation of neurochemistry – or simply due to overall developmental toxicity remains to be seen, however, alteration of neural

metabolism was, in subsequent metabolic profiling (discussed below), observed in the present study.

2.4.2. HRMAS NMR metabolic profiling of PFAS-exposed zebrafish embryos

Metabolic profiling of intact zebrafish embryos by HRMAS NMR was undertaken to elucidate targets and pathways associated with toxicity of three representative PFAS which were specifically selected to include a previously well studied representative (i.e., PFOA), and PFECA (i.e., GenX and PFO3TDA) with variable levels of embryo toxicity (i.e., low and high, respectively). More generally, the three compounds represent three levels of toxicity, i.e., PFO3TDA > PFOA > GenX (Figure.2.1 and Appendix A2).

Prior to exposures for metabolomics studies, embryo toxicity was reassessed (see Appendix A) to confirm relative toxicity, and establish appropriate concentrations and developmental stage for exposures. Based on these evaluations, sub-lethal concentrations for which embryos were generally indistinguishable from untreated controls, and severely moribund embryos as seen at higher exposure concentrations (Appendix A2), were not observed. Exposure concentrations consequently employed (50, 100 and 10 ppm for PFOA, GenX and PFO3TDA, respectively) were 2 to 3 orders of magnitude higher than levels typically measured in drinking water, and those measured in human serum which are typically ppb levels or less, although serum levels as high as 1 ppm have, in fact, been measured in occupationally exposed individuals (Sunderland et al., 2019; Worley et al., 2017). These relatively high (but sub-lethal) levels were selected, however, to assure a sufficient and significant alteration in metabolomics studies. An exposure window from 72 to 96 hpf was, furthermore, established. At this stage, most relevant organ systems (e.g.,

liver, kidney) are largely differentiated, and key aspects of CNS development occur, or have occurred, including formation of the midbrain-hindbrain boundary (~ 27 hpf), and elaboration of telencephalon, mesencephalon, hypothalamus and, importantly, primary and secondary motor neurons (~96 hpf). No apparent developmental effects with respect to these systems were observed (between 72 to 96 hpf) for any of the PFAS tested at these exposure concentrations.

Highly-resolved and reproducible NMR spectra were obtained from intact control and PFAS-treated embryos (Appendix A4), and quantitative analysis revealed significant alteration of at least 33 metabolites by one or more of the PFAS evaluated (Figure.2.4 and Appendix A7). Principal components analysis, furthermore, showed that metabolic profiles of the three PFAS, and untreated control, could be clearly discerned suggesting a significant quantitative difference in the metabolic profiles affected (Appendix A5). Notably, the percent of metabolites altered was positively correlated with LC_{50} of the PFAS (Figure.2.5), suggesting that metabolic effects paralleled acute toxicity. Qualitatively, however, there was considerable overlap in terms of the metabolites affected by exposure. Generally speaking, metabolite levels altered by PFAS could be grouped into several categories including (1) indicators of hepatotoxicity; (2) modulation of neural metabolites and/or pathways; (3) metabolites associated with oxidative stress; and moreover, (4) interrelated carbohydrate, lipid and amino acid metabolism, and associated cellular energetics.

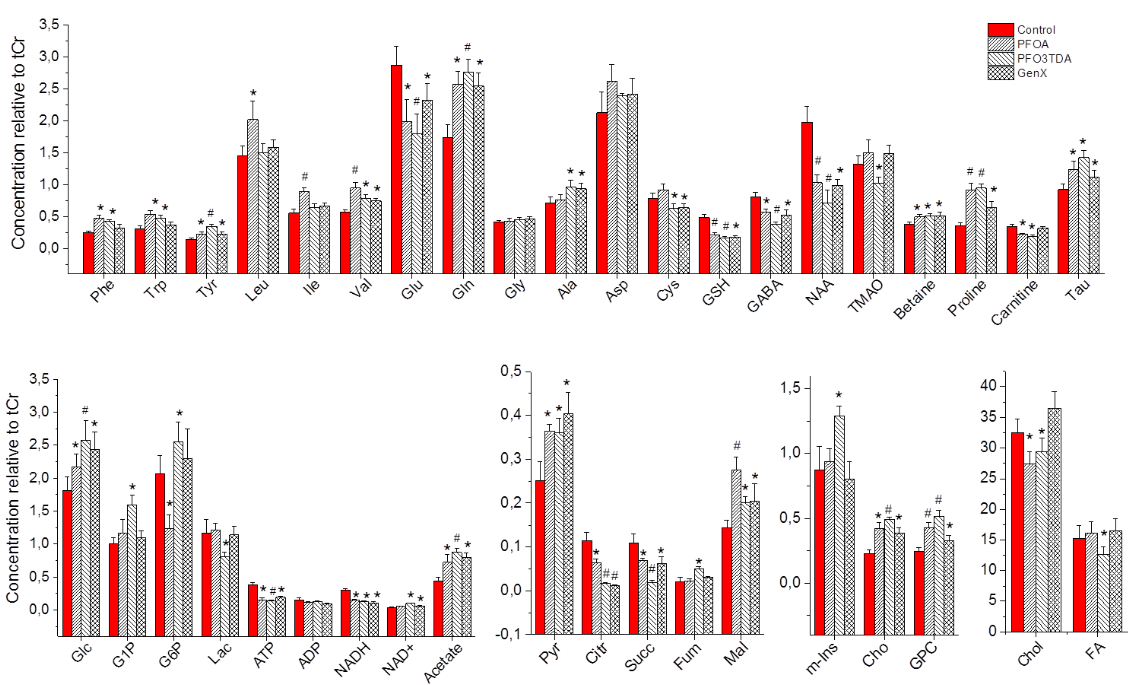

Figure 2. 4 Effect of PFOA, PFO3TDA and GenX treatment on the metabolic profile of intact zebrafish embryos. Zebrafish embryos (3 dpf) were exposed to 50 ppm PFOA, 10 ppm PFO3TDA or 100 ppm GenX for 24 hours. Shown are concentrations of metabolites relative to total creatine (tCr); values are average ± SE of mean. # $P<0.01$, * $P<0.05$. Abbreviations: Phe = phenylalanine; Trp = tryptophan; Tyr = tyrosine; Leu = leucine, Ile = isoleucine; Val = valine; Glu = glutamate; Gln = glutamine; Gly = glycine; Ala = alanine; Asp = aspartate; Cys = cysteine; GABA = g-aminobutyric acid; GSH = glutathione; Glc = glucose; G1P = glucose-1-phosphate; G6P = glucose-6-phosphate; Lac = lactate; ATP = adenosine triphosphate; ADP = adenosine diphosphate; NADH/NAD+ = reduced/oxidized nicotinamide adenine dinucleotide; m-Ins = myo-inositol; Cho = choline; GPC = glycerophosphocholine; Chol = cholesterol; FA = fatty acids.

2.4.3. An integrated system-level model of metabolic targets and pathways of PFAS toxicity

Taken together, the metabolic alterations observed in the present study enabled an integrated model of the metabolic pathways of PFAS toxicity in the zebrafish embryo (Figure.2.6). Although the current study is the first to utilize metabolomics of the zebrafish embryo to investigate PFAS - and the first omics study of any PFECA - previous *ex vivo*

transcriptomics (Peng et al., 2013), proteomics (Shao et al., 2018) and metabolomics (Peng et al., 2013; Shao et al., 2018; Yu et al., 2016) studies have been conducted for PFOA in other cellular and/or organismal systems. As such, metabolic profiles observed in the present study are perhaps best interpreted relative to this legacy PFAS. That said, metabolites altered by exposure to both PFO3TDA and GenX were quite similar to PFOA (Figure 2.4 and Appendix A7), and the differences between the three representative PFAS could be largely explained based on relative toxicity (Figure.2.5). Accordingly, this model could generally, therefore, be extended to the PFECA.

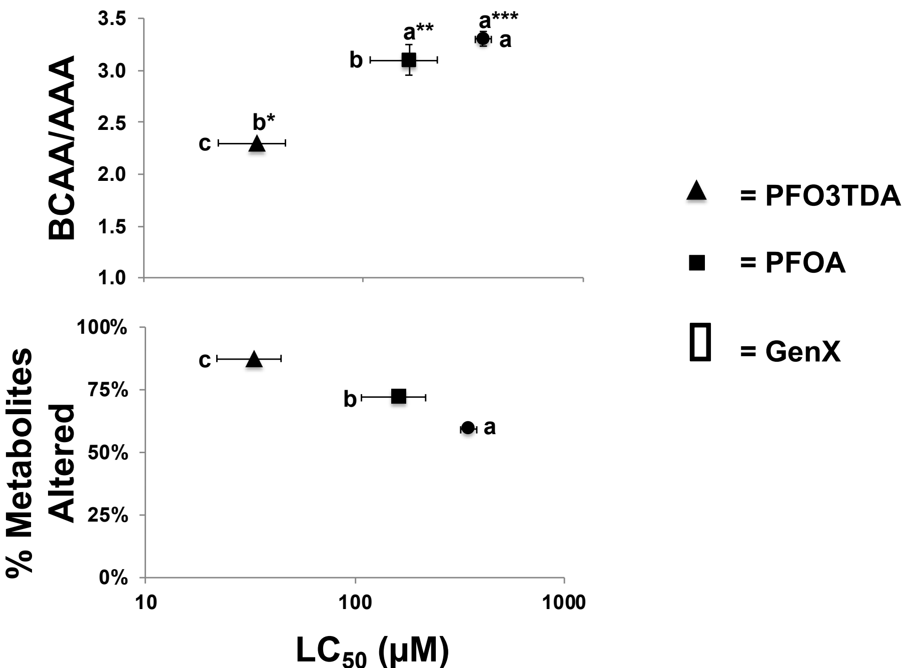

Figure 2. 5 Change in metabolite levels relative to acute toxicity. Acute toxicity is represented by LC_{50} values for PFAS. Hepatotoxicity (using BCAA/AAA ratio as biomarker) correlated with LC_{50} (A). Similarly, percentage of metabolites altered versus LC_{50} was correlated, suggesting a quantifiable coupling between acute toxicity and level of metabolic effects (B). Asterisks indicate significant difference between treatments and control with respect to BCAA/AAA ratio: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.000005$.

Letters associated with error bars indicate significant difference ($p < 0.05$) between treatments with respect to BCAA/AAA ratio (y-error bars) and LC_{50} (x-error bars).

At the organ system, and corresponding cellular level, several previous studies have suggested that PFAS target liver (i.e., hepatocyte) and kidney due, most likely, to the general role of these organ systems in detoxification and depuration of xenobiotics (Mortensen et al., 2011). Cellular uptake, and consequent tissue distribution, of PFOA has been shown in numerous studies to be specifically facilitated by solute carrier (SLC) transporters that are primarily localized to liver and kidney (Kimura et al., 2017; Popovic et al., 2014; Yang et al., 2010; Zhao et al., 2017). Consistent with targeting of hepatocytes, an observed stage-dependence of PFOA toxicity in the present study coincides (as discussed above) with differentiation (at ~ 72 hpf) of the liver as a target organ. Specifically aligned with hepatotoxicity, PFOA ($p < 0.005$), PFO3TDA ($p < 0.000001$) and GenX ($p < 0.05$) all showed a significant reduction in the molar ratio of branched-chain to aromatic amino acids (BCAA/AAA) relative to negative controls (Appendix A7). Altered BCAA/AAA ratio is a well-established *ex vivo* (i.e., plasma level) indicator of liver damage (Muratsubaki and Yamaki, 2011), and in similar studies, has been recently found to parallel hepatotoxicity in the intact zebrafish embryo model (Zuberi et al., 2019). Furthermore, BCAA/AAA ratio was significantly correlated with LC_{50}, underscoring hepatocytes as a target of acute toxicity (Figure.2.5, Appendix A7).

Presumably reflective of the relatively higher hepatotoxicity, a significant decrease in trimethylamine *N*-oxide (TMAO) was uniquely observed for PFO3TDA (as the most toxic PFAS tested). TMAO is the product of the oxidation of trimethylamine, exclusively by hepatic *flavin-containing monooxygenase 3* (FMO3), which is, in turn, derived from

metabolism of choline and betaine (among other diet-derived precursors) by enteric microbiota (Alfieri et al., 2008; Janeiro et al., 2018; Lang et al., 1998). Choline and betaine, in fact, are typically correlated - as companion biomarkers - with TMAO levels (Janeiro et al., 2018), and were both increased in the present study (for all PFAS treatments). Higher acute hepatotoxicity of PFO3TDA, therefore, is likely to result in a decrease of FMO3 function, and the consequent reduction in TMAO levels observed here. Moreover, TMAO (along with its metabolic precursors, i.e., betaine and choline) has been proposed as a biomarker for *metabolic syndrome* (Barrea et al., 2019; Janeiro et al., 2018), and as such, is perhaps not only reflective of hepatoxicity, but also correlates with the reported link between PFAS and metabolic syndrome (Christensen et al., 2019; Matilla-Santander et al., 2017), and the numerous metabolic alterations observed in the present study related to cellular energetics, glucose homeostasis and dyslipidemia (as discussed below).

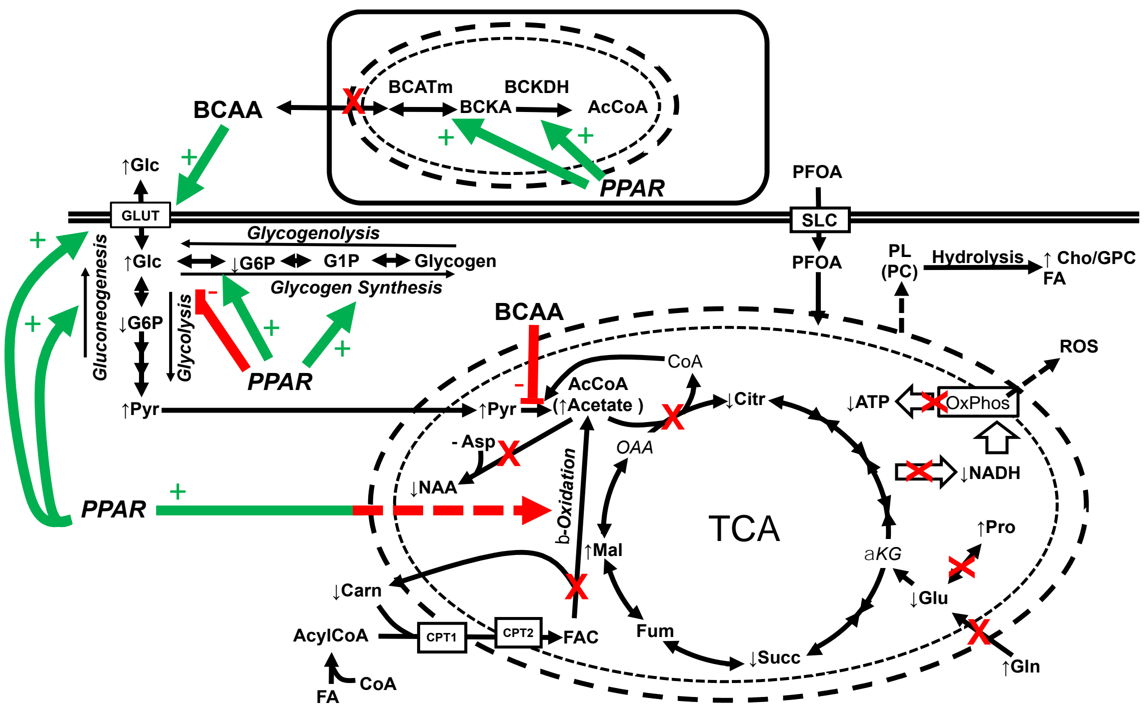

Figure 2. 6 A model of the targeting of mitochondria and PPAR by PFOA in relation to changes in metabolic alterations. Abbreviations: TCA = Tricarboxylic acid cycle, i.e., citric acid cycle; PPAR = peroxisome proliferator-activated receptor; BCAA = branch-chained amino acids; BCATm = branch-chained amino acid aminotransferase; BCKA = branch-chained keto acid; BCKDH = branch-chained alpha-keto acid dehydrogenase; AcCoA = acetyl CoA; CPT1 and CPT2 = carnitine palmitoyl transferase I and II; FAC = fatty acyl carnitine; OxPhos = oxidative phosphorylation; ROS = reactive oxygen species; PL = phospholipids; PC = phosphatidylcholine; SLC = solute carrier transport protein; α -KG = alpha-ketoglutarate; GLUT = glucose transporter; for other metabolite abbreviations, see Figure 2.4.

A significant decrease in GSH (Figure.2.4) may, furthermore, correlate with targeting of hepatocytes by PFOA. Although GSH is a ubiquitous antioxidant in biological systems, it is particularly associated with Phase II detoxification which is localized to the liver, and numerous studies have similarly reported decreases in GSH in association with hepatotoxicity (Maddox et al., 2009; Song et al., 2003). That said, a decrease in GSH may be more generally linked to the well-established induction of oxidative stress by PFAS (Hagenaars et al., 2013; Liu et al., 2007; Tang et al., 2018) mediated by mitochondrial impairment (as further discussed below).

Although liver and kidney have been most frequently linked to PFOA toxicity, there is a considerable body of evidence to suggest that neuronal dysfunction may contribute to deleterious effects (Johansson et al., 2008). Behavioral impairment (Figure.2.3B) observed in the current study is consistent with targeting of neural systems, and several metabolites affected by PFAS are, likewise, aligned with possible neurotoxicity and/or neurodevelopmental toxicity (Figure.2.4). Among these, levels of the metabolically interrelated neurotransmitters, Glu and GABA, were significantly reduced with exposure to PFAS. At the same time, the concentration of NAA which is uniquely associated with

(and one of the most abundant metabolites, second only to Glu, in) neural cells was significantly decreased. Biosynthesized from Asp and acetyl CoA in the mitochondria of neural cells, the observed decrease in NAA would be consistent with not only general targeting of neural cells, but specifically mitochondria (discussed below; Figure.2.5). Alterations of NAA have, furthermore, been recently proposed as a biomarker of disruption of the interaction between neurons and glial cells including myelination, i.e., oligodendrocyte/neuron interactions (Xu et al., 2016). Interestingly, the ratio of Gln/Glu (which was increased in PFAS-treated embryos) has been, likewise, suggested to be a metabolic indicator of impaired astrocyte-neuron interaction, based on the role of the "glutamate/glutamine cycle" in the shuttling and recycling of Glu/GABA between astrocytes and neurons (Pereira et al., 2008; Xu et al., 2016), and would be further suggestive of the disruption of the astrocyte-neuron interaction by PFAS .

At the subcellular and molecular level, two interrelated targets of PFAS (including PFOA) have been most frequently identified: namely, (1) disruption of mitochondria, and (2) pathways associated with activation of peroxisome proliferator-activated receptors (PPAR). Multiple studies in cell-based systems have shown that PFOA causes mitochondrial dysfunction including swelling and disruption of membrane integrity, membrane potential collapse with disruption of the electron transport chain, and consequent reduction in ATP levels (Choi et al., 2017; Mashayekhi et al., 2015; Suh et al., 2017). At the same time, mitochondrial dysfunction has been linked to the induction of oxidative stress by PFOA (including studies in the zebrafish model; Hagenaars et al., 2013). Molecular studies, on the other hand, have consistently documented the activation

of PPAR - and in particular, the PPARα and PPARγ subtypes - by PFOA (Abbott et al., 2012; Buhrke et al., 2013; Kennedy et al., 2004; Takacs et al., 2006; Vanden Heuvel et al., 2006). Collectively, the PPAR have diverse roles in lipid and glucose metabolism including β-oxidation, lipid biosynthesis and transport, ketogenesis, adipogenesis, and glucose homeostasis (i.e., glucose biosynthesis, catabolism, storage and transport) (Dubois et al., 2017; Peeters & Baes, 2010; Tan et al., 2005). Co-targeting of mitochondria and PPAR by PFOA is highly interrelated as both have key functions in cellular energetics as it relates, in particular, to carbohydrate (i.e., glucose) and lipid metabolism, and consequently, metabolic syndrome (Alderete et al., 2019; Cardenas et al., 2018; Christensen et al., 2019; Lin et al., 2009). And the alteration of numerous metabolites observed in the present study, likewise, points to the targeting of mitochondria and PPAR-regulated pathways by PFAS, and in turn, oxidative stress and cellular energetics including pathways relevant to metabolic syndrome (Figure.2.5). Alterations in metabolic profiles in relation to these two targets – as part of an integrated model -are discussed separately below.

2.4.3.1. Apparent disruption of mitochondrial pathways by PFAS

Several of the changes in metabolite profiles of PFAS-exposed zebrafish observed in the present study align with mitochondrial dysfunction in association with toxicity. Consistent with a compromise of mitochondrial membrane integrity, as previously established for PFOA (Hagenaars et al., 2013), significant increases in choline and glycerophosphocholine (GPC) were observed, and are possibly indicative of release and hydrolysis of phospholipids - following presumptive disruption of mitochondrial

membranes – including, in particular, phosphatidylcholine as one of the most abundant (and typically most abundant) classes of lipids in mitochondrial membranes(Almaida-Pagán et al., 2014; de Kroon et al., 1997).Increased concentration of polar head-groups of membrane phospholipids (e.g., choline, GPC, ethanolamine, myo-inositol) as, likewise, measured by HRMAS NMR of intact zebrafish embryos has, in fact, been similarly linked in previous studies to membrane disruption in this same sytem (Berry et al., 2016; Roy et al., 2017; Zuberi et al., 2019).

Most conspicuously, alteration of several metabolites associated with the tricarboxylic acid (TCA, or "citric acid") cycle, and associated electron transport chain and oxidative phosphorylation, is directly aligned with disruption of mitochondria. Key changes associated with PFAS exposure, in this regard, include significant decreases in citrate and succinate, and increase of malate, as well as apparent metabolic accumulation of acetate, which generally indicate disruption of the TCA cycle, and an inability of carbon (via acetyl CoA) to enter the cycle (Figure 2.6). Reductions in citrate and succinate, and increase in malate, which are associated with irreversible steps specifically point to inhibition of the aldol condensation of acetyl CoA and OAA as entry point (which links glycolysis and β-oxidation) to the TCA cycle. Exclusively for PFO3TDA, fumarate was additionally increased, and likely reflects consequent upstream metabolic "back-up" (from malate to fumarate) owing to the higher toxicity (and associated mitochondrial disruption) of this compound. Alongside changes in TCA cycle metabolites, decreases in the energetic currencies of NADH and ATP would correlate, in turn, with inhibited yield of the former,

and consequent lack of availability of this reducing agent for oxidative phosphorylation to produce the latter (Figure.2.6).

Upstream of the TCA cycle, the observed alteration of several metabolites associated with glycolysis and β−oxidation (which supply acetate to the TCA cycle) similarly indicate mitochondrial dysfunction and/or impairment of the transport of metabolic intermediates (from cytosol to the mitochondria) as part of these pathways (Figure.2.6). Aerobic glycolysis in the cytosol, for example, provides pyruvate that is, in turn, transported to mitochondria where conversion by pyruvate dehydrogenase (PDH) within the mitochondrial matrix provides acetyl CoA for entry to the TCA cycle. The observed increase in pyruvate, therefore, may extend the metabolic accumulation (from acetate) associated with loss of TCA cycle function, and PDH activity. With respect to β−oxidation, one particularly telling response for the two most toxic PFAS (i.e., PFOA and PFO3TDA) may be a significant decrease in carnitine. Carnitine is essential to the transport of fatty acids (via coenzyme A-activated, i.e., acyl CoA, intermediates) whereby carnitine palmitoyltransferases (CPT) located in the outer and inner mitochondrial membranes (i. e., CPT I and II, respectively), catalyze formation of acyl carnitines for transport into the mitochondrial matrix (where β−oxidation of FA occurs), and subsequent recycling of carnitine (back to the cytosol). Decreased carnitine, therefore, likely reflects a decrease in the mitochondrial capacity for β-oxidation, and consequently diminished recycling of free carnitine, for these two most toxic PFAS (Figure.2.6).

Alongside alterations of TCA cycle intermediates and precursors, auxiliary pathways are also seemingly affected by PFAS presumably due to impaired mitochondrial

function. For example, in addition to their role in neurotransmitter recycling, Glu and Gln are important metabolic intermediates for entry to the TCA: cytosolic Gln is transported to the mitochondria, and converted within the mitochondrial matrix to Glu (by glutaminase) which can, in turn, enter the TCA cycle via α−ketoglutarate (αKG). Accordingly, the observed increase in Gln/Glu ratio is consistent with disruption of mitochondria. At the same time, Pro derived from collagen in the extracellular matrix during times of metabolic stress can be catabolized to Glu (to supply anaplerotic αKG to the TCA cycle) by proline oxidase (POX) which is localized to the inner membrane of the mitochondria (Phang et al., 2008). Thus, the significant increase in Pro, likewise, aligns with mitochondrial dysfunction, and specifically loss of POX activity (Figure.2.6).

Finally, with respect to the role of mitochondria, previous studies have generally linked PFAS to increased production of reactive oxygen species (ROS) and consequent oxidative stress due to mitochondrial dysfunction (Liu et al., 2007; Suh et al., 2017; Wielsøe et al., 2015). Additionally, PFOA has been shown to decrease levels of nuclear factor erythroid 2-related factor (Nrf2) which is a key transcription factor required for induction of numerous genes involved in antioxidant response including GSH biosynthesis (Liu et al., 2015). The observed decrease in GSH for PFAS exposures would, therefore, generally align with both oxidative stress (i.e., production of ROS) and consequent depletion of the peptide, as well as reduced biosynthesis (via Nrf2). A similar decrease in GSH has, in fact, been consistently observed in association with PFOA-induced oxidative stress (Liu et al., 2007).In parallel, a marked increase in taurine was, furthermore, observed for all PFAS in the present study. Taurine has an established role in antioxidant defenses

(Schaffer et al., 2009), and likewise, may indicate an upregulation in response to oxidative stress in zebrafish embryos. Moreover, taurine is associated with Nrf2 translocation (Sun et al., 2018), and therefore, may be directly involved in the upregulation of antioxidant responses.

2.4.3.2. Role of PPAR in metabolic disruption by PFAS

Alongside mitochondria, PPAR has been consistently demonstrated to be a key target in PFAS toxicity. As transcription factors, PPAR are involved in the expression – specifically via PPAR response elements (PPRE) - of a wide range of genes, and accordingly, diverse cellular and biochemical pathways. However, the best described functions involve regulation of energy homeostasis including carbohydrate and lipid metabolism which in turn, would support a reported contribution of PFAS in increased rates of obesity, diabetes and other related metabolic disorders (Liu et al., 2018). Although PPAR expression was not directly measured in the present study, numerous alterations of metabolic profiles by PFAS are consistent with a role of PPAR-regulated pathways.

Fatty acids are recognized as endogenous ligands for PPAR, and thereby, represent a direct link between PPAR and lipid metabolism, specifically via β-oxidation. As a proposed mechanism of action, PFOA have been shown to bind and activate PPAR (Vanden Heuvel et al., 2006; Yamamoto et al., 2014), and induce β-oxidation of fatty acids (Kudo et al., 2006; Yu et al., 2016). That said, any PPAR-directed effects on β-oxidation associated with PFAS may be potentially confounded by concomitant disruption of mitochondria (where β–oxidation occurs; as discussed above). This may, for instance,

explain a lack of significant alteration (Figure.2.4) in fatty acids – except for the most toxic PFO3TDA – despite apparent effects on carnitine-based fatty acid transport pathways observed here (as suggested by elevated carnitine levels). Alongside a direct role in the catabolism (i.e., β–oxidation) of fatty acids, however, PPAR have been shown to regulate lipid metabolism via other direct and indirect routes. Most notably, PPAR have been shown to regulate multiple pathways associated with metabolism and transport of cholesterol (Li & Chiang, 2009). Significant decrease in cholesterol with PFOA and PFO3TDA treatment observed in the present study (Figure.2.4) may, therefore, be explained by the effect of these PFAS on PPAR-mediated pathways of cholesterol metabolism and uptake.

Although PPAR has been perhaps most widely investigated with respect to lipid metabolism, considerable evidence supports roles in the regulation of glucose metabolism including glycolysis, gluconeogenesis and glycogen metabolism (Peeters & Baes, 2010). And multiple studies have suggested a role of altered glucose metabolism in the adverse effects of PFOA (Yan et al., 2015; Zheng et al., 2017). Activation of PPARγ by the agonist fenofibrate was previously shown (Oosterveer et al., 2009) to reduce glycolytic flux of glucose (via G6P) through glucokinase (i.e., hexokinase IV), as a key step in glycolysis, with diversion toward gluconeogenesis. At the same time, studies (Im et al., 2011) identified a PPRE within glucose-6-phosphatase (G6Pase), and demonstrated that PPARα upregulates this enzymes as the key final step in liver gluconeogenesis. Accordingly, PFOA activation of PPAR would be, likewise, expected to reduce G6P and increase glucose – as was, indeed, observed in the present study - by way of reduced

glycolytic flux, and upregulation of gluconeogenesis (Figure.2.4). In the case of the more toxic PFO3TDA, a significant elevation in glucose was accompanied by a decrease in lactate which may reflect a decrease in glycolytic flux – and specifically anaerobic glycolysis (with lactate as end product) –or alternatively, increased hepatic gluconeogenesis from lactate (e.g., Cori cycle). That said, in contrast to PFOA, PFO3TDA was actually found to increase G6P (rather than decrease as observed for PFOA), and taken together with a decrease in glucose-1-phosphate (G1P) that was, likewise, exclusively observed for PFO3TDA exposures, points to a possible role of glycogen metabolism in the case of this more toxic PFAS.

Indeed, alongside glucose anabolism (i.e., gluconeogenesis) and catabolism (i.e., glycolysis), PFAS has been shown in multiple studies to alter glycogen metabolism. Specifically, PFOA was found to decrease liver glycogen with a concurrent increase in G6Pase as the final step in glycogenolysis, and decrease in glycogen synthase (GS) as the key regulatory step of glycogenesis (Zheng et al., 2017). Both of these enzymes, in turn, have been shown to be regulated by PPAR (Im et al., 2011; Mandard et al., 2007). More recently, PFOA was found to significantly decrease in glycogen (Hagenaars et al., 2013). Although a precise mechanistic role of PPAR in regulating glycogen metabolism remains unclear (Bandsma et al., 2004; Mandard et al., 2007; Oosterveer et al., 2009; Peeters & Baes, 2010), G1P is uniquely associated with glycogenolysis (and, in reverse, glycogenesis), such that the concurrent increase in G1P, alongside increases in both glucose and G6P, for PFO3TDA exposures is highly consistent with a role of increased glycogenolysis (in relation to the higher toxicity of this variant). The increase of G6P (from

glycogenolysis) could, moreover, offset any expected decreases of this metabolite resulting from reduced glycolytic flux and/or increased gluconeogenesis (as discussed above). Of further note, the increase in G6P (exclusively for PFO3TDA-exposed embryos) is mirrored by a similarly unique increase in *myo*-inositol (Figure.2.4) for which G6P is the rate-limiting biosynthetic substrate. Although exposure to GenX did significantly increase glucose, none of the intermediates associated with glucose or glycogen metabolism, on the other hand, were significantly altered. It is proposed that elevated glucose, in this case, may be related instead to intercellular glucose transport, and the established role of PPAR in the expression of glucose transporters.

Numerous studies have shown that PPAR activation upregulates expression and/or translocation of bidirectional glucose transport (GLUT) proteins including, in particular, GLUT2 in the liver as the primary transporter of glucose between liver and blood (Dasgupta & Rai, 2018; Im et al., 2005; Kim & Ahn, 2004; Liao et al., 2007; Variya et al., 2020; Wu et al., 1998). Consistent with this, PFOA exposure was previously found (in a mouse liver model) to decrease glucose in liver, while simultaneously increasing blood glucose, by directing hepatic glucose away from glycogen synthesis, and into the bloodstream toward other systems including various non-glycogen synthesizing cells, thus effectively increasing total "free" glucose (Zheng et al., 2017). Though only total glucose, and not cell-specific levels, in zebrafish embryos were measured in the present study, increased GLUT2 may, therefore, alternatively or additionally explain observed elevation of glucose as a result of "escape" from hepatic glycogenesis (Figure.2.6).

In parallel, one of the most revealing differences in metabolic profiles between PFOA and PFECA is the relative changes in BCAA which are, in fact, well documented regulators of GLUT (Holeček, 2018; Zheng et al., 2017), and in turn, known to be regulated by PPAR. As essential amino acids, levels of BCAA are exclusively regulated by catabolism which takes place by a two-step process involving (1) the reversible conversion of BCAA to corresponding a-keto acids by BCAA amino transferases (BCAT) in extrahepatic cells, and (2) the subsequent rate-limiting irreversible decarboxylation by branched-chain keto acid dehydrogenase (BCKDH), primarily in hepatocytes, following transport of α–keto acids to the liver (Li et al., 2017). Both enzymes have been shown to be upregulated by activation of PPARγ, and downregulated when PPARγ genes are knocked-out (Blanchard et al., 2018). Enzymes involved in both steps, however, are either exclusively (i.e., BCKDH) or primarily localized to mitochondria (García-Espinosa et al., 2007; Holeček, 2018). In the case of PFOA, all BCAA were significantly increased, and it is proposed that mitochondrial disruption, therefore, may supersede PPAR-mediated effects (i.e., increased BCAA catabolism) whereby impairment of mitochondrial BCKDH would reduce catabolism, and consequently lead to the observed accumulation, of BCAA (which could, in turn, upregulate GLUT, and contribute to the observed increase in glucose).

In contrast to PFOA, however, BCAA were either not significantly altered (i.e., leucine, isoleucine), or decreased to a lesser extent (i.e., valine), by both PFO3TDA and GenX (Figure.2.4). In light of presumptive mitochondrial disruption (and consequent loss of BCAT and BCKDH catabolic activity which would be expected to increase BCAA

levels), the lack of altered BCAA levels by PFECA may imply otherwise increased BCAA catabolism. In this regard, although BCAT is mostly localized to the mitochondria, cytosolic BCAT is abundant in certain cell types including, in particular, the CNS (García-Espinosa et al., 2007), where the first step of catabolism could, thus, consequently occur despite mitochondrial disruption (and loss of BCKDH activity). Further aligned with a possible increase in BCAA catabolism, alanine levels were also uniquely increased for both PFECA (but not PFOA); during transamination by BCAT, the amino group from glutamate (following deamination of BCAA) is transferred to alanine, and thus, increased BCAA catabolism would, indeed, be expected to increase alanine. At the same time, the observed increase in NAD^+ for PFECA-exposed embryos (and not PFOA) may reflect an upregulated BCAA catabolism by BCAT (in the absence of mitochondrial BCKDH). Specifically, it has been shown (Hutson et al., 2011; Islam et al., 2010) that in the absence of BCKDH activity, NAD^+ (which would, otherwise, be recycled to NADH in the presence of BCKDH) accumulates. Differences in the alteration of BCAA levels (and associated metabolites, i.e., alanine, NAD^+) between PFECA and PFOA may, therefore, point to differences in their effects on catabolic pathways including cell-specificity, or differential degrees of interaction (i.e., activation) with PPAR (increasing BCAA catabolism), compared to mitochondrial disruption (decreasing BCAA catabolism), or alternatively, toward particular subtypes, i.e., PPARα versus PPARγ. These hypotheses, however, remain to be investigated.

Finally, it is noteworthy that cysteine levels are uniquely decreased in embryos exposed to PFECA. Pathways targeted by PFECA with respect to the observed alteration

of cysteine are not abundantly clear. Cysteine has been linked to obesity, however, and depletion of cysteine was found to prevent induction of PPARγ and adipose differentiation (Haj-Yasein et al., 2017). At the same time, evidence suggests a role of PPARγ in the regulation of genes involved in biosynthesis including cystathionine γ-lyase (Yang et al., 2018) and cystathionine β-synthase (Mishra et al., 2010). It is possible that differential effects in relation to cysteine may, likewise, be linked to differences between PFOA and PFECA in terms of interactions with PPAR (and/or subtypes). However, these possibilities similarly remain to be investigated.

2.5. Conclusions

In conclusion, our findings confirm that PFECA are quantitatively and qualitatively (with respect to metabolic alterations) similar in toxicity to PFOA, as a "legacy" PFAS, in the zebrafish embryo system. Metabolic alterations are, furthermore, consistent with a previously reported link between PFAS and hepatotoxicity, neurotoxicity and metabolic syndrome including roles of mitochondria and PPAR, and may represent a model system for investigating this linkage. These findings most generally suggest that these next-generation PFAS need to be equally considered as environmental toxicants of potential concern.

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2.7 Appendix A

Zebrafish Embryo Toxicity Assays

Zebrafish embryo toxicity assays were primarily conducted in the laboratory at Florida International University (Miami, FL) as adapted from previously described methods ((Berry et al., 2007; Jaja-Chimedza et al., 2017; Weiss-Errico et al., 2017). Assays were conducted in polypropylene 24-well plates (Evergreen Scientific, Los Angeles, CA, USA) with five embryos (4- to 32-cell stage) per replicate, i.e., well (n = 4), in E3 medium for a total of 20 zebrafish embryos per treatment/concentration. Embryos were exposed via continuous static exposure (i.e., without replenishment) to a range of concentrations of PFAS, as determined (for each compound) in preliminary assays: 25, 50, 75, 100, 150, 200 ppm for PFMOBA, Gen X, PFO2HPA , PFO3DA and PFOA; 100, 150, 200, 250 and 300 ppm for PFDMMOBA; 5, 10, 50, 75 and 100 ppm for PFO2DA; and 1, 5, 10, 20, 30 and 40 ppm for PFO3TDA. Embryos were subsequently observed at 1, 2, 5 and 7 days post-fertilization (dpf) with a dissecting light microscope to assess mortality (i.e., lack of heartbeart, coagulation of embryo) and morphological developmental deformities. Median lethal concentration was calculated as the concentration corresponding to 50% mortality (LC_{50}); the LC_{50} values, and their 95% confidence intervals, were calculated via Probit Analysis in SPSS (version 22.0; IBM Corporation, Armonk, NY, USA, 2013). In addition to lethality and deformities, inhibition of embryo development was morphometrically assessed based on the *interocular* distance between eyes (as a proxy for body size) of 7 dpf embryos measured using Olympus DP2-BSW imaging software (Olympus, Center Valley, PA, USA, 2009). Apparent neurobehavioral effects were additionally measured as the

percent of 7-dpf eleuthero-embryos (per well) displaying listing behavior, as a measure of vestibular righting reflex, within a 30 s period following shaking of test plates (to stimulate embryos) between each measurement. All toxicity assays involving zebrafish were conducted under protocols approved by the Florida International University's Institutional Animal Care and Use Committee (IACUC), and performed by trained investigators.

Prior to exposures for NMR metabolomics studies, embryo toxicity for selected PFAS (i.e., PFOA, PFO3TDA and GenX) was reassessed in laboratories at the University of Leipzig to confirm concentration range, and optimal stage, for exposures. Assays were conducted with ≤ 5 dpf embryos (OBI/WIK line) in accordance with the local animal welfare regulations (Council of Europe Directive 86/609/EEC). Embryos (obtained from UFZ; see above) at 72 hpf were transferred to a sterile 6-well tissue culture plate, and exposed to PFOA and GenX (at concentrations of 0, 50, 100, 200, 300 and 400 ppm) and PFO3TDA (at concentrations of 0, 10, 20, 40, 50 and 100 ppm) for 24 and 48 hours. Embryos were observed using a light microscope to specifically assess percent mortality (i.e., lack of heartbeat, embryo coagulation) and record developmental deformities of each treatment. Based on these assessments of acute embryo toxicity, and primary assessments (as described above), relevant sub-lethal exposure concentrations for PFOA, PFO3TDA and GenX (i.e., 50, 10 and 100 ppm; see Figure S3) were established, and a suitable exposure window (72 to 96 hpf) was determined.

Multivariate statistical analysis

Multivariate statistical analysis of primary metabolites in the ^{1}H NMR spectra was performed using SIMCA software package (Version 14.0, Umetrics, Umeå, Sweden). The spectra, collected from embryos, were subdivided in the range between 0.3 and 9 ppm into buckets of 0.04 ppm (total 218 buckets), using MestReNova v.12.0.4 (Mestrelab research S.L., Santiago de Compostela, Spain). The region of 4.80 – 6.00 ppm was excluded from the analysis to remove the water signal. To compensate for the differences in the overall metabolite concentration between individual samples, the data obtained were mean centered, scaled to unit variance and then normalized by dividing each integral of the segment by the total area of the spectrum. The resulting data matrix was exported into Microsoft office Excel (Microsoft Corporation, USA). This was then further imported into SIMCA software for multivariate statistical analysis. A probability of p-value of <0.05 was taken to indicate statistical significance. Multivariate analysis specifically was done by supervised partial least squares discriminant analysis (PLS-DA) as previously described (Roy et al., 2017). This method gives both a score matrix and a loading matrix, with score matrix showing the relation between observations, while the loading matrix gives the individual contribution of each parameter, which is a peak in the case of NMR spectra. The HRMAS spectra were investigated by multivariate analysis to probe if control and PFAS-treated embryos can be discriminated and to determine the spectral regions and corresponding compounds mainly responsible for the separation. A clear clustering could be observed in the score plot of PLS-DA1 vs PLS-DA2 (Appendix A5 A-B). In order to determine the variables, i.e. metabolites assigned to the corresponding buckets that are

mainly responsible for the separation of three groups, the load values of the PLS-DA1 and PLS-DA2 were determined (Appendix A5 C).

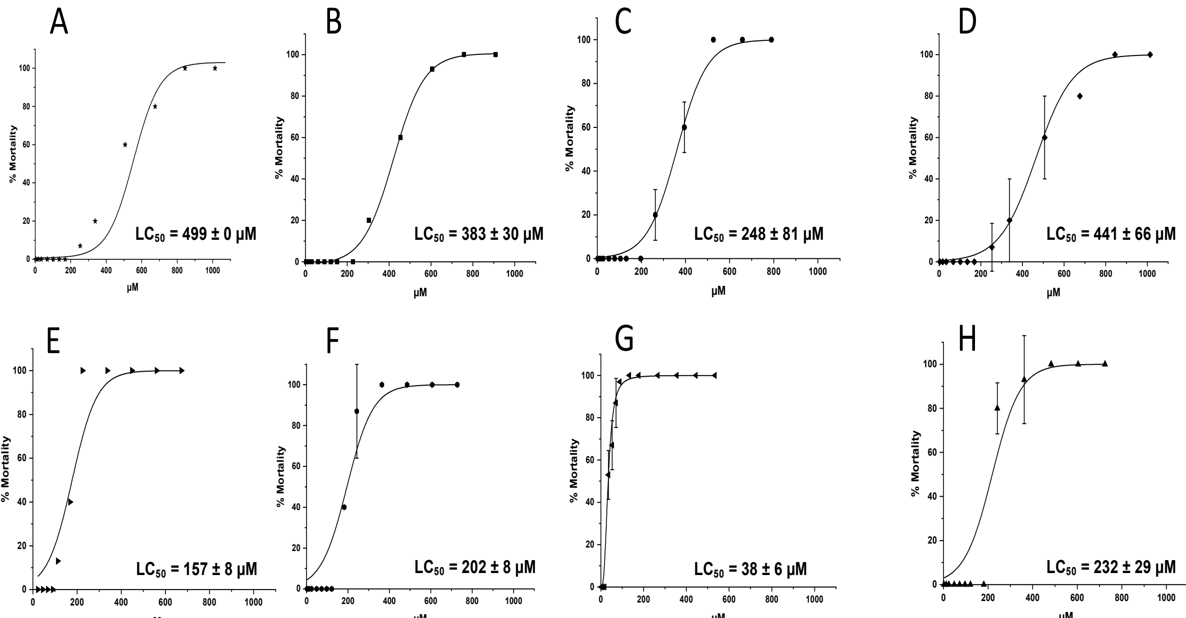

Figure.A1. Concentration-dependent acute toxicity of PFECA and PFOA. Given are curves for concentration-dependent toxicity (i.e., lethality) and calculated 50% lethal concentrations (LC_{50}) for PFMOBA (A), Gen X, (B) PFDMMOBA (C), PFO2HPA (D), PFO2DA (E), PFO3DA (F), PFO3TDA (G) and PFOA (H) at 24 hours post-fertilization (hpf). Error bar represents ± standard deviation (n=3).

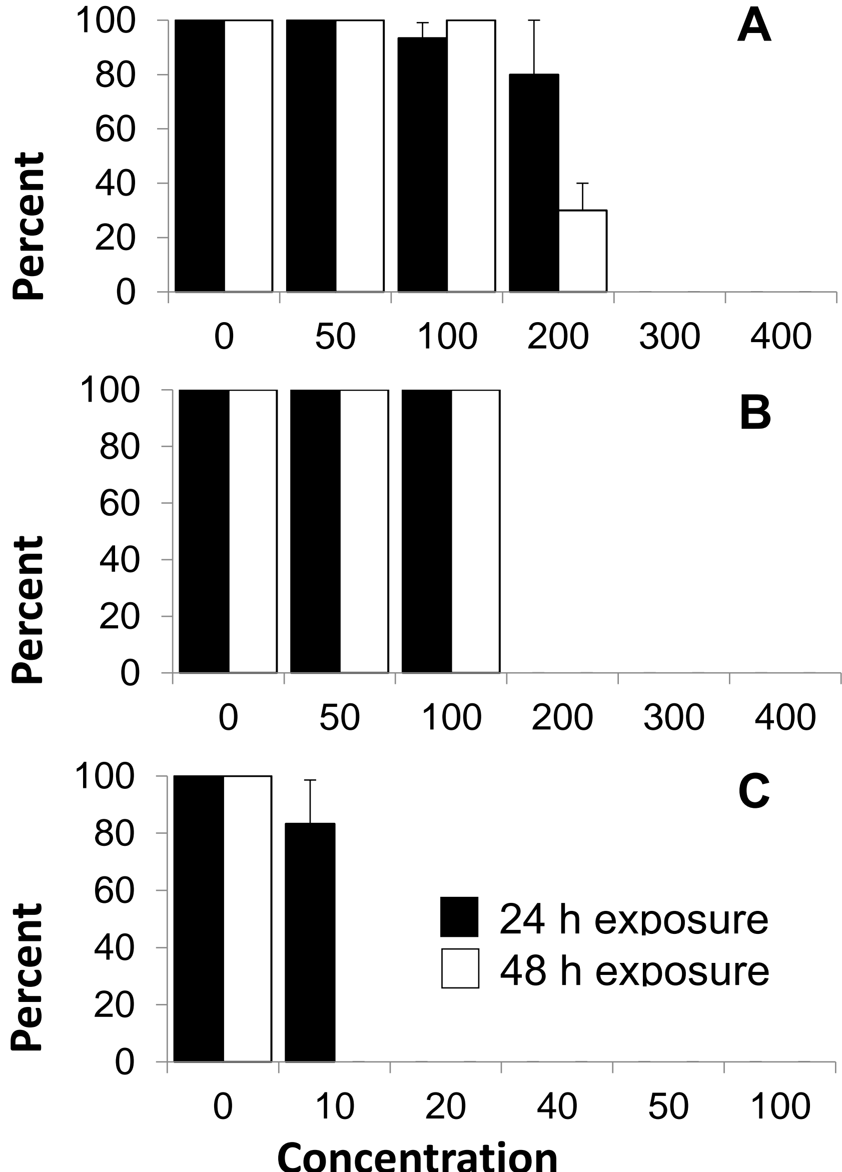

Figure.A2. Identification of sublethal concentrations of PFAS for 72 hpf zebrafish embryos exposed for 24 and 48 h. Given is percent survivorship for GenX (A), PFOA (B) and PFO3TDA (C). Error bars represents standard deviations.

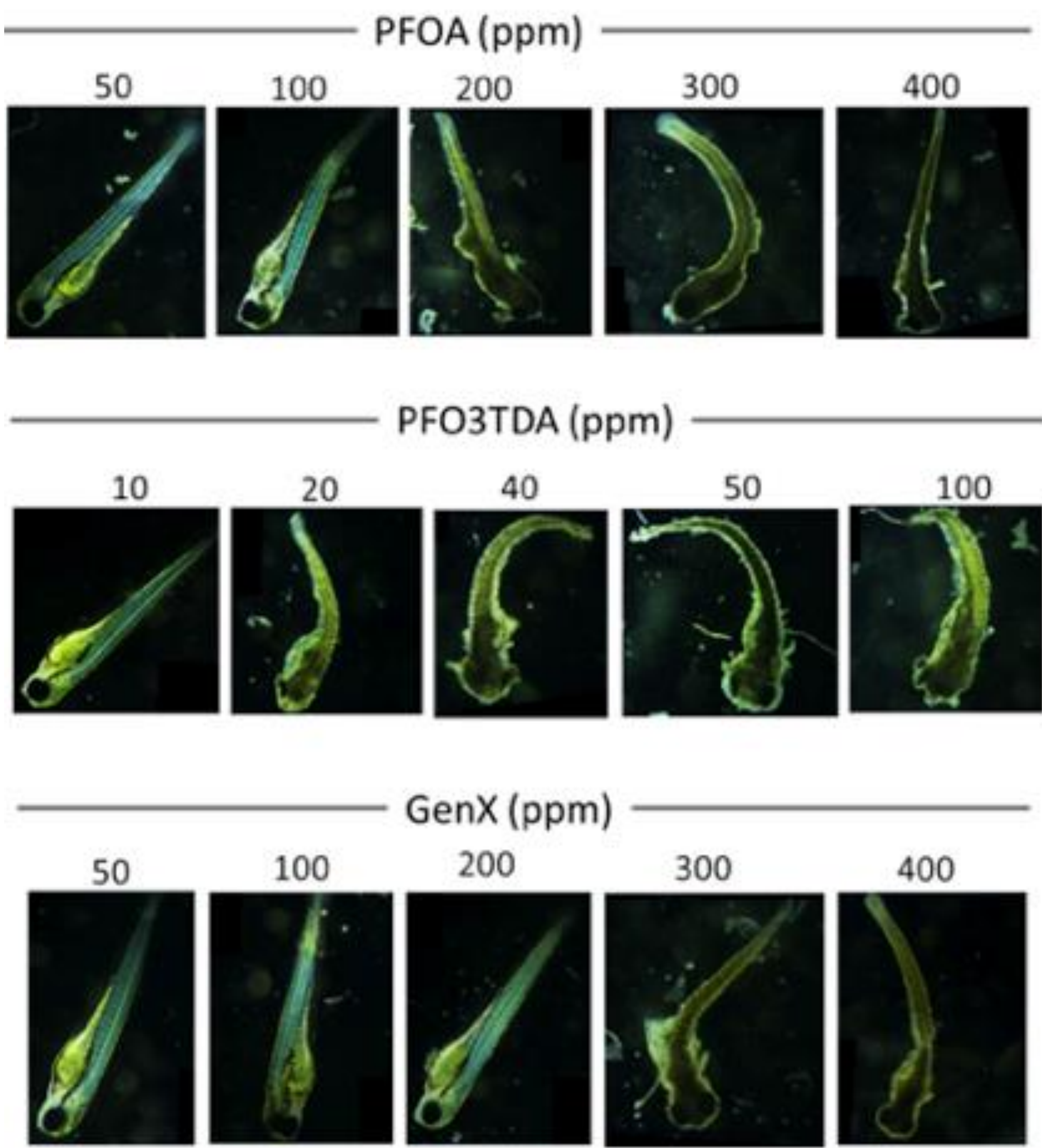

Figure.A3. Gradient of morphological abnormalities and moribundity observed for zebrafish exposed to representative PFAS, i.e., PFOA, PFO3TDA and GenX. Embryos shown were exposed for 24 h at 72 hpf. Deformities include malformation of head, bending of upper body and tail alongside dermal sloughing. Note: ~7% deformities were observed at and above 300 ppm of PFOA and GenX. For PFO3TDA, deformities were observed in ~7% embryos at 40 and 50 ppm, and ~10% at 100ppm.

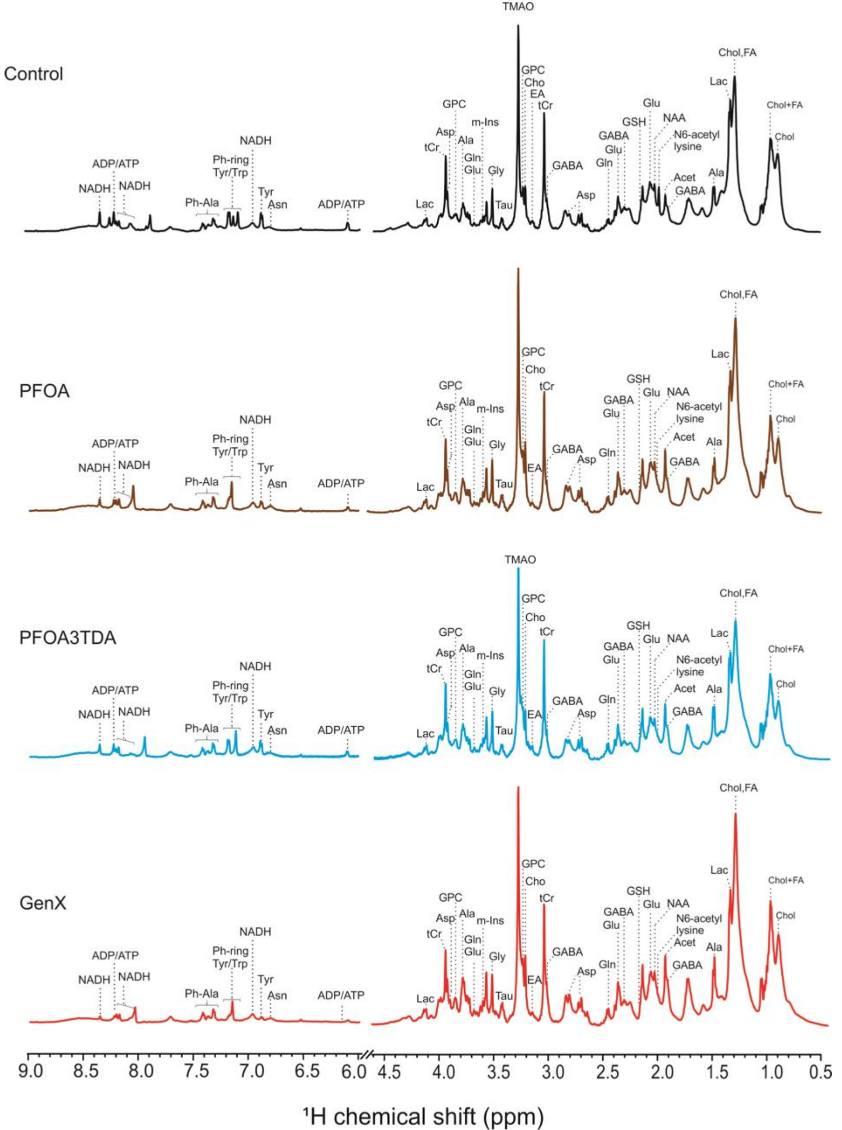

Figure.A4. Representative high-resolution magic angle spin (HRMAS) NMR spectra of control, PFAO (50 ppm), PFA3TDA (10 ppm) and GenX (50 ppm) treated zebrafish embryos exposed at 72 hpf for 24 h. Integrated peak areas and chemical shifts of 1-D NMR spectra were used to quantify and identify metabolites.

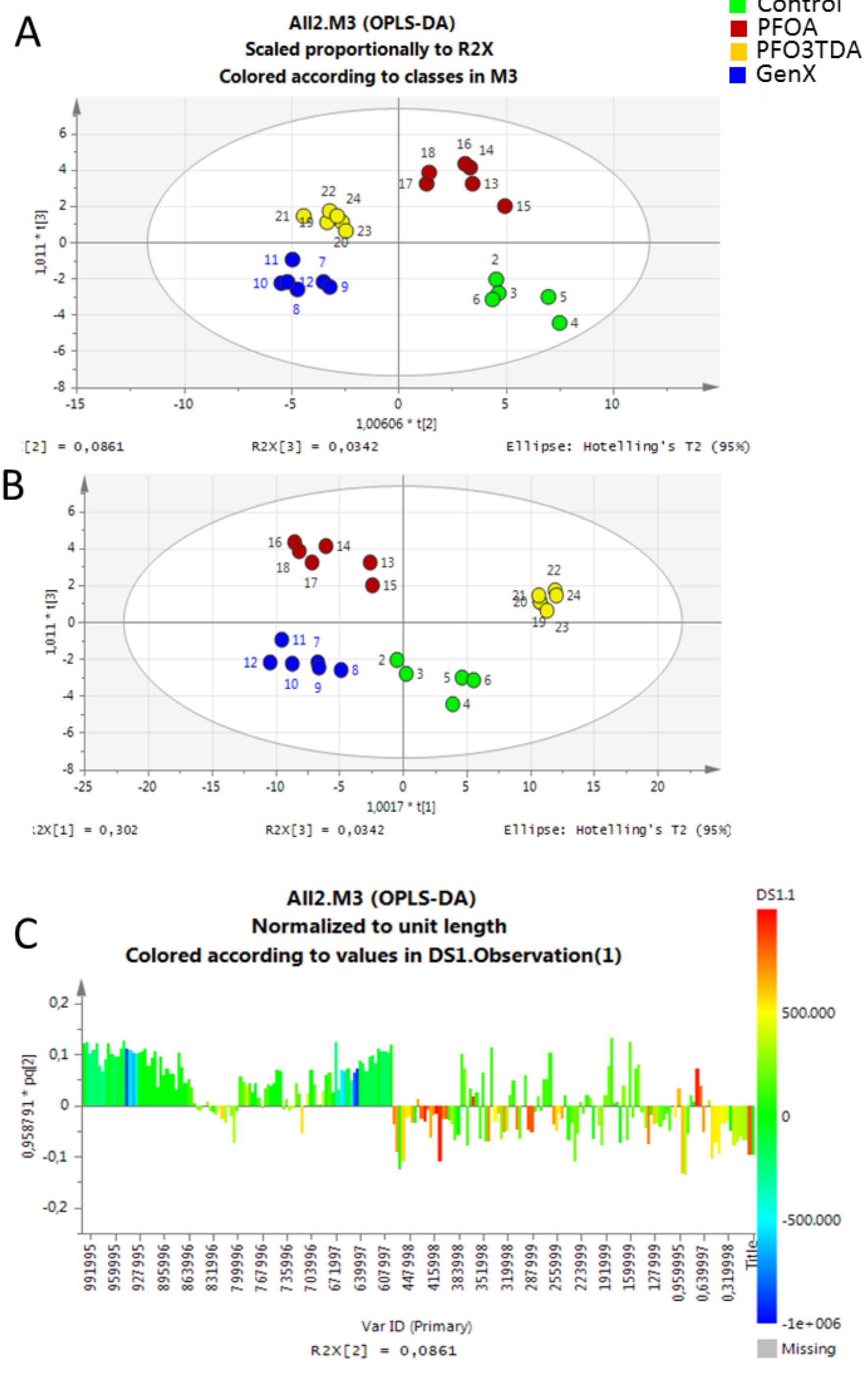

Figure. A5. (A-B) OPLS-DA scores plots for control (green), PFOA (red), PFO3TDA (yellow) and GenX (blue) treated embryos. Spectra derived from the same group have the same color. Total 42.2% variables are used to make this score plot. (C) Loading plots of OPLS-DA1 for all buckets.

Table A6. Change in metabolites observed for zebrafish embryos (72 hpf) exposed for 24 h to PFOA, GenX and PFO3TDA. Fold-change relative to untreated controls are given. Also shown is change in BCAA/AAA ratio, relative to controls, for each PFAS (p-values given for each).

Metabolite	Fold-Change, relative to Control (± s.d.) PFOA	PFO3TDA	GenX
Amino Acids			
AAA			
Phe	1.9 ± 0.3	1.7 ± 0.2	1.3 ± 0.3
Trp	1.7 ± 0.3	1.5 ± 0.3	1.2 ± 0.2
Tyr	1.5 ± 0.4	2.4 ± 0.5	1.5 ± 0.4
BCAA			
Leu	1.4 ± 0.2	1.0 ± 0.1	1.1 ± 0.1
Ile	1.6 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
Val	1.7 ± 0.2	1.4 ± 0.1	1.3 ± 0.1
Other			
Glu	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Gln	1.5 ± 0.2	1.6 ± 0.2	1.5 ± 0.2
Gly	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Ala	1.1 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
Asp	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
Cys	1.2 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Pro	2.6 ± 0.5	2.7 ± 0.4	1.8 ± 0.4
Neurometabolites			
GABA	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
NAA	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Oxidative Stress/Detoxification			
GSH	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
Tau	1.3 ± 0.2	1.5 ± 0.2	1.2 ± 0.2
TCA Cycle and Oxidative Phosphorylation			
Pyruvate	1.4 ± 0.2	1.4 ± 0.3	1.6 ± 0.3
Acetate	1.6 ± 0.4	2.0 ± 0.3	1.8 ± 0.3
Citrate	0.6 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Succinate	0.6 ± 0.1	0.2 ± 0.1	0.6 ± 0.2
Fumarate	1.0 ± 0.5	2.4 ± 1.0	1.5 ± 0.6
Malate	1.9 ± 0.3	1.4 ± 0.2	1.4 ± 0.3
ATP	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
ADP	0.7 ± 0.2	0.8 ± 0.1	0.6 ± 0.1
NADH	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
NAD+	1.9 ± 1.2	3.3 ± 2.0	2.0 ± 1.2
Carbohydrate Metabolism			
Glucose	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
G1P	1.2 ± 0.2	1.6 ± 0.2	1.1 ± 0.1
G6P	0.6 ± 0.1	1.2 ± 0.2	1.1 ± 0.3
Lactate	1.0 ± 0.2	0.7 ± 0.1	1.0 ± 0.2
Lipid Metabolism			
Cholesterol	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
Fatty Acid	1.1 ± 0.2	0.8 ± 0.1	1.1 ± 0.2
Carnitine	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
Phospholipid Polar Head Groups			
myo-Inositol	1.1 ± 0.2	1.5 ± 0.3	0.9 ± 0.2
GPC	1.8 ± 0.3	2.1 ± 0.3	1.3 ± 0.2
Choline	1.9 ± 0.3	2.2 ± 0.3	1.7 ± 0.3
Other			
TMAO	1.1 ± 0.2	0.8 ± 0.1	1.1 ± 0.1
Betaine	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.2

	Control	PFOA	PFO3TDA	GenX
BCAA/AAA	3.6	3.1	2.3	3.3
%Change	-	86%	64%	90%
p-value	-	< 0.005	< 0.000005	< 0.01

CHAPTER 3

Toxicity of perfluoroalkyl substances (PFAS) toward embryonic stages of mahi-mahi (*Coryphaena hippurus*)

(adapted from Gebreab, K., Benetti, D., Grosell, M., Stieglitz, J.D. and Berry, J.P., *Ecotoxicol.*, in press)

3.1 Abstract

Perfluoroalkyl substances (PFAS) are highly persistent organic pollutants that have been detected in a wide array of environmental matrices and, in turn, diverse biota including humans and wildlife wherein they have been associated with a multitude of toxic, and otherwise adverse effects, including ecosystem impacts. In the present study, we developed a toxicity assay for embryonic stages of mahi-mahi (*Coryphaena hippurus*), as an environmentally relevant pelagic fish species, and applied this assay to the evaluation of the toxicity of "legacy" and "next-generation" PFAS including, respectively, perfluorooctanoic acid (PFOA) and several *perfluoroethercarboxylic acids* (PFECA). Acute embryotoxicity, in the form of lethality, was measured for all five PFAS toward mahi-mahi embryos with median lethal concentrations (LC_{50}) in the micromolar range. Consistent with studies in other similar model systems, and specifically the zebrafish, embryotoxicity in mahi-mahi generally (1) correlated with fluoroalkyl chain length and hydrophobicity, i.e., log P, of PFAS, and thus, aligned with a role of uptake in the relative toxicity; and (2) increased with continuous exposure, suggesting a possible role of development stage specifically including a contribution of hatching (and loss of protective chorion) and/or differentiation of target systems (e.g., liver). Compared to prior studies in the zebrafish embryo model, mahi-mahi was significantly more sensitive to PFAS which may be related to differences in either exposure conditions (e.g., salinity) and uptake, or possibly differential susceptibility of relevant targets, for the two species. Moreover, when considered in the context of the previously reported concentration of PFAS within upper sea surface layers, and co-localization of buoyant eggs (i.e., embryos) and other early development stages (i.e., larvae, juveniles) of pelagic fish species to the sea surface, the

observed toxicity potentially aligns with environmentally relevant concentrations in these marine systems. Thus, impacts on ecosystems including, in particular, population recruitment are a possibility. The present study is the first to demonstrate embryotoxicity of PFAS in a pelagic marine fish species, and suggests that mahi-mahi represents a potentially informative, and moreover, environmentally relevant, ecotoxicological model for PFAS in marine systems.

3.2 Introduction

Perfluoroalkyl substances (PFAS) are environmental contaminants of growing scientific, health and regulatory interest, having been detected ubiquitously in diverse environmental matrices, and consequently, biota including plants, wildlife and humans (Giesy and Kannan 2001; Prevedouros et al. 2006; Wang et al. 2013). Owing to their surfactant properties (derived from their amphiphilic nature), and high chemical stability (due to their fluorocarbon backbone), PFAS have been extensively used over the past nearly 80 years in household products, cosmetics, firefighting foams, food packaging materials, and as water repellants for various consumer products (KemI 2015; Wang et al. 2013). Due, however, to this same chemical stability and amphiphilicity, PFAS are both highly persistent and bioaccumulative, and therefore, have been found to be widespread in the environment (Ankley et al. 2021; McCarthy et al. 2017). As the historically most widely used and, thus, both most environmentally prevalent, and best studied congeners, the so-called "legacy" PFAS including perfluorooctanoic acid (PFOA; Fig.1.1) and perfluorooctane sulfonate (PFOS) have been shown to exhibit considerable environmental persistence and bioaccumulation potential. And their presence in the environment has been putatively linked to adverse effects on human health including reproductive toxicity,

carcinogenicity, teratogenicity and possible neurotoxicity, as well as other organ-specific toxicity including liver (i.e., hepatotoxicity) and kidney (i.e., nephrotoxicity), and possible links to *metabolic syndrome* (Lau et al. 2007; Pérez et al. 2013; Prevedouros et al. 2006; Shrestha et al. 2017). In addition to human health concerns, a growing body of evidence including documented bioconcentration and bioaccumulation, and ecotoxicological assessments, points to potential impacts of PFAS on ecosystems (Ankley et al. 2021; Burkhard 2021).

Due to their prevalance in the environment, potential to bioaccumulate in biota, and associated health concern (Prevedouros et al. 2006; Wang et al. 2017), PFOA and PFOS have been largely phased-out from US and Europe, and replaced with several "next generation" alternatives. Most prominent of these alternatives are numerous *perfluoroether carboxylic acids* (PFECA; Fig. 3.1) that replace one or more fluorocarbon (-CF_2-) in perfluoroalkyl chains with ether oxygens (-O-), and thereby, retain similar physicochemical (i.e., surfactant, stability) properties. Perhaps the most notable examples of PFECA, in current use, include the proprietary compounds *GenX* (i.e., the ammonium salt of perfluoro (2-methyl-3-oxahexanoic), or hexafluoropropylene oxide dimer acid [HFPO-DA]) and ADONA™ (i.e., dodecafluoro-3H-4,8-dioxanonanoate). Although PFECA have been introduced, as replacements to legacy PFAS, in response to environmental health concerns (Bowman 2015), several recent toxicological studies have suggested quantitatively similar or, in some cases, greater toxicity and/or potential for bioaccumulation (Gomis et al. 2015; Wang et al. 2015; Gebreab et al., 2020). And PFECA, therefore, potentially represent "emerging" environmental toxicants of concern.

Of particular concern with regards to PFAS is contamination of aquatic systems including groundwater, freshwater bodies and marine systems (Xiao et al. 2017). Contamination of waterways not only raises concerns for human health (e.g., drinking water, contamination of seafood), but also has potential consequences for aquatic ecosystems and species. Therefore, a number of recent toxicological studies have utilized various aquatic vertebrate (i.e., fish) and invertebrate species to investigate potential ecotoxicity of PFAS (Giesy et al. 2010; McCarthy et al. 2017). Of the studies to investigate PFAS toxicity, those employing the zebrafish (*Danio rerio*), a well-established laboratory model, including both adult (Jantzen et al. 2016) and early life (i.e., embryos, larvae) stages are particularly notable. The zebrafish *embryo* model, in particular, has been employed to evaluate a wide range of legacy (i.e., PFOA, PFOS) and next-generation PFAS including PFECA (Shi et al. 2008; Ye et al. 2009; Hagenaars et al. 2011; Zheng et al. 2012; Jantzen et al. 2016; Godfrey et al. 2017; Weiss-Errico et al. 2017; Annunziato et al. 2019; Gebreab et al. 2020; Menger et al. 2020; Pecquet et al. 2020; Rericha et al. 2021; Wasel et al. 2021). While the zebrafish embryo model has been promoted as a general proxy for vertebrate toxicity, and as such, a possible model for human health (Bambino and Chu 2017; Bradford et al. 2017), toxicological studies in zebrafish, as a representative teleost fish species, also have potentially *direct* relevance to the impact of toxicants on aquatic biota. Toxicity studies of the early life (i.e., embryos, larvae) stages of fish, moreover, have specific *ecotoxicological* relevance as toxicity with respect to these development stages reveal implications for population recruitment, and in turn, aquatic ecosystems as a whole.

This said, while prior studies with well-established *laboratory* animal models, such as the zebrafish embryo system, have provided important insight to the potential

ecotoxicity of PFAS, such studies have perhaps limited applicability to wild-type ecological receptors in terms of both species, and moreover, as genetically non-wild type (i.e., laboratory bred) representatives of the species. In general, relatively few studies have assessed toxicity in *marine* species, compared to studies of freshwater species, and even fewer have focused on marine *vertebrates*. Indeed, a very recent and thorough review (Ankley et al. 2021) of the literature found that <5% of published toxicology studies cited marine (versus freshwater) fish. And among the limited studies that have investigated "marine" fish, most have actually focused on *anadromous* rainbow trout (*Oncorhynchyus mykiss* (Palmer et al. 2002a; Robertson and Gaines 1986), and *estuarine* species of sheepshead minnow (*Cyprinodon variegatus*; (Palmer et al. 2002b) and medaka (*Oryzias melastigma*; (Fang et al. 2014; Huang et al. 2011). The few studies that have evaluated truly marine species (i.e., rockfish, eel, cod; (Ankley et al. 2021) have generally measured sub-acute physiological effects on adult stages.

Impacts on early life stages of strictly marine (e.g., pelagic) fish may be especially relevant based on their natural history: while concentrations of PFAS in marine waters are typically low, and specifically below parts-per-trillion (ppt), recent studies have shown significant enrichment at or near the air-sea interface including, in particular, the *sea surface microlayer* (SSML) where buoyant, free-floating eggs (and embryos) of many marine fish species are localized. Ju et al. (2008), for example, reported an approximately 1.5-fold increase of PFOS and PFOA in surface water (< 20 cm) compared to subsurface water (> 30 cm), but as much as a 100-fold or more enrichment within the SSML, compared to subsurface water. More recently, Casas et al. (2020) similarly reported enrichment of a wide range of PFAS in the surface layer of near-shore waters, and although enrichment

factors for SSML were only up to 5-fold relative to seawater in this study, a nearly 5000-fold enrichment was observed for seawater aerosols, similarly suggesting a mechanism for surface concentration of PFAS.

To fill gaps in knowledge with respect to the ecotoxicology of PFAS in marine ecosystems, we developed an assay system based on early life (i.e., embryo) stages of the marine, ecologically, and commercially relevant fish species, mahi-mahi (*Coryphaena hippurus*). Mahi-mahi is a pelagic, high-trophic level, highly migratory species dispersed throughout the world's tropical and subtropical seas and oceans (Maggio et al. 2019; Palko et al. 1982; Perrichon et al. 2019), and highly valued in both sports fishing and commercial fisheries (Oxenford and Hunte 1999). Like many other pelagic marine fish, the species is characterized by both frequent spawning, and high fecundity (Beardsley Jr 1967; Maggio et al. 2019), with positively buoyant eggs localized to the surface of the water column (Perrichon et al. 2019), and embryos characterized by high growth and metabolic rates (Pasparakis et al. 2016). As an ecologically relevant species, early life stages of mahi-mahi have been recently developed as a model for environmental toxicology, and specifically demonstrated in relation to toxicity of crude oil fractions in association with oil spills in the Gulf of Mexico (Edmunds et al. 2015; Esbaugh et al. 2016; Heuer et al. 2019; Kirby et al. 2019; Nelson et al. 2016; Perrichon et al. 2018; Stieglitz et al. 2016). In the present study, we utilized assays based on the mahi-mahi embryo model to evaluate acute embryotoxicity of a representative sample (Fig.1.2) of both legacy PFAS (i.e., PFOA), and several PFECA (Fig.1.2) as emerging toxicants of concern. Embryotoxicity in mahi-mahi is compared, in turn, to our previously evaluated (Gebreab et al. 2020) toxicity of these same compounds in the zebrafish embryo model.

3.3 Materials and methods

3.3.1 Chemicals

Perfluorooctanoic acid (PFOA, 96% purity) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), and PFECA including perfluoro-3,6,9-trioxatridecanoic acid (PFO3TDA, 98% purity), perfluoro-3,6-dioxadecanoic acid (PFO2DA, 97% purity), 4-(heptafluoroisopropoxy) acid (PFDMMOBA) and perfluoro (2-methyl-3-oxahexanoic) acid (i.e., HFPO-DA, 97% purity) were purchased from SynQuest Laboratories (Dallas, TX U.S.A.). All chemicals were utilized without further purification. Stock solutions of PFECAs and PFOA were prepared in bio-filtered seawater (i.e., hatchery system water; see *Mahi-mahi rearing and breeding/spawning*) in polypropropylene tubes (to prevent adsorption to glass(Shafique et al. 2017), and sonicated until complete dissolution of the compounds was achieved. Stock solutions were diluted in filtered seawater over a relevant range of concentrations for assessment of mahi-mahi embryo toxicity (see *Mahi-mahi embryo toxicity assays*).

3.3.2 Mahi-mahi rearing and breeding/spawning

Embryos of mahi-mahi (*Coryphaena hippurus*) were obtained from wild-caught broodstock spawned in the University of Miami Experimental Hatchery (UMEH). The wild-caught fish were collected from water offshore of Miami, Florida and were transported, acclimated, and spawned according to protocols detailed by Stieglitz et al. (2017). Broodstocks of mahi-mahi were maintained in two 15,000-L fiberglass tanks outfitted with filtered and UV-sterilized seawater inflows (25–28 °C), and fed daily with a mixture of chopped squid, mackerel, and sardines (Kloeblen et al. 2018; Stieglitz et al.

2017). The seawater used for maintaining the mahi-mahi, and subsequent assays, was specifically pumped from nearby Bear Cut (Miami, Florida) through a series of settling tanks, sand filters, and bag filters before lastly passing through a UV-sterilizer prior to introduction into the fish rearing tanks. The filtered/sterilized seawater was assessed for PFAS including PFECA and PFOA by high-performance liquid chromatography/mass spectrometry (HPLC-MS; see *HPLC-MS measurement of PFAS in seawater medium*, below), and found in all cases (Supplementary Table B1) to be present at concentrations below 1 part-per-billion (ppb), and < 0.1% of the nominal exposure concentration range of toxicity assays. Captive spawning populations are maintained at a sex ratio of 1 male to 1 - 4 females. Fertilized eggs (i.e., embryos) produced from the volitional spawning events were collected 4 – 6 hours post-spawning, rinsed in UV-sterilized seawater, and transported in a cooler (~30 min transit time) to FIU laboratories for exposure and toxicity testing. All breeding of mahi-mahi was conducted under protocols approved by the University of Miami Institutional Animal Care and Use Committee (IACUC-18-052 LF).

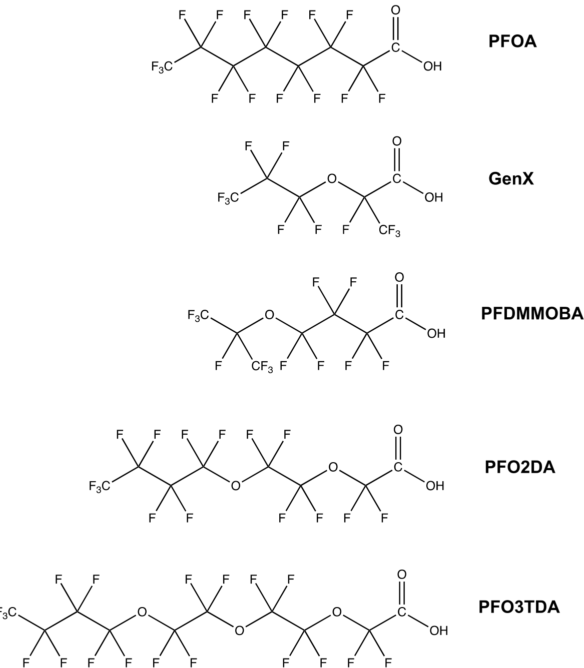

Figure 3. 1 Perfluoralkyl substances investigated in the current study, including PFOA (A) and the perfluoroether carboxylic acids, GenX (B), PFDMMOBA (C), PFO2DA (D) and PFO3TDA (E)

3.3.3 Mahi-mahi embryo toxicity assay

An assay to evaluate embryotoxicity of PFAS toward mahi-mahi embryos was developed, and specifically adapted from assay formats previously developed and validated for zebrafish embryotoxicity of PFAS (Berry et al. 2007; Gebreab et al. 2020; Weiss-Errico et al. 2017). Preliminary trials were conducted to establish a suitable exposure parameter including concentration range for each compound, and identified an effective percent weight (i.e., parts per million) concentration range of 1, 5, 10, 20, 30, 50, 100, 150, 200 and 250 parts-per-million (ppm) for all PFAS. In addition, toxicity (and specifically, lethality) was observed at 24 and 48 hours post-fertilization (hpf), corresponding to pre- and post-hatch embryos, respectively. Test solutions for exposures were prepared to the aforementioned nominal concentrations by dilution of sonicated

stocks (in filtered seawater in polypropylene tubes), and 1 mL of each was transferred to corresponding wells of 24-well polypropylene test plates with 6 wells (n = 6) for each treatment concentration, along with 6 control wells (on each assay plate) of filtered seawater only. Each treatment was tested in triplicate. Prior to assays, embryos in stock plates (< 8 h post-spawning) were evaluated using a dissecting microscope, and all unhealthy (i.e., dead, moribund or deformed) embryos were removed; only clearly viable embryos were, thus, selected for assays. For assays, one embryo was distributed into each well of 24-well test plates, and assay plates were maintained within an environmental control chamber at 27°C and 16:8 dark/light for 48 h. Plates were observed during static exposure to PFAS using a dissection microscope at 24 and 48 hpf, and lethality (based on mobility, response to stimuli and presence/absence of heartbeat) recorded as an endpoint of toxicity. All toxicity assays were performed under protocols approved by the Florida International University's IACUC (IACUC-19-085), and by trained investigators.

3.3.4 HPLC-MS analysis of PFAS in seawater medium

High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis of PFAS was performed according to a standardized and validated method (Li et al., 2022) using an Agilent 1290 Infinity II LC system connected to an Agilent 6470 Triple Quadrupole LC-MS/MS system equipped with an Agilent Jet Stream electrospray ionization source (Agilent 1200 Series; Agilent Technologies, Palo Alto, CA, USA). The LC was modified with PFAS-free tubing to avoid potential contamination. A delay column (Hypersil GOLD aQ C18, 20 × 2.1 mm, 12 μm) was placed between the mobile phase mixer and the sample injector. Then, PFASs were separated on a Hypersil GOLD

perfluorinated phenyl (PFP) column (150 × 2.1 mm, 3 μm) with a PFP guard column (Hypersil GOLD PFP 5 μm drop-in guards) at 50 °C with 5 mM ammonium formate and methanol, as mobile phases, at a flow rate of 0.4 mL/min. The gradient used for LC is given in Table B.2. The MS settings of the LC-MS/MS instrument included a drying gas temperature of 150 °C, a gas flow of 10 L/min, a nebulizer pressure of 15 psi, a sheath gas temperature of 300 °C, a sheath gas flow of 10 L/min, a capillary voltage of 2,000 V, a nozzle voltage of 0 V, and a cycle time of 500 ms. Sample acquisition was performed using a multiple-reaction monitoring (MRM) method in negative mode for the concurrent quantification of PFASs.

To assess *actual* concentrations, relative to *nominal* concentrations, of PFAS in assay exposure solutions, similarly prepared 1-ppm and 1-ppb solutions of PFECA and PFOA, as well as unadulterated seawater medium, were analyzed for PFAS by previously validated HPLC-MS method (Li et al., 2022). For both assessments, replicates of seawater (n=4), and prepared solutions (n=2), were filtered and analyzed directly. PFAS analysis was performed using an Agilent 1290 Infinity II LC system, modified with PFAS-free tubing to avoid potential contamination, connected to an Agilent 6470 Triple Quadrupole LC-MS/MS system equipped with an Agilent Jet Stream electrospray ionization source (Agilent 1200 Series; Agilent Technologies, Palo Alto, CA, USA). Detail of analysis are given in the Supplementary Information.

3.3.5 Data analysis

Median lethal concentrations (LC_{50}), and their 95% confidence intervals, for each (of 3) treatment replicates were calculated by Probit Analysis in SPSS (version 26.0; IBM

Corporation Armonk, NY, USA, 2015), as well as for pooled data (from triplicate measurements). Lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) were calculated for pooled data (i.e., mean percent survival from triplicate measurements of each treatment) by comparison to untreated (negative) controls using a t-test. Sigmoidal fit for concentration-response curves, based on pooled data from triplicates, were performed using origin 2019b (Origin Lab, Northampton, Massachusetts, USA). One-way ANOVA, followed by Tukey's post hoc test, was used to calculate the statistical significance of the LC_{50} values with significance level of $p < 0.05$.

3.4 Results and discussion

The current study is the first report of embryotoxicity of PFAS in a pelagic marine fish species. Dose-dependent embryotoxicity (Figure.3.2), and specifically, lethality, was observed within 24 for static exposure of mahi-mahi embryos to all PFAS evaluated in the nominal 10-100 ppm range (Table 3.1). Measured toxicity in the mahi-mahi embryo, thus, generally aligns with previous studies in the zebrafish model which have reported quantitatively similar embryotoxicity for both legacy PFAS (i.e., PFOA and PFOS) and PFECA (Gaballah et al. 2020; Gebreab et al. 2020; Godfrey et al. 2017; Hagenaars et al. 2011; Jantzen et al. 2016; Pecquet et al. 2020; Shi et al. 2008; Weiss-Errico et al. 2017; Ye et al. 2009; Zheng et al. 2012). Observations in both the present (i.e. mahi-mahi) and past (i.e., zebrafish) studies are notable as PFECA have been adopted as purportedly less toxic alternatives (Bowman 2015), and these results, thereby, agree with prior studies which have concluded that toxicity of these replacement compounds is, on the contrary,

potentially comparable to legacy PFAS (Gaballah et al. 2020; Gebreab et al. 2020).

Table 3. 1 Relevant measures of embryotoxicity including median lethal concentration (LC50), no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL), and comparison of median lethal concentrations to zebrafish embryo model (ZF LC50).

	24 hpf			**48 hpf [a]**			**ZF LC50 [95% CI][a,c]**	
	LC_{50} [95% CI]	**NOAEL[b]**	**LOAEL**	**LC_{50} [95% CI]**	**NOAEL[b]**	**LOAEL**	**24 hpf**	**7 dpf**
Compound	**(ppm)**	**(ppm)**	**(ppm)**	**(ppm)**	**(ppm)**	**(ppm)**	**(ppm)**	**(ppm)**
PFOA	55 [40,73]	10	20	4 [2,6]*	1	5	96 [82,111]	51 [43,59]*
HFPO-DA	84 [64,106]	--	100	20 [11,31]*	--	100	127 [109,147]	84 [72,99] *
PFDMMOBA	17 [9,28]	5	10	10 [4,19]	5	10	95 [81,110]	80 [68,95]
PFO2DA	34 [15,69]	5	10	1.1 [0.2,3.8]*	--	1	71 [62,82]	11 [8,15]*
PFO3TDA	19 [9,35]	5	10	7 [4,10]	1	5	22 [18,26]	10 [7,14]*

[a] Statistical significance of difference between LC_{50} at 24 and 48 hpf for mahi-mahi, and 24 hpf and 7 dpf for zebrafish, based on 95% confidence interval ($p < 0.05$), is indicated by asterisk (*)
[b] "--" indicates that NOAEL was below the exposure concentration range.
[c] Zebrafish LC_{50} values from Gebreab et al. (2020).

Relative embryotoxicity for mahi-mahi, based on calculated 24-h LC_{50} values (converted to micromolar concentrations for mole-to-mole comparison, Figure. 3.2 and 3.3), was found to be HFPO-DA < PFOA < PFO2DA < PFDMMOBA < PFO3TDA, and was generally correlated with PFAS chain length (inclusive of all fluorocarbon and ether groups) with the notable exception of the relatively high toxicity of PFDMMOB. Indeed, LC_{50} was significantly correlated ($p < 0.001$) when PFMMOBA was not included (Figure. 3.3). This trend and, furthermore, the exception of the PFMMOBA is noteworthy as a similar significant correlation between embryotoxicity and chain length of PFAS has been

consistently demonstrated in numerous, previous studies in the zebrafish embryo model (Buhrke et al. 2013; Gaballah et al. 2020; Menger et al. 2020; Ulhaq et al. 2013; Wasel et al. 2021) including a recent assessment of the same PFECA (Gebreab et al. 2020; see Table 3.1). These studies collectively demonstrate a highly reproducible, quantitative toxicity of PFAS in embryonic fish models. It has, likewise, been recently shown that bioconcentration factors (BCF) of PFAS (alongside relative toxicity) in zebrafish are, likewise, significantly correlated with chain length, and concluded that toxic potential is likely correlated with relative uptake potential (Menger et al. 2020; Vogs et al. 2019). Moreover, a recent metabolomics analysis in the zebrafish embryo model (Gebreab et al. 2020) identified nearly identical patterns of altered metabolic profiles between embryos exposed to both PFOA, and long- and short-chain PFECA (i.e., PFO3TDA and HFPO-DA, respectively). This observation suggests shared mechanistic pathways of toxicity, despite observed quantitative differences, and correlations between embryotoxicity and chain-length, and thus, further supports a role of differential uptake (rather than difference in mechanism) in the relative toxicity of perfluorocarboxylic acids. Relative uptake is presumably a function of hydrophobicity, and indeed, a similar correlation between toxicity and calculated log P values for PFAS (with similar exception of PFDMMOBA) was observed in the present study (Figure.3.3). That said, while hydrophobicity and, in turn, uptake, of PFAS is expected to primarily increase with chain length,

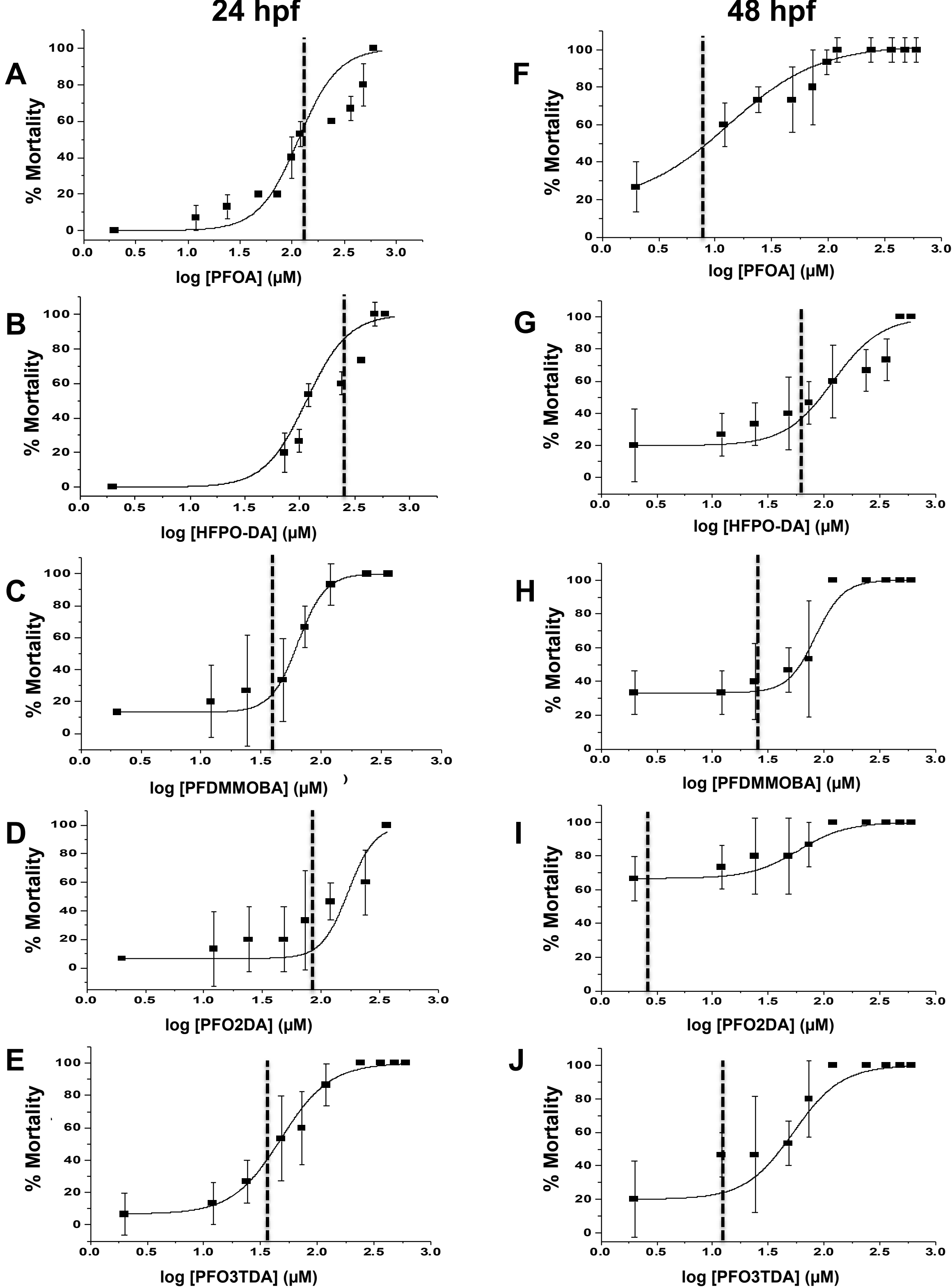
24 hpf
48 hpf
A
B
C
D
E
F
G
H
I
J
% Mortality
log [PFOA] (μM)
log [HFPO-DA] (μM)
log [PFDMMOBA] (μM)
log [PFO2DA] (μM)
log [PFO3TDA] (μM)

Figure 3. 2 Concentration-dependent toxicity of PFOA, HFPO-DA, PFDMMOBA, PFO2DA and PFO3TDA at 24 hpf (A-E, respectively) and 48 hpf (F-J, respectively). Mean percent mortality pooled for each concentration from triplicate measurements. Error bar represents ± standard deviation (n = 3). Concentrations converted to molar concentrations (µM) for mole-to-mole comparison of PFAS; relevant values (i.e., LC50 and NOAEL/LOAEL) are given in ppm units in Table 3.1.

previous studies have suggested other structural features including, a role of terminal functional groups (e.g., carboxylic versus sulfonic acids) in the relative uptake and, thus, toxic potential (Menger et al. 2020; Vogs et al. 2019). Thus, correlations as observed here are likely to only apply to perfluorinated carboxylic acids.

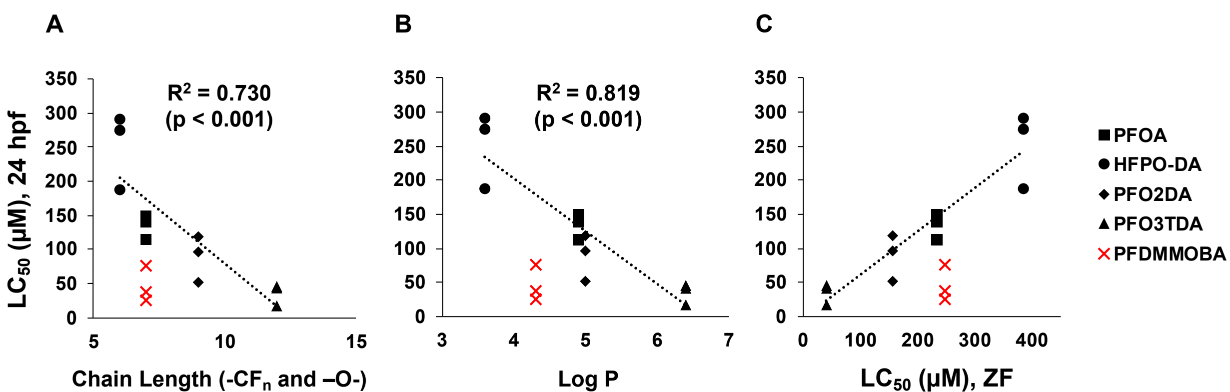

Figure 3. 3 Correlation between mahi-mahi 24-h embryotoxicity, as measured by median lethal concentration (LC50), and (A) PFAS fluoroetheralkyl chain-length, (B) log P, and (C) embryotoxicity, i.e., 24-h LC50, previously reported for zebrafish (Gebreab et al. 2020). Log P values from National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/, retrieved June 22, 2021). Chain-length inclusive of all fluorocarbons, ethers and terminal carboxylic acid group. Concentrations converted to molar concentrations (µM) for mole-to-mole comparison of PFAS; relevant values (i.e., LC50 and NOAEL/LOAEL) are given in ppm units in Table 3.1.

Although the reason for the higher than expected toxicity of PFDMMOBA in the present study is unclear, and remains to be investigated further, it is worth noting that this congener is among the only two (with the other being the most lipophilic, long-chain PFO3TDA) for which LC_{50} and LOAEL/NOAEL (Table 1) did not significantly change

following an additional 24 h of exposure (discussed further below). And, in fact, a similar lack of significant increase in toxicity during an equivalent development window (7 dpf, i.e., fully hatched, early larval stage) was, likewise, observed for PFDMMOBA in a previous study of zebrafish embryotoxicity (Gebreab et al. 2020). It is, therefore, proposed that more rapid uptake may explain higher than expected toxicity observed for this congener.

With the exception of PFDMMOBA, as well as most toxic PFO3TDA, lethality significantly increased for all compounds tested over 48 h of static exposure, as evidenced by significantly ($p < 0.05$) lower LC_{50}, and NOAEL/LOAEL (Table 3.1). Similarly increased toxicity, based on both lethality and other toxicological endpoints, with continuous exposure has been observed in previous studies of PFAS including PFOA and PFECA in zebrafish embryos (Gebreab et al. 2020; Hagenaars et al. 2011; Ye et al. 2009; Zheng et al. 2012). With respect to developing embryos, increased toxicity at 48 h is notable as mahi-mahi embryos typically hatch within approximately 36-48 hpf (Perrichon et al. 2019), and nearly all (96%) of the embryos in the current study were, in fact, fully hatched at 48 h. Increased toxicity, therefore, may be associated with loss of the protective chorion as a barrier for uptake of PFAS. A role of the chorion would, in turn, have ecotoxicological implications for environmental exposure of post-hatch eleutheroembryos and larvae of mahi-mahi. A similarly significant increase in toxicity coincident with hatching (~ 72 hpf) has previously been observed for the same compounds (except PFMDMMOBA) in zebrafish (Gebreab et al. 2020). A recent study (Vogs et al. 2019) has documented a “biphasic” pattern of slower uptake prior to hatching, and accelerated uptake post-hatch, in the zebrafish embryo model generally concluding that the chorion, indeed,

serves as a protective barrier. The current results are consistent with such a bi-phasic pattern, yet reflect differential effects of the chorion for PFAS congeners: specifically, rapid uptake (and little effect of chorion) of PFO3TDA and PFDMMOBA is seemingly reflected in the observation of a near maximum toxicity of these congeners within 24 h, whereas the chorion limits uptake of PFOA, HFPO-DA and PFO2DA until hatching. Notably, the correlation between toxicity and chain-length is not maintained after 48 h exposure (Appendix Figure B2), and the differential role of the chorion may, furthermore, explain this lack of correlation: in short, as hindrance of the chorion does not exist post-hatch, equivalent uptake of congeners leads to a convergence of lethal concentrations (in the low ppm range) after hatching.

Alternatively, it is possible that increased toxicity with exposure time might relate, at least in part, to the development of relevant cellular and molecular targets of PFAS. As recognized hepatotoxins, for example, both liver (i.e., hepatocytes) and enzymes associated with phase I hepatic detoxification including, in particular, cytochrome P450 have been well documented as targets of PFAS (Bassler et al. 2019; Cheng and Klaassen 2008; Dale et al. 2020). Studies in the zebrafish have recently suggested a role of the differentiation of the liver and hepatic enzymes in the observed stage-dependent increase in embryotoxicity of both PFOA and PFECA (Gebreab et al. 2020), as well as other hepatotoxins (Zuberi et al. 2019). Expression of genes associated with differentiation of hepatocytes, and associated liver enzymes, in mahi-mahi has been similarly found (Xu et al. 2017) to occur over a timeframe (i.e., 36 to 48 hpf) coincident with increased embryotoxicity in the current study, suggesting a possibly similar contribution of hepatic development in this toxicity. Elevated mortality may, of course, be simply due to the cumulative exposure to the

compounds during the continuous exposure period. Whether the observed increased toxicity is due to the loss of the protective chorion barrier, development of the liver (or possibly other targets), or simply, to the prolonged duration (and cumulative effects) of exposure remains to be clarified.

Although observed toxicity of PFOA and PFECA in the mahi-mahi embryo system was generally comparable (i.e., ppm range) to that previously observed in the zebrafish model (Table 3.1), and similarly, significantly correlated fluoroalkyl chain length (with the exception of PFDMMOBA; Figure.3.3), comparison of the current data to our previous assessments of the same compounds in the zebrafish embryo model (Gebreab et al. 2020) demonstrated consistently higher toxicity, as evidenced by LC50 and LOAEL values (Table 3.1). The consensus of previous studies (Gaballah et al. 2020; Godfrey et al. 2017; Hagenaars et al. 2011; Pecquet et al. 2020; Ye et al. 2009; Zheng et al. 2012) of PFAS including PFOA and PFECA in the zebrafish embryo model have, likewise, generally observed lower toxicity, i.e., LC_{50}, compared to that currently reported for mahi-mahi. Higher relative toxicity for mahi-mahi, compared to zebrafish, may be related to a number of factors including differences in toxicokinetics (i.e., uptake) and susceptibility of relevant biochemical, molecular or cellular targets between the two species, as well as respective assay parameters including, in particular, exposure media (e.g., seawater versus non-saline medium, pH, etc.). A positive correlation between salinity and BCF in fish has, for example, been previously demonstrated (Jeon et al. 2010), suggesting a possible role of both exposure medium (i.e., salinity), and consequent toxicokinetics, in the higher toxicity among mahi-mahi embryos. Alternatively, however, higher sensitivity of mahi-mahi may relate to the targeting of interrelated pathways of cellular energy metabolism by PFAS.

Numerous previous studies have implicated metabolic dysfunction including lipid, amino acid and carbohydrate among the adverse effects of PFOA and PFECA including early life (i.e., embryo and larval) stages of zebrafish (Yu et al. 2016; Alderete et al. 2019; Chen et al. 2020; Gebreab et al. 2020; Sant et al. 2021). At the same time, it has been shown that rapidly developing mahi-mahi embryos are among the most metabolically active of marine fish species (Pasparakis et al. 2016), and much higher than zebrafish embryos. Taken together, it is possible that targeting of energy metabolism by PFAS may accentuate toxicity in mahi-mahi embryos, alongside any contributions of differential uptake and toxicokinetics.

Although, in the present study, the concentration of PFAS was not directly measured in exposure media of assays, concentrations of PFECA and PFOA in the seawater medium (utilized in assays studies) were subsequently assessed by HPLC-MS. For 1-ppm and 1-ppb solutions, concentrations measured by HPLC-MS were substantially lower than nominal concentrations, ranging from as low as 7-52% and 4-77%, respectively (Appendix Table B1). Aligned with these observations, a recent study (Menger et al., 2020) of bioaccumulation and toxicity in the zebrafish embryo model has, in fact, similarly measured concentrations which were consistently < 50% of nominal concentration for 4- to 8-fluorocarbon PFAS (including PFOA), and specifically suggested that high potential of sorption of PFAS to surfaces was likely responsible for concentrations measured in exposure solutions. Both studies, thus, indicate that nominal exposure concentrations likely over-estimate toxic concentrations, and under-estimate toxicity, substantially. Notably, the percent recovery of PFECA from seawater in the present study was significantly correlated with fluoroalkyl chain length (Appendix Figure B2), suggesting a

likely even greater reduction in exposure concentration for long-chain PFAS, and further supporting a possible role of sorption (due to the fluoroalkyl chain). Taken together, these observations suggest that effective toxic concentrations (i.e., LC_{50}, LOAEL/NOAEL) calculated from nominal concentrations, therefore, may represent significant over-estimates of actual concentrations, and in the case of the PFECA and PFOA evaluated in the current study, actual toxic concentrations may be as much 100-fold lower (i.e., ppb concentrations) than nominal values reported (Table 3.1).

Low toxic concentrations, and correspondingly high toxicity in early life stages of mahi-mahi, measured in the present study may have implications for potential exposure of embryos to toxic concentrations of PFAS in ecologically relevant (i.e., marine) waters. Indeed, a lingering ecotoxicological question, in this regard, is whether environmentally relevant concentrations of PFAS in aquatic systems are sufficient for toxicity. With 48-h LOAEL (for lethality) in the low ppm range for PFAS (Table 3.1), it is possible that sub-acute toxicity may, indeed, effectively extend into the parts-per-billion range, particularly since calculated values (as discussed above) may represent significant over-estimates. While typically in the sub-ppt range, PFAS concentrations approaching nanomolar concentrations (e.g., 200 ppt PFOA) have been measured, particularly in nearshore marine waters (Yamashita et al. 2004). Moreover, 100-fold concentration factors of PFAS within the SSML – and as much as 5000-fold in aerosols – have been reported (Casas et al. 2020; Ju et al. 2008), suggesting potentially ppb concentrations within ecologically relevant upper-layer surface waters where buoyant eggs (i.e., embryos) and larval stages of mahi-mahi, and many other marine fish species, are distributed. Whether these early life stages

of marine fish are, in fact, exposed to effectively toxic concentrations, however, remains to be investigated in future studies.

3.5 Conclusion

In conclusion, embryos of mahi-mahi, as a representative pelagic marine fish species, were found to be a quantitative model of the toxicity of PFAS, largely comparable in this regard to the zebrafish embryo as an established laboratory model. Alongside quantitative potential of this model with respect to structure-activity (i.e., chain-length and relative toxicity), these studies point to interactive effects of uptake and development stage (e.g., hatching/loss of chorion, development of relevant target organs). Moreover, these studies identified toxicity at exposure concentrations sufficiently low to approach environmentally relevant concentrations, particularly in marine waters, and especially the sea surface where both PFAS and buoyant eggs of many marine fish species are generally concentrated. These findings provide a baseline of toxicity in early life stages of marine fish species and opens the door to future studies to evaluate ecotoxicological impacts of PFAS on marine fish populations.

3.6 References

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3.7 Appendix B

Table. B1 Concentration of PFOA and PFECA in filtered seawater (n = 4) used to rear and spawn mahi-mahi, and as exposure medium for assays, and concentrations measured (n =2) for solutions of PFOA and PFECA with nominal concentrations of 1 ppm and 1 ppb. PFAS were measured by HPLC-MS using the method of Li et al. (2022). All concentrations are given in ppm, aligned with nominal exposure concentration range of toxicity assays.

	Seawater **[PFAS], ppm (± s.d.)**	***1 ppm* solution in seawater** **[PFAS], ppm (± s.d.)**	***1 ppb* solution in seawater** **[PFAS], ppb (± s.d.)**
PFOA	0.00025 (± 0.00046)	0.52 (±0.06)	0.18 (±0.13)
HFPO-DA	0.000013 (± 0.000025)	n.d.[a]	0.77 (±0.03)
PFDMMOBA	0.000026 (± 0.000026)	0.17 (±0.01)	0.28 (±0.01)
PFO2DA	0.000043 (± 0.000043)	0.07 (±0.01)	0.12 (±0.01)
PFO3TDA	0.00043 (± 0.00049)	0.07 (±0.01)	0.04 (±0.01)

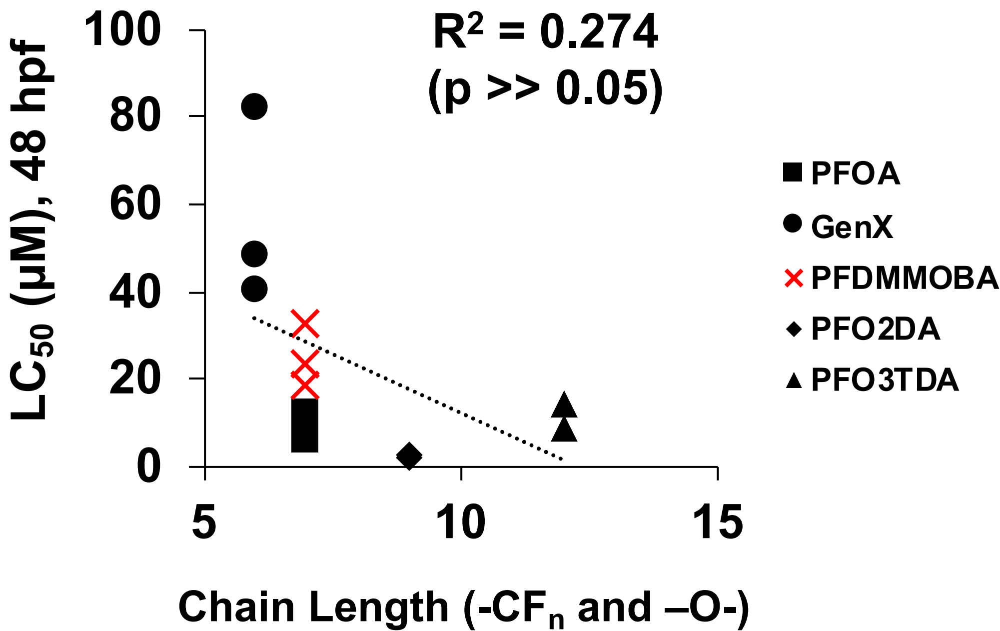

Figure.B1 Correlation between mahi-mahi 48-h embryotoxicity, as measured by median lethal concentration (LC_{50}), and PFAS fluoroetheralkyl chain-length. Chain-length inclusive of all fluorocarbons, ethers and terminal carboxylic acid group.

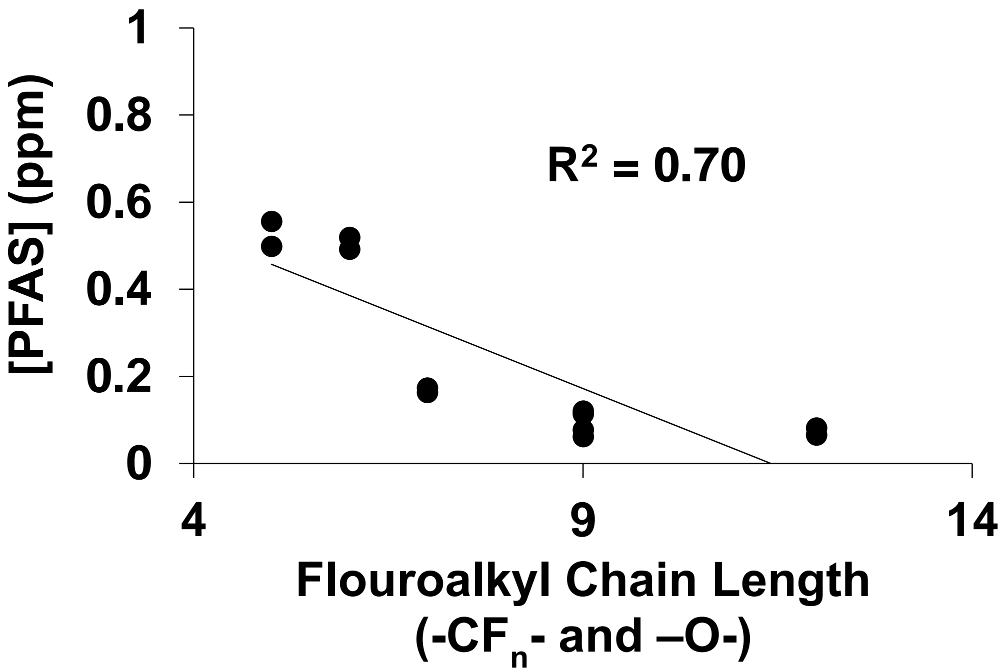

Figure. B2 Correlation between concentration of six PFECA measured by HPLC-MS for a solution with nominal concentration of 1 ppm in seawater medium, and fluoroetheralkyl chain length. Chain-length inclusive of all fluorocarbons, ethers and terminal carboxylic acid group. PFECA measured include PFDMMOBA, PFO2DA and PFO3TDA (evaluated for toxicity in the present study; see main text), and perfluoro-4-methoxybutanoic acid (PFMOBA), perfluoro-3,6-dioxaheptanoic acid (PFO2HPA) and perfluoro-3,6,9-trioxadecanoic acid (PFO3DA).

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CHAPTER 4

Bioconcentration of Legacy and Next-Generation Perfluoroalkyl Substances (PFAS) in Early Life Stages of Zebrafish (*Danio rerio*) and Olive Flounder (*Paralichthys olivaceus*)

4.1Abstract

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic organic surfactants that have emerged as environmental contaminants of concern, owing to their widespread industrial use, and consequent prevalence and persistence in the environment, as well as potential for bioaccumulation, and demonstrated toxicity. Given their nearly ubiquitous presence in aqueous matrices, ranging from groundwater and surface water to marine and freshwater bodies, the impact of PFAS on aquatic organisms and ecosystems has been increasingly found to be of particular concern. The aim of this study is to investigate the bioconcentration potential of the legacy PFAS, perfluorooctanoic acid (PFOA), and moreover, perfluoroether carboxylic acids (PFECA) - as "next generation" PFAS - in early life (i.e., embryonic) stages of representative freshwater and marine fish, and specifically the zebrafish (*Danio rerio*) and Olive Flounder (*Paralichthys olivaceus*), respectively. Liquid chromatography/mass spectrometry (LC-MS) was used to measure PFAS including PFOA, and seven representative PFECA, in experimentally exposed embryos, and determine bioconcentration factors (BCF), as a relevant hazard assessment metric of environmental uptake and exposure, for both species. Bioconcentration (BCF > 1) was observed for all PFAS in both species with values ranging from 22- to more than 300-fold. Notably, BCF values were consistently higher for flounder compared to zebrafish. Bioconcentration was observed at both high (1 ppm) and low (1 ppb) nominal exposure concentrations and based on measured exposure concentrations suggest that bioconcentration can occur at environmentally relevant concentrations. Calculated BCF values were correlated with fluoroalkly chain length, and corresponding lipophilicity (log P) values, aligned with previously observed correlation between chain length, and

lipophilicity, and embryotoxicity in the zebrafish embryo model. Notably, the bioconcentration and toxicity of PFECA, as next-generation PFAS replacements, and PFOA was comparable overall. This is notable as PFECA, and other alternatives have been introduced as less toxic and safer replacements to bioaccumulative and toxic legacy PFAS. As the first study to demonstrate bioconcentration in an ecologically and commercially relevant marine fish species, these results provide framework for assessing impacts of PFAS on aquatic habitats and biota.

4.2 Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic organofluorinated compounds that have been widely used since the 1950s. Chemically, PFASs are characterized by short (4 to 6 $–CF_n$-) or long (> 6 $–CF_n$-), hydrophobic fluorocarbon moieties, coupled to polar headgroups imparting amphilic, and thus, surfactant, properties. In addition, strong C–F bonds make PFAS resistant to chemical degradation including photolysis and hydrolysis, and biological and thermal degradation (Prevedouros et al., 2006; Wang et al., 2015). Their unique surfactant properties, alongside their high stability, have allowed them to be used in a myriad of applications ranging from cosmetics, food packaging and various household products including water- and stain-resistant clothing, carpeting and upholstery to firefighting foam, plastics and various industrial applicatoins (Prevedouros et al., 2006; Wang et al., 2013). Because of their wide use, and consequently prevalent distribution in the environment, however, they have been detected in diverse plant and animal wildlife, as well as humans (Giesy & Kannan, 2001; Martin et al., 2003; Prevedouros et al., 2006; Wang et al., 2017).

Due to their stability (and consequent persistence in the environment) and potential bioaccumulation, as well as documented toxicity, and links to adverse health effects, many of the so-called "legacy" long-chain PFAS have been phased out – as part of the 2009 Stockholm Convention - and replaced by short-chain PFAS and other alternatives, such as perfluoroether carboxylic acids (PFECA) and perfluoroether sulfonic acids (PFESAs) (Giesy & Kannan, 2001; Cadore et al., 2009; EPA, 2010; Pérez et al., 2013; Wang et al., 2013). Among the legacy PFAS, two of the most widely used compounds, namely perfluorooctanoic acid (PFOA) and perfluorosulfonic acid (PFOS), starting in the early 2000s, were phased-out and replaced with a short-chain analogs, as well as other chemically modified variants.

Perfluoroether carboxylic acids (PFECAs) in which one or more of the fluorocarbons in the backbone are replaced by an ether have, in particular, emerged as important "next-generation" PFAS. As an example, perfluoro(2-methyl-3-oxahexanoic) acid, under the trade name, GenX, is produced at a large industrial scale in the US (DuPont, 2010) and Europe, and subsequently, have also been increasingly detected in water bodies in the US (631 ng/L), Germany (107.6 ng/L), and China (3825 ng/L) (Heydebreck et al., 2015; Sun et al., 2016). Recently, several PFECAs, including perfluoro-4-methoxybutanoic acid (PFMOBA), perfluoro-3-methoxypropanoic acid (PFMOPrA), and perfluoro-2-propoxypropanoic acid (PFPrOPrA), have, likewise, been detected in large amounts in the Cape Fear River watershed in North Carolina, USA (Sun et al., 2016). Although these short-chain substitutes were *assumed* to be safe, they share the same environmental stability as PFAS, and moreover, a growing body of evidence suggests they,

indeed, have quantitatively and qualitatively similar toxicity as legacy long-chain PFAS, and may pose a similar risk to the environment and human health (Gebreab et al., 2020; Gomis et al., 2018; Wang et al., 2013).

Over the last two decades, numerous studies have revealed widespread occurrence and bioaccumulation of PFAS in the environment, and moreover, plants and animals including humans (Giesy & Kannan, 2001; Wang et al., 2015). Their persistence in the environment and bioaccumulative potential in various tissues are, in turn, linked to adverse effects including documented neurological, behavioral, morphological, and embryogenic effects (Coperchini et al., 2020; Menger et al., 2020; Prevedouros et al., 2006). Humans, it is believed, are predominantly exposed to PFAS through drinking water, food and consumer products. And consequently, a number of PFAS have been detected in blood and serum including maternal-cord blood, and urine (Cai et al., 2020; Worley et al., 2017). Both PFOA and PFOS, which are the most studied PFAS, have been found in considerable concentrations in human blood ($12.4\ \mu g\ L^{-1}$ and $37\ \mu g\ L^{-1}$) (Kärrman et al., 2006) and breast milk (Post, 2022). Recently, PFECAs received significant attention, in this regard, after being detected in drinking water near a fluorochemical production facility in North Carolina (Sun et al., 2016), and in surface water in China, Germany and the Netherlands (Heydebreck et al., 2015).

Given their ubiquitous presence in aquatic matrices (e.g., groundwater, surface water and marine and freshwater bodies), considerable research has specifically studied the presence of PFASs in aquatic environments and biota. High concentrations of PFASs were found in flounder in the Nordic environment which implies that PFAS have potential to

accumulate in aquatic habitats including fish species (Kallenborn, 2004). Subsequently, several PFASs have been detected in the brain and liver of fish, suggesting that they have the potential to cause behavioral and metabolic alterations (Cui et al., 2015; Jantzen et al., 2016; Menger et al., 2020; Tu et al., 2019). However, the current understanding of the presence and fate of PFAS, and particularly, next-generation variants - such as PFECA - in aquatic ecosystems and biota is still very limited.

Toward improved understanding of PFAS in aquatic organisms and ecosystems, numerous studies have employed the zebrafish (*Danio rerio*), and particularly, early life (i.e., embryo, larval) stages of this established vertebrate model to assess toxicity of PFAS, and have demonstrated acute toxicity including lethality and morphological, behavioral, and developmental effects (Prevedouros et al., 2006; Weiss-Errico et al., 2017; Gebreab et al., 2020; Menger et al., 2020). As an established *laboratory* vertebrate model, early life stages of the zebrafish embryos benefit from numerous practical advantages including small size (≤ 1mm diameter), nearly transparent embryo and rapid embryogenesis (< 7 days), as well as high fecundity with a single breeding pair generating thousands of eggs/embryos in a given day (Berry et al., 2007; Gebreab et al., 2020). That said, studies utilizing the zebrafish as a species, and particularly, genetically non-wild type laboratory lines, have relatively limited relevance to assessing impacts on wild populations of fish, and moreover, ecologically relevant marine species. Accordingly, developing alternative – and particularly marine – species of fish as models for toxicological studies of aquatic pollutants, such as PFAS, is needed.

One such group of fish that has been investigated, as a potential sentinel for environmental toxicants, are flounder. As demersal (i.e., bottom-dwelling) species with potential to be exposed contaminants in sediments, studies have investigated a wide range of environmental pollutants and toxicants. Studies have, likewise, identified acute toxicity, alongside morphological and endocrine changes, as well as oxidative stress, in larval and juvenile stages of flounder exposed to several pollutants including components of oil and nitrogenous compounds, e.g., ammonia, nitrates (Alkindi et al., 1996; Carls, 1987; Kim et al., 2019). However, the literature on the toxicity of PFAS in flounder is rather limited with the exception of a few studies that have assessed accumulation in *adult* stages (Järv et al., 2017; Kallenborn, 2004; Marchand et al., 2004).

The aim of the present study is to assess the potential for bioconcentration, and corresponding toxicity, of several relevant PFECA and PFOA – as a representative legacy PFAS - in early life (i.e., embryo) stages of fish including freshwater and marine representatives. Whereas embryos of zebrafish, employed in the present study, represent both an established laboratory model (which has been widely used in past studies of PFAS), and a potentially relevant freshwater species, the current study additionally developed and employed embryonic stages of the Olive Flounder (*Paralichthys olivaceus*), as a potentially novel model for ecotoxicology of marine fish, and specifically utilized this system to evaluation of PFAS. Because of their considerable commercial value, as a food fish, as well as potential for scientific research, Olive Flounder are, indeed, cultured in hatcheries worldwide including the U.S. and Asia, e.g., Japan, Korea, and China (Washio et al., 2015). As a research model, Olive Flounder share multiple advantages with zebrafish including

rapid embryogenesis, small embryo size and excellent aquaculture performance (Benetti et al., 2001; Kim et al., 2002; Stieglitz et al., 2021). In addition, however, Olive Flounder represents a both ecologically (i.e., marine) and commercially relevant species, giving direct environmental and economic relevance to studies.

4.3 Materials and Methods

4.3.1 Chemicals

All PFECAs, including GenX (97% purity), PFMOBA, perfluoro-3,6,9-trioxatridecanoic acid PFO3TDA (98% purity), 4-(heptafluoroisopropoxy)hexafluorobutanoic acid (PFDMMOBA), perfluoro-3,6-dioxaheptanoic acid (PFO2HpA), perfluoro-3,6-dioxadecanoic acid (PFO2DA, 97% purity), perfluoro-3,6,9-trioxadecanoic acid (PFO3DA), and perfluoro-3,6,9-trioxatridecanoic acid, were purchased from SynQuest Laboratories (Dallas, TX, USA). PFOA (96% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, water, and ammonium formate, all Optima LC/MS Grade, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All chemicals were used without further purification. Stock solutions of PFECAs and PFOA were prepared in polypropylene tubes with biofiltered seawater for flounder and with deionized water for zebrafish to prevent adsorption to glass (Shafique et al., 2017) and then sonicated until complete dissolution of the compounds was attained. The stock solutions were then diluted in biofiltered seawater over a relevant range of concentrations to assess the level of toxicity in flounder. Internal standards of PFAS, for LC-MS analyses (Gen X IS), were kindly provided by the laboratory of Dr. Quinete (Florida International University, Miami, FL, USA).

4.3.2 Fish rearing and breeding/spawning

To perform toxicity and bioconcentration studies, fertilized eggs (i.e., embryos) of zebrafish and Olive Flounder were obtained, respectively, from the University of Miami Zebrafish Facility and UM Experimental Fish Hatchery (UMEH) at the Rosenstiel School of Marine and Atmospheric Chemistry. In both cases, rearing and breeding of fish were performed according to protocols approved by the University of Miami's Institutional Animal Care and Use Committee (UM IACUC), and executed by trained personnel.

Fertilized eggs of Olive Flounder were obtained from naturally spawning broodstock at UMEH. Rearing and breeding of flounder were done following previously described procedures and protocols (Benetti et al., 2001; Geng et al., 2019). Briefly, to collect the eggs, a 500 L tank was connected to a 15 m^3 broodstock tank with an overflow pipe. Subsequently, after spawning, eggs were collected, and placed in a 5 L beaker for 10 min to separate floating, fertilized eggs from sinking (and potentially non-viable) eggs. Floating, viable eggs were collected, and maintained in a 400 L incubator at a density of 300 eggs/L. The incubator was equipped with a central standpipe fitted with a 300-µm mesh, and supplied with pure oxygen and gentle ventilation through an air ring placed at the bottom of the standpipe. The temperature was maintained at 17 °C, and dissolved oxygen was maintained between 6.5 and 8.5 mg/L.

Zebrafish (*Danio rerio*) were reared and bred according to previously described procedures (Gebreab et al., 2020). Briefly, adult zebrafish were maintained in 30 L tanks at 28 °C with a 14 h:10 h light/dark cycle, and bred in 10 L tanks in mesh enclosures. Eggs were collected from the bottom of the tanks within 1 h of the end of the dark cycle, washed

with system water, and transferred to Petri dishes containing an E3 medium (Brand et al., 2002). Dead embryos were removed, and the remaining embryos were used within 3 h after fertilization for bioconcentration assays. Rearing and breeding were performed according to protocols approved by the UM IACUC and executed by trained investigators.

4.3.3 Determination of bioconcentration factor (BCF) of PFAS in zebrafish and flounder embryos

Uptake and bioconcentration were evaluated for zebrafish and flounder embryos exposed individually to each PFECA, and PFOA. Bioconcentration of PFAS by both zebrafish and flounder embryos was assessed at a nominal exposure concentration of 1 part-per-million (ppm) in E3 medium or biofiltered seawater, respectively. This concentrations was selected on the basis of prior assessments of embryotoxicity, and represents a sublethal concentrations (< median lethal concentration, i.e., LC_{50}) of PFAS in embryos (Gebreab et al., 2020). Bioconcentration of all PFAS by flounder embryos was additionally evaluated at an exposure concentration of 1 part-per-billion (ppb, in biofiltered seawater) which approaches environmentally relevant concentrations of these compounds in marine waters (Sinclair et al.2020).

For each exposure, ≥15 embryos were exposed in duplicate to 5 mL of PFAS solution (1 ppb or 1 ppm) in plastic 35-mm Petri dishes for 48 h. Duplicates of exposure medium without PFAS, as controls, for each exposure. During the exposure, dead or moribund embryos were removed. At 48 h of exposure, embryos were collected from the solution, and rinsed three times with deionized water before being transferred into 7-mL lysis tubes . Lysed embryos samples were frozen (at -20°C), and subsequently, freeze-dried

over 48 h. Samples of exposure solutions (1 mL) were collected, in parallel, to measure actual exposure concentrations (versus nominal, i.e., 1ppm or 1 ppb, concentration). Vials with freeze-dried embryos and solutions were stored in a freezer (−20 °C) until extraction.

All embryos were extracted as described by Menger et al. (2020) with some modifications. Briefly, freeze-dried samples, including the controls, were transferred into 15 mL polypropylene tubes. To each tuber, 100 µL of an internal standard (GenX IS) was added, and samples were extracted with 5 mL of methanol using a PowerGen 125 Homogenizer (Thermo Fisher Scientific). The polypropylene tubes were centrifuged for 10 min at 4,500–5,000 rpm, and the supernatant was transferred into other 15 mL polypropylene tubes. The extract supernatant was reduced to 1 mL with gentle nitrogen blowdown, and 50 mg of carbon black (CB) was added to each tube. Tubes were vortexed for 2 min, and centrifuged for 10 min at 4,500 rpm. Finally, the samples were filtered with syringe filters, transferred into amber glass LC/MS vials, and stored at −20°C until analysis. For samples of exposure solutions, 100 µL of an IS was added, and samples were filtered into amber glass vials, and stored at −20 °C, and analyzed directly with no further sample preparation.

4.3.4 LC/MS analysis of PFAS

PFAS analysis was performed according to a standardized, and previously validated method (Li et al., 2022). Embryo extracts and exposure solutions were filtered prior to analysis. For 1-ppm exposures, embryo extracts and exposure solutions were diluted 100-fold prior to analysis. Analyses were done using an Agilent 1290 Infinity II LC system connected to an Agilent 6470 Triple Quadrupole LC-MS/MS system equipped with an

Agilent Jet Stream electrospray ionization source (Agilent 1200 Series; Agilent Technologies, Palo Alto, CA, USA). The LC was modified with PFAS-free tubing to avoid potential contamination. A delay column (Hypersil GOLD aQ C18, 20 × 2.1 mm, 12 µm) was placed between the mobile phase mixer and the sample injector. Then, PFASs were separated on a Hypersil GOLD perfluorinated phenyl (PFP) column (150 × 2.1 mm, 3 µm) with a PFP guard column (Hypersil GOLD PFP 5 µm drop-in guards) at 50°C with 5 mM ammonium formate and methanol, as mobile phase, at a flow rate of 0.4 mL/min. The MS settings of the LC-MS/MS instrument included a drying gas temperature of 150°C, a gas flow of 10 L/min, a nebulizer pressure of 15 psi, a sheath gas temperature of 300°C, a sheath gas flow of 10 L/min, a capillary voltage of 2,000 V, a nozzle voltage of 0 V, and a cycle time of 500 ms. Table C2 (of the Appendices) shows the LC gradient conditions used. Sample acquisition was performed using a multiple-reaction monitoring (MRM) method in negative mode for the concurrent quantification of PFASs.

4.3.5 Data and statistical analysis

One-way analysis of variance (ANOVA) was performed to calculate statistically significant differences between the treatment groups at a significance level of $p = 0.05$. In addition, Tukey's post hoc test was used to compare each treatment group to the control group. The bioconcentration factor (BCF) was calculated as:

$$\mathrm{BCF} = \frac{\mathrm{C\ Embryo}}{\mathrm{C\ Solution}}$$

where C_{Embryo} is the individual PFAS concentration (in mg/g wet weight of the embryo), and $C_{Solution}$ is the exposure concentration measured at 48 h (in mg/mL), as measured by LC/MS.

4.4. Result and Discussion

4.4.1 Measured concentration of PFAS in exposure solutions and media

To assess bioconcentration, the concentration of PFAS in exposure solutions for each PFECA, and PFOA, were measured by LC/MS. The *measured* concentrations of the PFAS, as determined by LC/MS, in fact, were low relative to the *nominal* concentration of the prepared solutions, ranging from 4-77% of nominal concentrations (i.e., 1 ppm and 1 ppb; Table 4.1). A similar, recent study of PFAS toxicity in zebrafish (Menger et al., 2020), in fact, found, concentrations in exposure solutions to be equally low, relative to nominal concentrations, and likewise, consistently measured < 50% of expected concentrations; these authors attributed this to the high potential for sorption of PFAS to surfaces. Notably, the relative (to nominal) concentration of PFECA was significantly negatively correlated (Figure 4.1) with both fluorocarbon chain-length and log P, as a measure of lipophilicity, and may relate to the sorption potential, as previously proposed (Menger et al., 2020).

Table 4. 1 Concentration of PFOA and PFECA in filtered seawater (n = 4) used to rear and spawn Olive Flounder, and as exposure medium for assays, and E3 medium used as exposure medium for zebrafish assay, and concentrations measured (n =2) for solutions of PFOA and PFECA with nominal concentrations of 1 ppm and 1 ppb (in filtered seawater and E3 medium). PFAS were measured by LC/MS using the method of Li et al. (2022).

	Seawater [PFAS], ppm (± s.d.)	***1 ppm* solution in seawater [PFAS], ppm (± s.d.)**	***1 ppb* solution in seawater [PFAS], ppb (± s.d.)**	***E3 medium* [PFAS], ppm (± s.d.)**	***1 ppm* solution in E3 medium [PFAS], ppb (± s.d.)**
PFOA	0.00025 (± 0.00046)	0.52 (±0.06)	0.18 (±0.13)	0.00048(± 0.000008)	0.46 (±0.02)
HFPO-DA	0.000013 (± 0.000025)	n.d.[a]	0.77 (±0.03)	0.00004 (± 0.0)	n.d.[a]
PFDMMOBA	0.000026 (± 0.000026)	0.17 (±0.01)	0.28 (±0.01)	0.00007 (± 0.0)	0.17 (±0.01)
PFO2DA	0.000043 (± 0.000043)	0.07 (±0.01)	0.12 (±0.01)	0	0.10 (±0.0)
PFO3TDA	0.00043 (± 0.00049)	0.07 (±0.01)	0.04 (±0.01)	0.00066 (± 0.000001)	0.14 (±0.0)
PFMOBA	0	0.53 (±0.03)	0.46 (±0.01)	0	0.57 (±0.0)
PFO2HPA	0.000028 (±0.000012)	0.51 (±0.02)	0.48 (±0.05)	0.00086 (± 0.000001)	0.47 (±0.01)
PFO3DA	0.000013 (±0.000013)	0.12 (±0.01)	0.15 (±0.02)	0.00004 (± 0.000001)	0.14 (±0.0)

In addition, exposure media including both filtered seawater and E3 medium (without added PFAS) were analyzed. As shown in Table 4.1, both PFECA and PFOA were measured above the limit of detection and quantitation of the LC/MS method. This observation is aligned with the ubiquitous presence of PFAS in natural waters (e.g., seawater), as well as treated water (as in the case of the prepared E3 medium). Concentrations in media were not measured above 1% of the measured concentration of 1 ppm treatments. Background concentrations of PFAS in media, however, exceeded 1% of measured concentration for 1-ppb solutions (in seawater) in several cases and thus, may contribute significantly to the actual (measured) exposure concentration. Notably, high concentrations in both seawater and laboratory water (i.e., E3 medium) were most regularly measured for legacy PFOA, presumably due to the historically widespread use of this legacy PFAS.

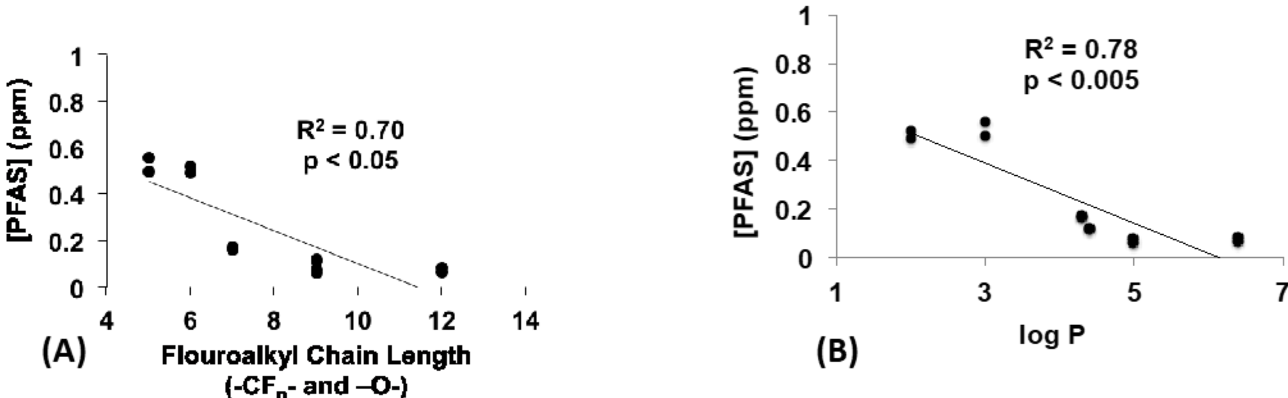


Figure 4. 1 Correlation between concentration of six PFECA, measured by LC/MS, for a solution with nominal concentration of 1 ppm in seawater medium, and either (A) fluoroetheralkyl chain-length, or (B) log P values. Chain-length is inclusive of all fluorocarbons, ethers and terminal carboxylic acid group.

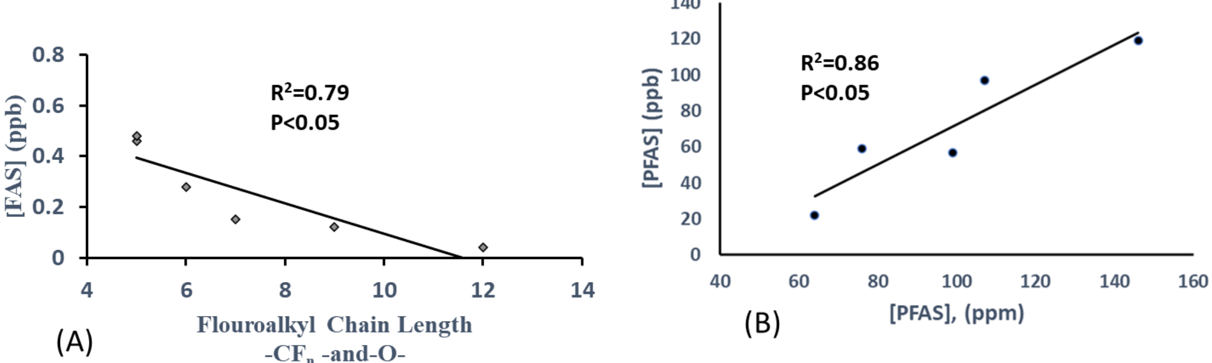


Figure 4. 2 Correlation between concentration of six PFECA, measured by LC/MS, for a solution with nominal concentration of 1 ppb in seawater medium, and fluoroetheralkyl chain-length (A), and BCF comparison between low-dose (1 ppb) and high-dose (1 ppm) exposures for PFECA (B). Chain-length is inclusive of all fluorocarbons, ethers and terminal carboxylic acid group.

4.4.2 Bioconcentrations of PFAS by zebrafish and Olive Flounder embryos

Concentration of PFAS was measured in embryos by LC/MS, and subsequently calculated BCF (relative to exposure solution concentrations) indicated uptake, and moreover, accumulation above exposure concentrations (i.e., BCF > 1), for all PFECA and PFOA (Table 4.2). In all cases, bioconcentration was greater than 10-fold. The BCF values for zebrafish at high-dose treatment (1 ppm) ranged from 83- to 262-fold: 83.33 ± 28.07 (PFMOBA), 164.25 ± 21.91 (PFDMMOBA), 161.66 ± 26.06 (PFO2HPA), 191.73 ± 45.99 (PFO2DA), 214.25 ± 37.04 (PFO3DA), 262.04 ± 45.25 (PFO3TDA), and 104.27 ± 22.67 (PFOA). Notably, the BCF determined for zebrafish embryos in the present study are largely comparable (in range) to a previous study which similarly evaluated bioconcentration in zebrafish embryos (Menger et al., 2020), and the value for PFOA (as the one PFAS evaluated in both studies) was, for example, nearly identical (i.e., 104 and 100, respectively) in the present and previous study. The BCF values in flounder at the

highest exposure concentration (1 ppm) ranged, on the other hand, from 56- to 145-fold: 64.12 ± 6.22 (PFMOBA), 75.59 ± 4.23 (PFDMMOBA), 98.60 ± 53.93 (PFO2HPA), 106.99 ± 20.77 (PFO2DA), 145.49 ± 81.91 (PFO3DA), 131.26 ± 30.43 (PFO3TDA), and 55.85 ± 2.85 (PFOA). Most importantly, these results suggest that BCF were consistently higher, for *all* PFAS tested, in zebrafish compared to flounder (Table 4.2). And suggest a higher bioconcentration potential with respect to PFAS. That said, although BCF values for Olive Flounder were consistently higher than zebrafish (Table 4.2), significant pairwise correlation ($R = 0.93$, $p < 0.001$; Figure 4.3) was observed between the two species, and (as discussed below) bioconcentration for both species was correlated with fluorocarbon chain length, and relative lipophilicity.

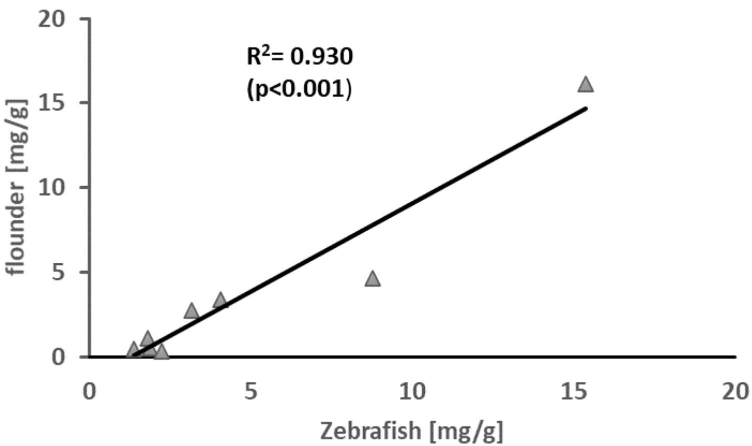


Figure 4. 3 Positive Pair-wise correlations (p<0.001) of PFAS concentrations in flounders against zebrafish at 1 ppm

In the low-dose exposure (1 ppb), BCF for flounder were, likewise, all > 1, and ranged from 22- to 329-fold concentration (Table 4.2) relative to exposure solution concentration:22±11(PFMOBA),49±42(GenX),59±12(PFDMMOBA),57±4(PFO2HPA),

97±6(PFO2DA),119±19(PFO3DA),329±68(PFO3TDA), and 151±16(PFOA).Biocon centration at 1 ppb nominal (and sub-ppb measured; see Table 4.1) concentration suggests that fish embryos in marine waters may bioconcentrate PFAS: concentrations in seawater are typically measured in the parts-per-trillion (ppt) to ppb concentration range (Sinclair et al. 2020). When BCF were compared between low-dose (1 ppb) and high-dose (1 ppm) exposures for PFECA (and not including PFOA), values were found to be lower for lower exposure concentrations with the exception of PFO3TDA (that has the highest log P value, i.e., lipophilicity), and excluding this compound, a linear relationship was observed between BCF of the high and low exposure concentration (Figure 4.2B).

In the highest exposure (1 ppm) concentration for both zebrafish and flounder, GenX was not detected in exposure solutions by LC/MS, so BCF values could not be determined (Table 4.2), even for diluted samples. This may be described by the fact that GenX has a low uptake potential in zebrafish and likely also in flounder fish embryos as compared to other PFASs in this study and thus, the concentration in the solution would be much higher than the limit of detection (Siddiqui et al., 2022). Measurement of GenX in the low exposure (1 ppb) concentration was possible, and bioconcentration was approximately 50-fold (i.e., corresponding BCF = 49). This value is on par with other similarly short-chain PFECA (Figure 4.2A), and likewise, aligned with correlation between BCF and chain-length (see below). In previous studies of adult fish, however, bioaccumulation potential for GenX was generally low (Burkhard, 2020; Siddiqui et al., 2022).

Table 4. 2 Calculated bioconcentration factors (BCFs) of PFAS. Data is displayed ± the standard deviation for n = 2 replicates. (A) Zebrafish, 1 ppm, (B) Olive Flounder, 1ppm, (C) Olive Flounder, 1 ppb.

Zeb-[1ppm]	**BCF [AVE] (± s.d.)**	**BCF [Control, E3 medium] (± s.d.)**	***p*-value**	**Log. BCF (± s.d.)**
PFMOBA	83 ±28	0	0.102	1.91±0.15
PFDMMOBA	164 ±22	8.6±1	0.0001	2.21±0.06
PFO2HPA	162 ±26	10±1	0.0001	2.21±0.07
PFO2DA	192±46	0.2±0	0.0001	2.28±0.11
PFO3DA	214 ±37	27.2±8	0.0001	2.33±0.08
PFO3TDA	262±45	16.8±5	0.0001	2.42±0.08
PFOA	104 ±23	18.9±7	0.088	2.01±0.1
FL-[1ppm]	**BCF [AVE] (± s.d.)**	**BCF [Control, sea water] (± s.d.)**	***p*-value**	**Log. BCF (± s.d.)**
PFMOBA	64 ±6	0	n.d	1.80±0.10
PFDMMOBA	76 ±4	0.7±0	0.577	1.87±0.13
PFO2HPA	99 ±54	3.3±0	0.268	1.96±0.25
PFO2DA	107±21	8±3	0.228	2.03±0.08
PFO3DA	146 ±82	19.7±21	0.061	2.13±0.26
PFO3TDA	131.3±30	10.2±5	0.078	2.09±0.21
PFOA	56 ±3	5.1±1	0.931	1.75±0.04
FL-[1ppb]	**BCF [AVE] (± s.d.)**	**BCF [Control, sea water] (± s.d.)**	***p*-value**	**Log. BCF (± s.d.)**
PFMOBA	22 ±11	8.7±1	1.000	1.32±0.22
HFPO-DA	49 ±42	0	0.675	1.59±0.43
PFDMMOBA	59 ±12	4.4±0	0.508	1.77±0.09
PFO2HPA	57 ±4	9.1±1	0.699	1.75±0.03
PFO2DA	97 ±6	18±14	0.103	1.98±0.03
PFO3DA	119 ±19	49±13	0.190	2.07±0.07
PFO3TDA	329 ±68	57.9±1	0.0001	2.51±0.09
PFOA	151 ±16	5.7±5	0.0001	2.18±0.05

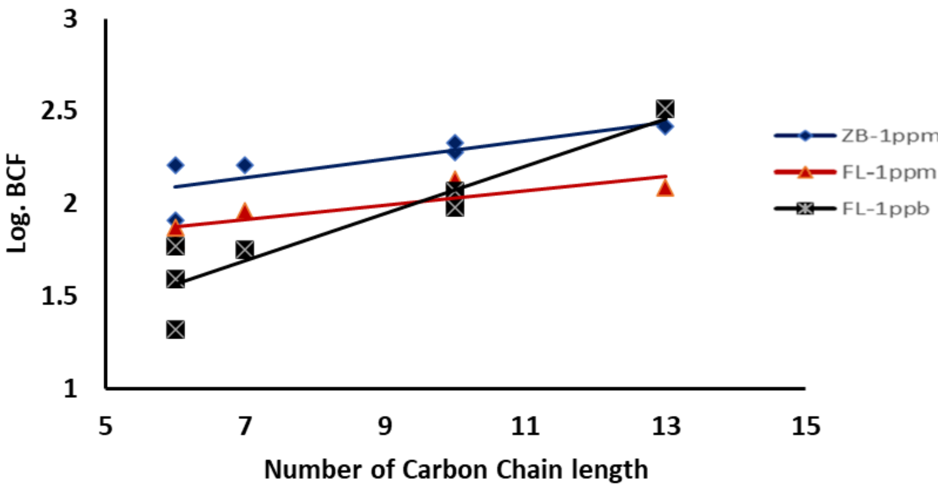
Interestingly, PFAS were also measurable in negative control (i.e., exposure medium only) embryos as well, and moderate bioconcentration (BCF > 1) – ranging from 3- to 58-fold - was observed for most exposure controls. Indeed, as discussed above, most PFAS were detectable and measurable (by LC/MS) in exposure media (i.e, seawater and laboratory-prepared E3 medium). This result suggests that even at lower "background"

concentrations of parts-per-trillion (ppt), PFAS including PFOA and PFECA can be bioconcentrated (into the low ppb concentration range) by both zebrafish and flounder embryos. This observation further supports potential for bioconcentration of PFECA, and PFOA, at or near environmentally relevant concentrations.

Bioconcentration potential generally correlated with fluorocarbon chain length. For zebrafish exposed to nominal 1-ppm concentration of PFAS, a non-significant correlation between log BCF and fluorocarbon chain length (inclusive of all fluorocarbon and ether groups) was observed ($R^2 = 0.646$, $p = 0.5$). The lack of statistical significance is perhaps related to the lower bioaccumulation of PFAS by zebrafish, as evidenced, in this study, by comparison of BCF at the nominal 1-ppm concentration that was consistently lower for zebrafish compared to flounder (Table 4.4). However, for flounder embryos, at both low and high exposure concentrations (i.e., 1 ppb and 1 ppm), a significant correlation between log BCF and chain length ($R^2 = 0.864$, $p = 0.002$ and $R^2 = 0.745$, $p = 0.03$) was observed (Figure. 4.4). Similar trends of increased BCF values with increased fluoroalkyl chain length have been reported in earlier PFAS studies of both adult and embryonic fish, as well as marine invertebrates (Martín et al., 2019; Martin et al., 2003; Menger et al., 2020; Naile et al., 2013). Similalry, increased toxicity, likely due to increased uptake, of PFAS (including PFECA) with increasing fluorocarbon chain length has also been reported in earlier studies on zebrafish (Gebreab et al., 2020; Ulhaq et al., 2015; Chapter 2 and 3).

However, the BCF of GenX was not determined as the measured concentration in the solution (i.e., solution taken after 48 h of PFAS exposure) was above the limit of detection. This may be explained by the fact that GenX has a low uptake potential in zebrafish and

likely also in flounder fish embryos as compared to other PFASs in this study (Siddiqui et al., 2022).



ZB-1ppm	FL-1ppm	FL-1ppb
R^2=0.646	R^2=0.745	R^2=0.864
P=0.5	P=0.03	P=0.002

Figure 4. 4 Correlation of log bioconcentration factor (BCF) as a function of perfluorocarbon chain length in zebrafish (p=0.5, blue), Olive flounder ,1 ppm (p=0.03, red) and Olive flounder, 1 ppb (p=0.002, black) embryos.

A number of recent studies have focused on the bioaccumulation and bioconcentration of PFAS including PFECAs (e.g., GenX and PFO3TDA) in marine and freshwater fish as indicators of the fate of these compounds in aquatic systems (Han et al., 2021; Pan et al., 2017a; Menger et al., 2020; Siddiqui et al., 2022). Likewise, bioaccumulation in humans exposed to contaminated waterways has, likewise, identified potential for bioaccumulation: a significant concentration of GenX in human blood was, for instance, detected in residents living near a fluoropolymer production plant in China

(Pan et al., 2017b). In this previous study, the BCF value of GenX (2.18) was, in fact, found to be considerably higher than that of PFOA (1.93) in human blood, suggesting that emerging PFASs may accumulate equally, or in even greater amounts, compared to legacy PFAS (Pan et al., 2017b). Unlike lipophilic chlorinated organic compounds, PFASs do not accumulate in adipose tissues. Being hydrophobic and lipophilic in nature, earlier studies have highlighted instead a strong protein-binding property of PFASs in biological systems which may explain the bioaccumulation potential (Jones et al., 2003; Siddiqui et al., 2022). In particular, due to the high protein-binding affinity, PFAS most commonly accumulate in the blood and liver (Guy et al., 1976). The results obtained in the present study are in good agreement with previous studies such as Han et al. (2021), who similarly reported a high bioaccumulation potential of PFO3TDA in zebrafish embryos, for example, and Menger et al. (2020) who identified a nearly identical BCF value for PFOA in zebrafish. Several recent studies have, in turn, suggested that the embryotoxic potential of PFAS – and, therefore, potential adverse effects - in early life stages of marine and freshwater fish most likely correlates with the uptake and bioconcentration potential (Vogs et al., 2019; Gebreab et al., 2020; Menger et al., 2021; Gebreab et al., 2022; Chapters 2 and 3).

As next-generation replacements to legacy PFAS, shorter chain PFAS and other chemical variants such as PFECA are expected to pose a lower bioconcentration potential, and thus, toxic potential, than that of long-chain PFASs. As demonstrated by several studies including the present study, the bioconcentration of PFECA in zebrafish embryos is equal to or even greater than that previously reported for legacy PFAS (e.g., PFOA) (Menger et al., 2020; Pan, Zhu, et al., 2017; Vogs et al., 2019), implying that the inserted

ester oxygen does not alter bioconcentration potential of these compounds. Both legacy PFAS and PFECAs have the potential to accumulate in fish tissues, including the liver, muscles, and blood (Pan et al., 2017a; Siddiqui et al., 2022). A similar trend was observed for Olive Flounder in the present study (the first marine species for which bioconcentration by embryonic stages is reported), suggesting that this can be extended to ecologically and commercially relevant fish species, and populations. In both cases, it is expected that this pattern of uptake and bioconcentration of PFECA will, in turn, translate to increased toxic potential, and potential adverse outcomes on marine and freshwater fish populations.

4.5 Conclusion

The results obtained in this study provides important information regarding both legacy PFAS (i.e., PFOA) and PFECA, as emerging pollutants of concern, in zebrafish and Olive Flounder. These studies identified bioconcentration of PFAS, based on BCF values, with values for zebrafish - as an established laboratory model - comparable to those obtained in earlier studies. In addition, the present study is the first to report bioconcentration of PFAS including legacy and next-generation representatives in early life stages of an ecologically and commercially relevant species (i.e., flounder). And notably, BCF values were consistently higher for flounder compared to zebrafish embryos, a finding that has direct implications for ecotoxicology of PFAS in marine fish species. The results obtained in this study can, thus, serve as a feasible reference for further studies of fate and effect of PFAS in aquatic environments.

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4.7 Appendix C

Table C1. Measured concentration of PFAS at 1 ppm. Data is displayed ± the standard deviation for n replicates. (A)Zebrafish 1 ppm, (B)Flounder 1ppm, (C) Flounder 1ppb.

Zeb [1ppm]	Zerafish_ext (n=2) [mg/g]	Control_Zeb_ext (n=2) [ng/L]	Solution-Zeb (n=2)[mg/L]	Control_Zeb_Sol (n=2)[ng/L]
PFMOBA	47.6 ± 16	0	0.57 ± 0.001	0
Gen X	230.7 ± 229	45 ± 0.4		44.46 ± 0.1
PFDMMOBA	28.2 ± 6	73.92 ± 0.37	0.17 ± 0.015	71.86 ± 0.21
PFO2HPA	75.9 ± 8	859.39 ± 0.15	0.47 ± 0.008	858.9 ± 0.06
PFO2DA	18.7 ± 8	158.51 ± 97.21	0.1 ± 0.004	0
PFO3DA	30.2 ± 8	84.02 ± 17.59	0.14 ± 0.004	44.98 ± 1.27
PFO3TDA	36.8 ± 12	1818.86 ± 39.43	0.14 ± 0.002	658.07 ± 0.58
PFOA	47.7 ± 5	686.83 ± 14.53	0.46 ± 0.02	478.53 ± 7.8

FL-[1ppm]	Flounders-ext (n=2)[mg/g]	Control_Fl_ext (n=2)[ng/L]	Solution-FL (n=2)[mg/L]	Control_FL_sol (n=2)[ng/L]
PFMOBA	34 ± 6.5	808 ± 5.7	0.53 ±0.04	44 ±0.7
GenX	242 ± 56	127 ± 4.6		
PFDMMOBA	13 ± 0.4	261 ± 23.6	0.17± 0.01	77± 5.2
PFO2HPA	50 ± 29	950 ± 31.6	0.51± 0.02	859 ± 0.2
PFO2DA	7.5 ± 0.3	1858 ± 124.6	0.07 ± 0.01	72 ± 79
PFO3DA	17.5 ± 8.9	725 ± 107.6	0.12 ± 0.01	48 ± 4.6
PFO3TDA	9.0 ± 1.5	3189 ± 419.2	0.07 ± 0.01	675 ± 17
PFOA	29 ± 5.7	1970 ± 561.4	0.52 ± 0.06	503 ± 83

FL-[1ppb]	Flounders-ext (n=2) [ng/g]	Control_Fl_ext (n=2) [ng/g]	Solution-FL (n=2) [ng/L]	Control_FL_sol (n=2) [ng/L]
PFMOBA	10214 ± 2933	48.49 ± 2.98	461.3± 3.8	5.64 ± 1.01
GenX	37261 ± 11945	27.80 ± 1.91	766.9± 31	0
PFDMMOBA	16799 ± 3048	96.77 ± 6.19	283.7 ± 7.4	2.79 ± 0.0
PFO2HPA	27452 ± 732	61.96 ± 8.78	483.7 ± 53	6.81 ± 0.1
PFO2DA	11892 ± 798	119.40 ± 12.43	123 ± 10.6	10.04 ± 8.63
PFO3DA	17463 ± 932	95.02 ± 54.86	146.2 ± 19.1	1.36 ± 1.33
PFO3TDA	12519 ± 5938	324.01 ± 16.80	38 ± 10.2	5.6 ± 0.15
PFOA	26800 ± 2192	2048.07 ±704.14	178±125	540.75 ± 383.66

Table C2. The LC gradient conditions.

Time (min)	**A [%]**	**B [%]**	**Flow (ml/min)**	**Max.Pressure Limit[bar]**
0.00	90	10	0.4	1300.00
8.00	5	95	0.4	1300.00
11.00	5	95	0.4	1300.00
12.00	90	10	0.4	1300.00

A=5mM Ammonium formate and B=Methanol

CHAPTER 5

General Conclusion

Per- and Polyfluorinated substances (PFASs) are problematic organic compounds that have been globally incorporated in various industrial and customer products since 1950s. They have been widely detected in humans, animals, and the environment. They are cause of many health problems including, neurological, behavioral, morphological, and developmental disorders. Due to their significant effects in humans, aquatic life, and the environment, many of the long chain PFASs have been phased out from the manufacturing plants and swapped with emerging and short chain alternatives. Per-fluoroether carboxylic acids (PFECAs) are a class of an alternative PFAS who have been manufactured since the 2000s. Their effect in humans, wildlife, aquatic life, and the environment are not well explored. Their presence in the water bodies, soil and in air have been corroborated. With their potential accumulation in the environment and in animals, it is critical to evaluate their effect in animal models. The focus of this dissertation is to develop PFECAs toxicity models in environmentally relevant fish species and then evaluate their toxicity effect in these fish models. We demonstrated that emerging PFAS have an acute developmental, locomotory and neurological effect in zebrafish embryo. PFAS causes metabolic profile alterations in zebrafish embryos and thus, triggers glucose and amino acid metabolic alternations and mitochondrial dysfunction. We developed PFECAs toxicity models in zebrafish, Mahi Mahi and flounder embryos. Mahi Mahi are environmentally relevant pelagic species that are more sensitive to PFAS comparing to zebrafish embryos. Finally, we measured the bioconcentration of PFAS in zebrafish and flounder embryos. A significant amount of PFAS was detected. In all fish models, the toxicity effect was proportionally correlated to PFAS chain length. Generally, the bioconcentration factors (BCF) of PFECAs were higher than PFOA in zebrafish and flounder embryos, and this

implied that emerging PFAS are, likewise, or more accumulative than long chain PFAS. It is our anticipation that this finding will somewhat fulfill the data gap in emerging PFASs. Future research should be directed toward the development of toxicity assays in human specific organ models.

Compound name	Precursor Ion	Product Ions	Retention Time (min)	Delta Retention Time	Fragmentor	Collision Energy	Cell Accelerator Voltage
GenX	**285**	**169**	**5.36**	**3**	**108**	**12**	**5**
GenX I.S	**287**	**185**	**5.4**	**3**	**108**	**20**	**5**
PFDMMOBA	**378.9**	**184.9**	**6.02**	**3**	**135**	**12**	**5**
PFMOBA	**279**	**279**	**4.3**	**3**	**135**	**4**	**5**
PFO2DA	**445**	**235**	**6.88**	**3**	**135**	**24**	**5**
PFO2HPA	**295**	**200.9**	**4.96**	**3**	**135**	**4**	**5**
PFO3DA	**410.9**	**317**	**6.5**	**3**	**135**	**4**	**5**
PFO3TDA	**560.9**	**466.9**	**7.44**	**3**	**135**	**4**	**5**
PFOA	**413**	**369**	**6.4**	**3**	**69**	**8**	**5**
PFOA	**413**	**169**	**6.4**	**3**	**69**	**16**	**5**

Table C3. Summary of the MRM method for the analysis of PFAS

Abbreviation	Compound Name	Molecular Formula	Molecular Weight	Structure
HFPO-DA (GenX)	2,3,3,3-Tetrafluoro-2-(heptafluoropropoxy)-propanoic acid	$C_6HF_{11}O_3$	330.05	
PFDMMOBA	4-(Heptafluoroisopropoxy)hexafluorobutanoic acid	$C_7HF_{13}O_3$	380.06	
PFMOBA	Perfluoro(4-methoxybutanoic) acid	$C_5HF_9O_3$	280.05	
PFO2HPA	Perfluoro-3,6-dioxaheptanoic acid	$C_5HF_9O_4$	296.04	

PFO2DA	Perfluoro-3,6-dioxadecanoic acid	$C_8HF_{15}O_4$	446.07	
PFO3DA	Perfluoro-3,6,9-trioxadecanoic acid	$C_7HF_{13}O_5$	412.06	
PFO3TDA	Perfluoro-3,6,9-trioxatridecanoic acid	$C_{10}HF_{19}O_5$	562.08	
PFOA	Perfluorooctanoic acid	C8HF15O2	414.07	

Table C4. List of PFAS with their Structures used in this study

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PUBLICATIONS AND PRESENTATIONS

Gebreab, K. Y., Eeza, M. N., Bai, T., Zuberi, Z., Matysik, J., O'Shea, K. E., Alia, A., & Berry, J. P. (2020). Comparative toxicometabolomics of perfluorooctanoic acid (PFOA) and next-generation perfluoroalkyl substances. Environmental pollution, *265*, 114928.

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