# THESIS

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# RT-qPCR examination of the inflammation and immune system gene markers in the liver of Zebrafish after exposure to PFOA

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# 1 Introduction:

## 1.1 What is PFOA and where are they used:

Perfluorooctanoic acid (PFOA) is a part of a class of compounds called perfluoroalkyl substances (PFAS). These synthetic compounds provide many benefits in industrial use. Most importantly, they are resistant to water and lipids. Due to their capacity to deter water and grease, they have been used in a wide range of products (Vierke et al., 2012). The unique properties of PFAS are due to their many carbon-fluorine (C-F) linkages (Verma et al., 2022). Direct fluorination, such as electrochemical fluorination, and oligomerization, such as fluorotelomerization, are the two techniques used to create PFAS (Evich et al., 2022). The greater the amount of carbon-fluorine (C-F) bonds present and the longer the C-F chains increases their bioaccumulation ability and toxicity (Renner R, 2006).

PFAS have been detected in jackets, upholstery, carpets, paper, building materials, food contact materials, impregnation agents, cleansers, polishes, paints, and ski waxes, among many other items commonly found in offices, households, and cars (Sunderland et al., 2019). Other sources include PFAS that are produced and applied to textiles and painting/printing facilities (Danish EPA, 2014). In food, fish species at the top of the food chain and shellfish are significant sources of PFAS exposure. Livestock raised on contaminated land can accumulate PFAS in their meat, milk and eggs (Ingelido et al., 2018; Numata et al., 2014). Direct exposure to humans may also come via skin creams and cosmetics (Danish EPA, 2018; Schultes et al., 2018).

PFOA is extremely resistant to environmental degradation processes and thus persists indefinitely (Post, G. B et al., 2012). PFOA has an average half-life in humans of 2.4 years (Russel et al.,

2015). Animal studies on the effects of PFOA have indicated that it is likely hepatotoxic as well as likely to cause developmental issues in humans (Kennedy et al., 2004). The route of action of PFOA was determined to be connected to receptor activation rather than genotoxicity, making it a potential human carcinogen (Tardiff, R. G., 2009).

### 1.2 Environmental effect and health risks associated with PFAS:

PFAS either are, or degrade to, persistent chemicals that accumulate in humans, animals, and the environment. This adds to the total burden of chemicals to which people are exposed and increases the risk of health impacts (Evans et al., 2016). PFAS are ubiquitous in the environmental matrix, and their ecotoxicity on wildlife and the suspected health risks to humans are increasing concerns (Ao et al., 2019). Exposure to PFAS has been associated with adverse human health impacts on female and male fertility, metabolism during pregnancy, endocrine function which includes pancreatic dysfunction and the risk of developing Type 2 diabetes, lipid metabolism and a risk of childhood adiposity, hepatic and renal function, immune function, cardiovascular health (atherosclerosis), bone health including a risk of dental cavities, osteoporosis, vitamin D deficiency, neurological function, and a risk of developing breast cancer (Jane et al., 2022). Some PFAS bioaccumulate and bind to proteins (but not to lipids) in biota and humans due to their surface-active characteristics and very low solubility in water and fat (Tansel et al., 2022).

The production and use of PFAS in products has resulted in the contamination of drinking water supplies in several European countries. In some highly polluted areas, concentrations of PFOA and perfluorosulfonic acid (PFOS) in drinking water were above the limit (EC, 2017). Higher concentrations of PFAS were observed in municipal tap water as compared to bottled water (Ao et al., 2019). Due to their high mobility and endurance, PFOA compounds frequently migrate from

soils to the surrounding surface water and/or groundwater (Jha et al., 2021). PFAS can also be found in dust as fine particles of solid matter, including building materials, furnishings and consumer products used in typical indoor spaces (Savvaides et al., 2021). Human exposure can also occur during the processing steps for materials recovery and scavenging at disposal sites, resulting in PFAS intake through inhalation, ingestion, and dermal routes (Tansel et al., 2022).

In biota, PFOS (C8 fluorocarbon) is typically the dominant PFAS, and the PFOS concentration increases along the food chain, showing its high bioaccumulation potential. In contrast, PFOA (C7 fluorocarbon) has a low bioaccumulation potential and is relatively similar among species from different trophic levels (Giesy et al., 2001). For example, PFOS and PFOA have the same maximum concentration in invertebrates, but in fish, reptiles, birds, and mammals PFOS has a higher concentration, up to three times that of PFOA (Ahrens et al., 2014). In contrast to PFAS precursors, which can be converted into PFCAs and PFSAs, PFAS can be thought of as persistent in the environment as a whole (Butt et al., 2014).

PFOA's widespread use, presence and persistence in the aquatic environment has led to an increasing number of studies focusing on its toxicological effects (D'Hollander et al., 2010). The chronic exposure to PFAS can have negative impacts on the health and reproductive success of aquatic species, as well as potentially posing a risk to human health through consumption of contaminated fish and shellfish (Ahrens et al., 2014). Therefore, it is important to monitor and regulate the release of PFAS into the environment to minimize their potential impacts (Guelfo et al., 2018). PFAS exposure in Japanese medaka (Oryzias latipes) was shown to have a negative impact on both parental generations and subsequent offspring, highlighting the potential for these chemicals to cause long-term harm in aquatic ecosystems (Ji et al., 2008).

### 1.3 The hormonal effect of PFAS compounds and PFOA:

PFOS and PFOA are thought to act as endocrine disruptors, potentially inducing physiological alterations and damaging reproductive health. PFOS and PFOA act on steroidogenic ovarian cells as endocrine disruptors, which could affect the dependent functions of sexual steroids (Chaparro-Ortega et al., 2018). The elevated levels of several androgen indicators in postmenopausal women, particularly in those who are overweight or obese, were positively correlated with linear perfluorooctane sulfonate (n-PFOA) and perfluorohexane sulfonic acid (PFHxS) (Lopez-Espinosa et al., 2011). This increased risk of cardiometabolic diseases is linked to elevated levels of androgens in postmenopausal women (Wang et al., 2021). Long (carbon-fluorine) chain PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), have been associated with various reproductive effects in both humans and animals (Grandjean et al., 2015). These effects include a decrease in fertility, reduced fetal growth and birth weight, pregnancy-induced hypertension and preeclampsia, thyroid hormone disruption during pregnancy, and preterm birth. These findings suggest that exposure to long-chain PFAS during pregnancy can have negative impacts on both maternal and fetal health (Chambers et al., 2021). Perfluorohexanoic acid (PFHxA) has been recognized as an alternative to the wide usage of PFOA and PFOS in the fluoropolymer industry for years. PFHxA has been frequently detected in the environment due to its wide application (Zhou et al., 2022). One study found that exposure to PFHxA caused slow body growth and changes in thyroid hormone levels in zebrafish larvae, indicating an interference effect on larval development, the study also observed upregulation of the gene expression level of thyroid hormone receptor β in a dose-dependent manner, suggesting that exposure to PFHxA can disrupt thyroid status and impair growth in zebrafish larvae (Zhang et al., 2022). Thyroid hormones (THs) play an important role in the normal development and

physiological functions in fish. Environmental chemicals may adversely affect thyroid function by disturbing gene transcription (Walter et al., 2019). One study found that PFOS exposure could alter gene expression in the HPT axis and disrupt thyroid function (Shi et al., 2009). Male Murray River rainbowfish (*Melanotaenia fluviatilis*) were exposed to different concentrations of PFOA, and the study found altered levels of thyroid hormones and the presence of vitellogenin in the plasma, which are indicators of endocrine disruption (Dolomatov et al., 2013 ; Chidakel et al., 2005). The fish also exhibited increased activity of certain antioxidant enzymes in the gills and reduced activity in the liver, along with lipid peroxidation in both tissues indicating oxidative stress (Hoque et al., 1998). These results suggest that PFOA exposure can cause hormonal and oxidative stress disturbances in aquatic organisms, which can have negative impacts on their health and survival in natural populations (Miranda et al., 2020).

## 1.4 Inflammation and PFOA:

### 1.4.1 Genes associated with inflammation and the immune system

From several studies, there are a lot genes known to be associated with inflammation and the immune system in zebrafish (Levraud et al., 2022 ; Leiba et al., 2023). Four such genes are: TNFA, Il1B, Il8, and Il10. These genes are frequently chosen in different immunological studies because they play an important role in immune responses and inflammation (Jin et al., 2010 ; Wibiwo et al., 2020).

TNF-α

The cytokine tumor necrosis factor (TNF-α) is a pleiotropic polypeptide cytokine that plays an important role in brain immunological and inflammatory activity (Lee et al., 2003). TNF-α is present early in neuronal cells in and around the ischemic tissue and it's produced in the brain in response to a variety of pathological conditions, including infectious agents [e.g., HIV and malaria], ischemia, and trauma (Mogi et al., 2011). TNF-α mRNA expression precedes the infiltration of inflammatory cells into the damaged zone and corresponds in time to other cytokines such as interleukin (IL)-6, cytokine-induced neutrophil chemoattractant (KC), and IL-1(Feuerstein et al., 1994). TNF-α was one of the primary signals expressed transiently by polarized macrophages during the early stages of regeneration, and TNF-α /TNF-r1 signaling was required for stromal cell proliferation (Iribarne., 2021).

IL-1β

Interleukin-1β is a proinflammatory cytokine that affects neurotoxic neurotransmission and increases the duration of kainate-induced seizures by increasing glutamatergic neurotransmission (Vezzani et al., 2015). IL-1β also inhibits stomach acid output. Polymorphisms in the IL-1B

gene have been linked to an increased risk of hypochlorhydia, gastritis1, and non-cardia stomach cancer development (Murphy et al., 2009). IL-1ß has been discovered as a marker for life stress, and IL1B variations have frequently been employed in gene environment studies to evaluate stress and depression across the life span (Michael et al., 2018).

Il-8

Interleukin-8 (IL-8) is an essential neutrophil activator and chemoattractant, it belongs to the CXC chemokine family and has been linked to several inflammatory disorders (Bickel M., 1993). Numerous different cell types release IL-8 in response to different stimuli, and its regulation predominantly occurs at the level of gene transcription (Kawasaki et al., 2001). Only 100 nucleotides of 5'-flanking DNA upstream of the TATA box are needed for IL-8 transcriptional responses to proinflammatory mediators to occur quickly (Roebuck., 1999). IL8 is also in charge of directing neutrophils through the tissue matrix until they reach areas of injury (Harada et al., 1994). CXCL8 homologs in zebrafish are Cxcl8-l1 and Cxcl8-l2. Cxcl8-l1 has been shown to be upregulated in inflammatory situations produced by bacterial or chemical insults (De Oliveira et al., 2013).

Il-10

Interleukin-10 (IL-10) is often regarded as the most effective anti-inflammatory cytokine. Almost all innate and adaptive immune cells generate it (Mosser et al., 2008). These cells are also its targets, showing that IL-10 production and function are tightly controlled and possibly compartmentalized (Saraiva et al., 2010). IL-10 has the ability to stimulate humoral immune responses by increasing class II expression on B cells and triggering immunoglobulin (Ig) synthesis (Saxena et al., 2015). In vitro, IL-10 is a cytokine released by a wide range of cell types that possesses pleiotropic stimulatory and suppressive actions on lymphoid and myeloid cells

(Lee et al., 2022). IL-10 is produced by activated T cells, B cells, macrophages, and a range of other cell types and has both immunostimulatory and immunosuppressive effects (Groux ey al., 1999). IL-10 inhibits NF-κB, a transcription factor involved in the production of inflammatory cytokines (*e.g.*, IL-1β, IL-6, IL-8, and TNF-α) (Im et al., 2004).

### 1.4.2 PFOA to inflammation

The physiologic response to injury brought on by a wound, chemical irritation/damage, or infection is inflammation. Acute inflammation starts as a chain reaction of cytokines and chemokines that draw neutrophils and other immune and non-immune cells into disturbed and damaged tissue. Because the production of pro-inflammatory cytokines is replaced by anti-inflammatory cytokines as healing continues, acute inflammation is typically self-limiting (Sethi et al., 2008).

The widespread presence of PFOA in the marine environment raises serious concerns about its ecotoxicological impact on marine mammals and biodiversity (Houde et al., 2006). One study showed that in male Japanese medaka (*Oryzias latipes*) exposed to PFOA, there was an increase in the mRNA levels of proinflammatory cytokines such as IL-6, TNF-α, and IL-1β, indicating a potential role in inflammation and tissue injury (Ji et al., 2008). This was observed in a study that investigated the effects of PFOA exposure on gene expression in the liver of the fish. The results suggest that PFOA exposure may have harmful effects on aquatic organisms and their health (Yang et al., 2010). The NHANES 2005-2012 study found that PFAS exposure is associated with markers of chronic inflammation and oxidative stress, based on the results of generalized linear models used to analyze the data (CDC, n.d. 2023). The study examined the association between PFAS and inflammatory markers such as ferritin, alkaline phosphatase, C-reactive protein, absolute neutrophil count, and lymphocyte count, as well as oxidative stress markers such as serum

bilirubin, albumin, and iron (Omoike et al., 2021). The analysis showed that increased exposure to PFAS was associated with increases in the serum concentration of these markers, indicating a link between PFAS exposure and both chronic inflammation and oxidative stress (Omoike et al., 2021). The models were adjusted for covariates to account for potential confounding factors (Omoike et al., 2021). In a related study, PFAS exposure increased hepatic injury, demonstrated by increased liver weight, hepatic inflammation, and plasma alanine aminotransferase levels (Wang et al., 2022). Females displayed increased lobular and portal inflammation compared to the male PFAS-exposed mice (Roth et al., 2021). PFOA, classified as a possible human carcinogen, may cause hepatic, testicular, and pancreatic toxicities and cancers. In yet another study, PFOA exposure altered oxidative stress parameters and gene expression level-related inflammation in the human pancreatic cell line PANC-1, suggesting the role of oxidative stress in PFOA-induced pancreatic toxicity and highlighting the incidence of inflammation (Abudayyak et al., 2021).

## 1.5 Zebrafish as a toxicological model:

Zebrafish (Danio rerio) have long been the genetic model of preference for researchers researching the development of organs and tissues in vertebrates. Zebrafish have become a valuable model organism for studying the molecular and cellular pathways by which environmental contaminants affect normal developmental processes. Zebrafish are a useful model for studying processes in young and old animals, such as carcinogenesis, aging, and the effects of environmental pollutants on these processes. Zebrafish are an increasingly popular model organism in laboratory research due to their advantageous traits, which include their small size, transparent embryos, external embryonic development, and short reproductive cycles (Feitsma et al., 2008). With the development of sophisticated genetic tools and genome sequencing programs, which have elevated zebrafish as a viable model to effectively study human sickness and pathophysiology, a new era of comparative biology and medicine has begun (Norton & Bally-Cuif, 2010). Zebrafish are increasingly being used as an animal model for cardiovascular illness. For instance, zebrafish fed a diet rich in cholesterol (4%) developed blood vessel plaques that resembled atherosclerosis in people (Powell et al., 2015). The zebrafish is an ideal model organism for genetic studies, as it has a small genome and a short generation time. Methods for producing homozygous diploid zebrafish, such as hydrostatic pressure or thermal shock, make it easier to study gene function and genetic interactions (Feitsma et al.,2008). Zebrafish are a valuable model organism for studying vertebrate gene function and human genetic disorders. Their transparent embryos and the ability to manipulate gene expression make them useful for genetic investigations by gene knockdown or overexpression. (Howe et al., 2013).

## 1.6 Objective of the study:

The objective of this study was to determine whether PFOA exerted an inflammatory effect in a zebrafish model by assessing the expression patterns of several genes of interest.

## 2 Materials and Methods:

### 2.1 Chemicals:

All reagents were an analytical grade.

### 2.2 Animal protection:

The Animal Protocol was approved in accordance with the Hungarian Animal Welfare Law, and all research was finished before the treated animals would have been able to eat on their own.

### 2.3 Test Organisms:

The Department of Environmental Toxicology at the Hungarian University of Agriculture and Life Sciences (Gödöllő, Hungary) provided the zebrafish facility with the zebrafish (AB wildtype) used in this study. In a recirculating zebrafish housing system made by Tecniplast ZebTec (Buguggiate, Italy), fish were kept at constant water quality parameters (25 ± 0.5 °C; pH 7.0 ± 0.2; conductivity 500 ± 50 μS; alkalinity < MDL, 0 mM CO32−, 0.4 mM HCO32−; hardness < 0.5° dH; DOC > 90%; system water) in a Tecniplast ZebTec (Buguggiate, Italy) recirculating zebrafish housing system. The photoperiod was set to a cycle of 14 hours of light and 10 hours of darkness. The fish were fed brine shrimp (Ocean Nutrition > 230,000 NPG) twice a week in addition to ZEBRAFEED (Sparos, 400-600 m) twice a day.

### 2.4 Experimental Design:

Zebrafish aged 9 to 12 months old were put into 15 experimental tanks. To eliminate any possible confounding effects of vitellogenin and estrogenic expression due the reproductive cycle, we assessed inflammatory response genes in male fish only.

Each tank contained 3 liters of the test solution (with nominal concentrations of 0, 10, 20, 40, or 80 g/L of PFOA). These tests lasted for 28 days and were intended for adults. Each treatment included three replicates, each containing six fish. The lowest and highest PFOA concentrations tested, 10 and 80 g/L, respectively. Based on the results of the VTG, the EC50 was calculated to assess the nonlinear response caused by the combined effect of PFOA and P4.

The expected outcome of the test chemicals led to the selection of VTG. Each replicate contained six fish, eighteen fish total for each mixture. The theoretical toxic effect in all mixtures was predicted to be 50%. This setup made it simple to identify nonlinear mixture effects, whether they were antagonistic or synergistic, and to observe the impact of various concentration ratios. Fish were exposed for 28 days and were given ZEBRA FEED (Sparos, 400-600 m) once daily in a quantity equal to 2% of the fish weight in the aquarium. Every three days, the exposure media were completely renewed. The ranges for the water quality parameters were maintained as described in the "Test Organisms" section above. To ensure that the nominal and actual test compound concentrations in the aquaria were identical, water samples were examined by LC-MS/MS throughout the experimental period. After renewing the test solutions for 1 and 36 hours, samples from the aquaria were taken from the test medium. The average PFOA and DMSO concentrations in the water samples were consistently within 20% of the targeted concentrations.

At the 7th, 14th, 21st and 28th days of exposure, 6 fish from each exposure concentration and replicate were sacrificed after an anesthetic overdose (0.04% MS-222 (tricaine-methane-sulphonate) (Sigma-Aldrich, Darmstadt, Germany)). The liver of each fish was isolated and stored in microtubes at −80 °C for later biochemical analyses.

### 2.5 Expression analysis of inflammatory response genes:

Vitellogenin is upregulated during the inflammatory response in zebrafish. However, in female fish, it also is a key component in the reproductive cycle (egg production). Therefore, to eliminate any possible confounding effects of vitellogenin expression due the reproductive cycle, we assessed inflammatory response genes in male fish only. Livers of 3 fish selected randomly from each diet treatment were pooled to ensure sufficient tissue (approximately 30 mg wet) for each replicate. Four replicates including control (each consisting of 6 livers) were obtained for each treatment.

### 2.5.1 RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR):

Reverse transcriptase-polymerase chain reaction (RT-qPCR) was used to analyze the expression of the chosen target genes. Zebrafish livers were transferred into 10.5 mL microcentrifuge tubes (2liver/tube;6 tubes per replication) following PFOA exposure, homogenized in 200 L Trizol reagent, and then kept at -80 °C. By using the chloroform/isopropanol extraction and ethanol-precipitation method, RNA from the embryos was isolated. A NanoDrop One spectrophotometer was then used to measure the quantity and quality of RNA. Applied Biosystems' High-Capacity cDNA Reverse Transcription kit was used to create cDNA. On a LightCycler 480 Instrument II, the PCR assays were carried out using 5x HOT FIREPol EvaGreen qPCR Supermix. Target gene expression levels were compared to that of the housekeeping gene, ef1a, to determine their normalized levels.

## 2.6 Statistical Analysis:

Data derived from RT-qPCR were tested for normality using the Shapiro-Wilk test. Differences in normalized mRNA level between the control and treatment groups were assessed by one-way ANOVA followed by Dunnett's test or Kruskal Wallis test and then by Dunn's test using GraphPad Prism software. Results were presented as mean ± SD. Statistically significant differences were considered at $p < 0.05$.

## 3 Results:

After every 7 days of exposure to PFOA (up to four weeks of age), we harvested a subgroup of zebrafish and measured each zebrafish's total weight and liver weight. Figure 1 shows the results of total weight and liver weight change in the zebrafish overtime. From morphologic observations we observed that each week of exposure to PFOA reduced the movement speed of the zebrafish. Additionally, some of the zebrafish died during the 4$^{th}$ week of exposure, prior to tissue harvesting.

Figure 1: Zebrafish total weight average (grams) after PFOA treatment

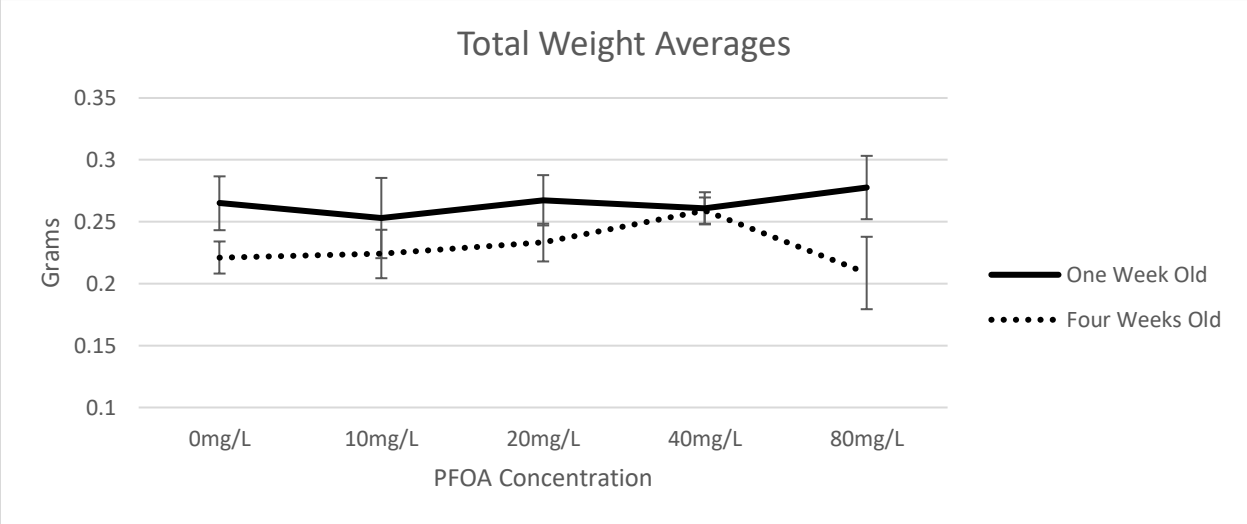


*Figure 1 shows the changes of zebrafish weight between the first week and the fourth week after PFOA treatment in a different concentration. Error bars are representations that show the variability of the data of the total weight different within the group (Small SD bars = more reliable, Big SD bars = less reliable).*

As the results show, the total weight difference from the first week and the last week changed slightly, with the first week having a higher total weight average at 0.26 grams and after four weeks of exposure the weight went down to at 0.21 gram. We observed that some of the fish died during the experiment period from the third week and progressively slowed in their swimming motion.

Figure 2: Zebrafish liver weight average (grams) after PFOA treatment

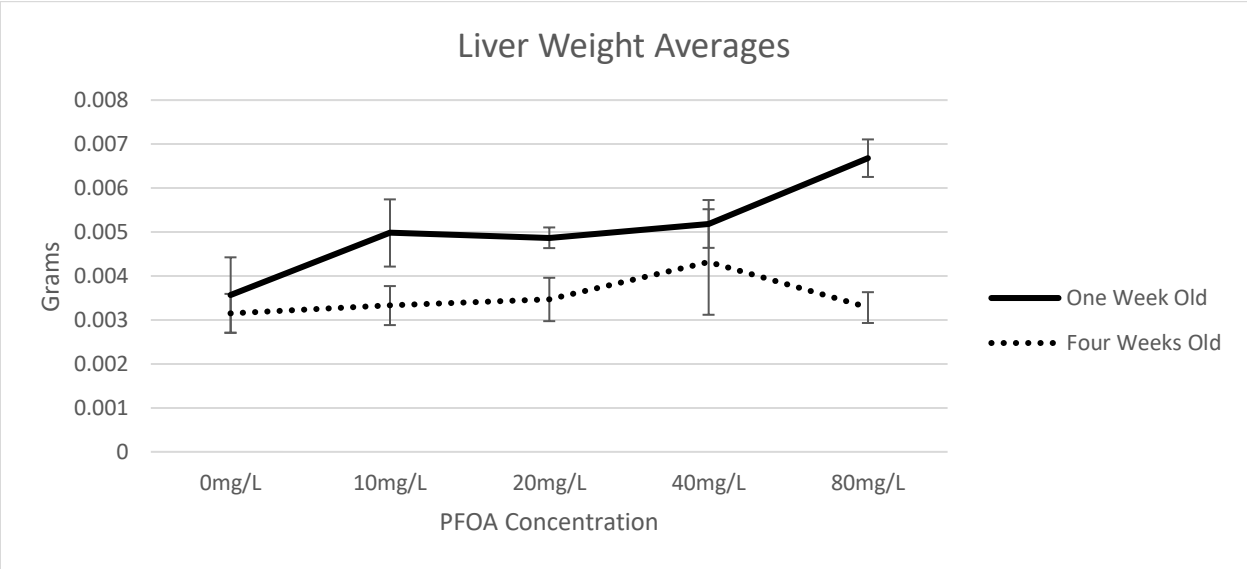


*Figure 2 shows the average liver weight between one week and four-week PFOA exposure at varying concentrations. Error bars are representations that show the variability of the data of the liver weight different within the group (Small SD bars = more reliable, Big SD bars = less reliable).*

In the present study, we also analyzed the effect of PFOA exposure on the liver weight of zebrafish over a period of four weeks at varying concentrations. The results presented in Figure 2 show the average liver weight of the zebrafish for each week of PFOA exposure. Interestingly, we observed a noticeable decrease in the liver weight of zebrafish exposed to 80mg/L PFOA after four weeks of exposure. Specifically, the average liver weight decreased from 0.0065 gram to 0.0031 gram in the treatment group, indicating a considerable loss in liver weight in the PFOA-exposed zebrafish.

Gene expression and RT-qPCR results

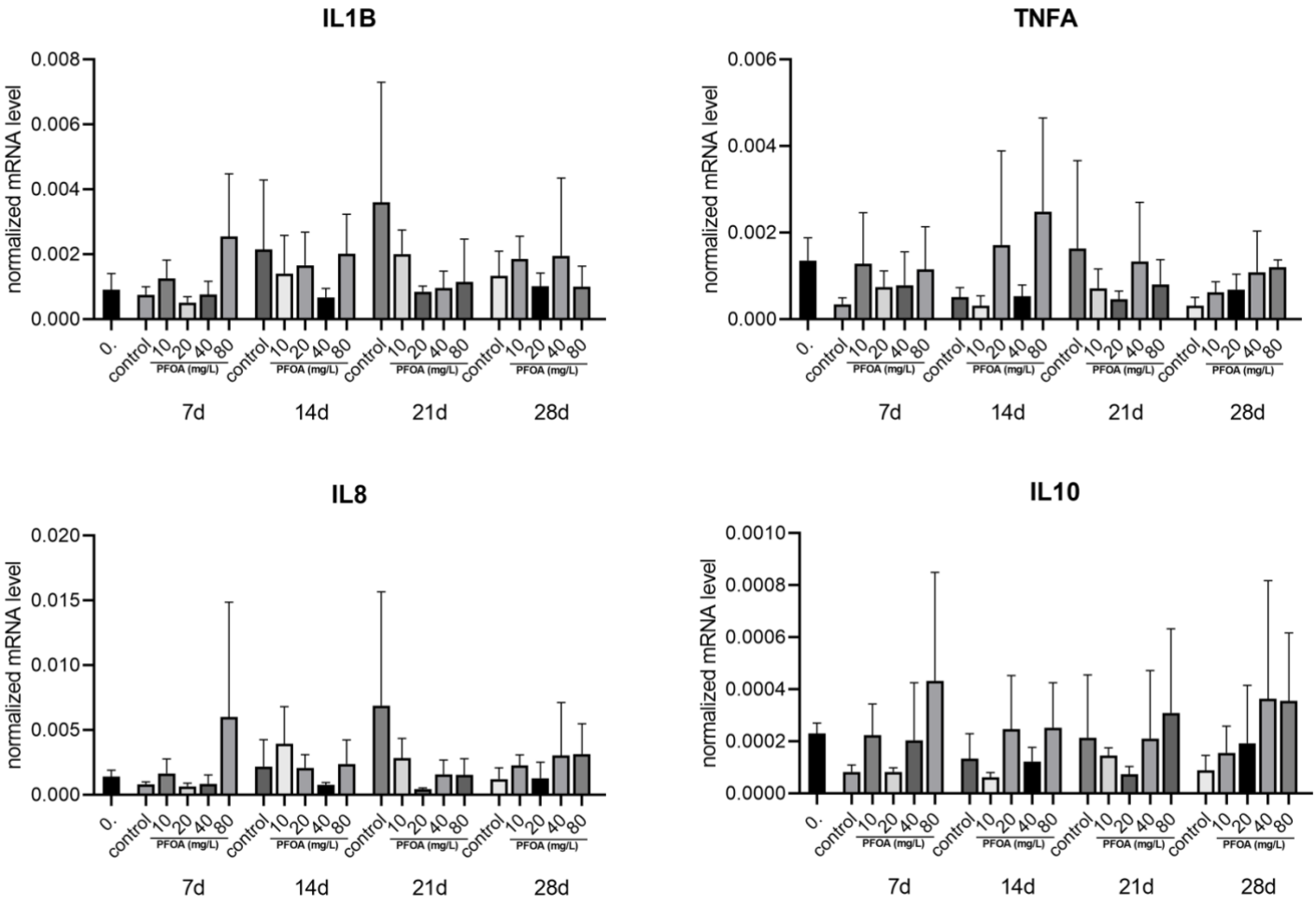


*Figure 3: Changes in different targeted gene (TNFA, IL1B, IL8, IL10) activity in the liver of Danio rerio exposed to PFOA after each week of exposure.*

To evaluate the pro-inflammatory effects of PFOA-exposure in zebrafish, we selected different immune response- and inflammation-related genes (*tnfα*, *il8, il1β* and *il10*) and measured their expression. The differences of the normalized mRNA levels between the control and treatment groups changed considerably week-by-week during the four-week PFOA exposure. We detected a statistically significant increase in the expression of *il1β* at the end of the first week of exposure and in the expression *tnfα* and *il10* at the end of the fourth week of exposure at 80 mg/L PFOA. Besides, the expression levels of *tnfα*, *il8* and *il10* also showed a considerable, but statistically not significant, increase at the end of the first week of exposure at 80 mg/L PFOA.

Taken together, PFOA-exposure resulted in altered expression of immune response- and inflammation-related genes at relatively high concentration of 80 mg/L. We could see strong upregulation in the treated groups as compared to the control treatment. Nevertheless, we detected a large variance between replicates in the treatment groups, resulting in statistically insignificant results.

## 4 Discussion:

The purpose of this study was to determine whether PFOA exerted an inflammatory effect in a zebrafish model by assessing the expression patterns of several genes of interest. Unfortunately, the RT-qPCR, body and liver weight results of our study were statistically non-significant. We chose *tnfα*, *il8, il1β* and il10 because they are related to inflammation and immune system functions that are easily detected and widely used with RT-qPCR. The qPCR results we obtained were unclear and we weren't able to conclude any significant changes. However, the data indicates that there was a difference in gene expression levels for each gene of interest between the highest concentration of PFOA exposure (80mg/L) and the control samples, beginning from the first week of the experiment.

In this study, we used Dimethyl sulfoxide (DMSO) as a solvent to maintain the concentration of PFOA. DMSO is a common chemical agent for DNA denaturation (Xu et al., 2022). Interestingly, a publication came out after the completion of our studies that indicated a negative effect of DMSO on PFOA structure (Trang et al., 2022). It was found that DMSO can affect the chemical structure of PFOA by the method for mineralizing using reactive perfluoroalkyl anions under mild conditions (80 to 120 °C) (Trang et al., 2022). PFOA are decarboxylated in dipolar aprotic solvents at low temperatures in the presence of NaOH in mixtures of water and dimethyl sulfoxide (DMSO) (Trang et al., 2022). In the described conditions, PFOA undergoes complete degradation with over 90% defluorination and minimal generation of fluorocarbon by-products (Trang et al., 2022). Trang found that this can result in changes in its biological activity and toxicity but the specific effects of DMSO on PFOA may depend on the concentration of DMSO and the specific experimental conditions used (Trang et al., 2022). The results of this publication by Trang could apply to our study and explain the large variance in data results we obtained.

Further investigations are necessary to fully understand the effects of PFOA on inflammation-related genes. While we cannot discount the accuracy of changes in liver weight averages, it should be noted that there was no random selection of fish based on body weight for the control and experimental groups. Therefore, total weight differences may not be a reliable comparison between the groups.

The next steps in the research will be to reassess the effect of PFOA in a zebrafish model, without the use of DMSO. Current efforts in the lab are focused on another PFAS compound, GenX, and its physiological effects in zebrafish. Overall, the results of this study were inconclusive as to the effect of PFOA and its ability to induce an inflammatory response in zebrafish. With the new knowledge related to DMSO and PFOA, we hope to obtain different and clear results in a future repeat of this experiment.

## 5 Conclusion:

In recent years, there has been growing concern about the presence of polyfluoroalkyl and perfluoroalkyl compounds, such as PFOA, in the aquatic environment. These compounds have been found to be highly persistent and can accumulate in living organisms, potentially causing harm to their immune systems. One species that has been widely used as a model for studying the effects of PFOA is the zebrafish. Studies have shown that exposure to PFOA can cause organ abnormalities and is toxic to this species.

In order to better understand the effects of PFOA on the developing liver, we conducted a study using the zebrafish model. Our results showed that the expression of proinflammatory cytokines, such as tnfα, il8, il1β, and il10, increased significantly in response to PFOA exposure at various doses. Interestingly, the differences in mRNA expression between the control and treatment groups changed over time, with significant changes seen week by week.

It is important to note that PFOA is often found in environmental samples along with other potentially harmful substances, which may increase the risk to human health. Therefore, it is crucial to investigate the impact of these other compounds, which can affect the mobility and survival of mature zebrafish, on PFOA exposure. Additionally, repeating the experiment without the use of DMSO as a water-soluble agent is necessary to detect statistically significant results from the inflammation and immune system-related genes of interest.

Overall, our findings emphasize the need for continued research on the effects of PFOA on aquatic organisms and their potential implications for human health. By better understanding the mechanisms by which PFOA affects the immune system and identifying ways to mitigate its harmful effects, we can work towards protecting the health of both wildlife and humans alike.

## 6 Summary:

Polyfluoroalkyl and perfluoroalkyl compounds (PFAS) are emerging environmental contaminants that have been detected in various environmental matrices, including air, water, and soil. These compounds are widely used in a range of industrial and consumer products due to their unique properties such as water and oil repellency, non-stick properties, and heat resistance. PFOA is a common PFAS compound that has been found to be the most persistent and prevalent in the environment and is known to bioaccumulate in the tissues of living organisms.

The toxicity of PFOA has been extensively studied, and it has been shown to have negative effects on various physiological processes in many species, including mice and zebrafish. The immune system is one of the most commonly affected systems by PFOA exposure, and it has been found to be associated with chronic inflammation and altered immune responses.

One of the most common methods used to assess the effects of PFOA exposure is to observe the morphology and behavior of the exposed organisms. In the case of zebrafish, exposure to PFOA has been shown to cause a gradual decrease in their movement, which can be observed over time. Additionally, some of the exposed zebrafish have been found to die, which suggests that PFOA exposure can be fatal.

After PFOA exposure, the mRNA levels of proinflammatory cytokines such as tnfα, il8, il1β, and il10 have been found to be increased, which suggests that PFOA may be involved in inflammation and tissue injury. However, more research is needed to determine the precise mechanisms underlying these effects, and to establish a causal relationship between PFOA exposure and inflammation.

In conclusion, PFOA is a highly persistent and prevalent environmental contaminant that has been shown to have toxic effects on many species, including zebrafish. It is important to continue studying the effects of PFOA exposure to better understand its impact on the environment and human health. Further research is needed to elucidate the molecular mechanisms underlying the observed effects of PFOA exposure on the immune system and to develop effective strategies for mitigating its adverse effects.

## 7 Acknowledgement:

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# 8 Literature review:

## 8.1 PFAS compounds case studies:

**PFAS**

Per- and polyfluoroalkyl substances (PFAS) is a lifetime chemical that has a long carbon chain bond and because of the high energy carbon-fluorine bond can make it resistant to hydrolysis, photolysis, microbial degradation, and vertebrate metabolism (Liang et al. 2022). PFAS are ubiquitous in the environment, are widely detected in human biomonitoring studies, and are of growing regulatory concern across federal, state, and local governments (Gaballah et al. 2020).

**PFHxA:**

perfluorohexanoic acid (PFHxA) studied with zebrafish embryos exposed in PFOS and PFOA in PCB126, done qPCR on the 24-26 hpf aged embryos examine the change in gene expression of metabolism of xenobiotics (ahr2, cyp1a), oxidative stress (gpx1a, tp53), lipids metabolism (acaa2, osbpl1a), and epigenetic mechanisms (dnmt1, dnmt3ba) and might have a role in increasing cell membrane permeability and solubilizing chemicals. PFHxA could affect gene expression with the help of PFOS and PCB126 (Blanc et al. 2017).

**PFHxS**

Environmental exposure of PFBS and PFHxS shows a negative significance with sperm count and sperm concentration show negative association with total motility. PFHpS was positively significant with sperm concentration and sperm count (Luo et al. 2022).

**PFHpS**

Studied in Norwegian ADHD and ASD patients, measured PFAS (PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonic acid (PFHpS), and perfluorooctane sulfonate (PFOS)) in maternal plasma in mid pregnancy. PFOA is associated with increased odds of ASD and ADHD (Skogheim et al. 2021).

Climate sensitive factors affecting PFAS, studying the concentration of the common goldeneye eggs, feeding compounds might have PFAS compounds, higher exposure in birds feeding at upper trophic levels. Egg laying date was positively associated to perfluoroheptane sulfonic acid (PFHpS), perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Bustnes et al. 2022).

Environmental effects of PFHpS and heavy metal were associated with the reduction of kidney function in adults. Quantified 13 PFAAs in serum and 14 heavy metals in plasma results associated with declined kidney function in single-pollutant models. major contributors with reduced kidney function in adults and PFHpS (Su et al. 2022).

**PFOS**

Perfluorooctanesulfonic acid and PFOA are the most common PFAS compounds in living things (human and biota). Environmental persistence and bioaccumulation have a toxic effect on the immune system in many species such as mice and zebrafish (Liang et al. 2022).

PFOS exposure in the farmed flatfish (Cynoglossus semilaevis) in china. 1695 and 5244 genes were identified as significantly increased and depressed, respectively. Significant expression changes were observed in immune-related genes. Also altered the gene expression levels of hormone (inhibin, insulin, somatostatin, and glucagon), which could lead to severe metabolic and endocrine dysfunction (Zhang et al. 2020).

PFOS are suspect to the increasing the risk of liver tumorigenesis. A hepatocellular carcinoma (HCC) model that may generate liver carcinogenesis by doxycycline (DOX) induction, was utilized to evaluate the influence of PFOS exposure on HCC advancement. The combination of PFOS and DOX can generate Kras-induced liver enlargement, and studies show that PFOS may accelerate liver tumor growth, as well as a drop in vitamin D levels and increased fatty acid intake. (Zhu et al. 2021).

The effect of chronic PFOS exposure on thyroid shape and function was studied using a zebrafish model from 8hpf to 120hpf. Thyroid hormone levels, thyroid follicular cell structure, and thyroid hormone-related gene expression were all affected by chronic PFOS exposure. (Chen et al. 2018).

Estrogenic effect of PFOS in swordtail (Xiphophorus helleri). Males with xiphoid caudal fins were used to study vitellogenin (VTG) mRNA expression in response to a variety of PFOS doses. To assess developmental damage, juveniles (20-30 days old) were exposed to 0 and 0.1mg/l PFOS for 90 days. VTG mRNA expression was dramatically reduced after one and two weeks but increased after three weeks. (Han et al. 2010).

For a comparative developmental toxicity assessment, zebrafish embryos were acutely exposed to varying doses of OBS and the positive control PFOS. Further research revealed that both OBS and PFOS impacted ciliogenesis. OBS and PFOS may act on ciliary motor proteins to disrupt ciliogenesis, resulting in ciliary dysfunction and offering a unique probable action pathway connected to developmental toxicity (Huang et al. 2021).

PFOS in the natural water ecosystem can affect the immune system and immune related genes of male zebrafish. After 21 days of exposure to PFOS accumulated in livers, growth of the adult zebrafish in the experiments was significantly inhibited, and the microstructures of the liver were seriously damaged. The expressions of immune-related mRNA were significantly affected (Guo et al. 2019).

PFASs accumulated in larvae in the order of F-53B > PFOS > OBS, with the bioconcentration factors ranging from 20 to 357. Point of Departure (PoD) indicates that metabolic end points at the molecular and organismal level are most sensitive to F-53B followed by PFOS and OBS. Collectively, F-53B has the highest bioconcentration potential and the strongest metabolism-disrupting effects, followed by PFOS and OBS (Tu et al.2019).

PFOS found in aquatic environment, chronic PFOs exposure lead to female biased sex ratio and in male by decreased sperm quality of zebrafish. the sex differentiation period, we observed elevated estradiol (E2) and decreased testosterone (T) levels in whole tissue homogenates from PFOS exposed juveniles. The estrogen receptor alpha (esr1) was significantly elevated in PFOS treated male gonads (Chen et al. 2016).

PFOS exposure in aquatic organisms on lipid metabolism. The effect of chronic exposure to low levels of PFOS in the zebrafish(F0) and their offspring(F1), There were a significant ultrastructure changes associated with metabolism in the liver and intestine of F0 and the transgenerational effect were also detected by quantitative PCR the results show that some genes(lepa,kiss1,xdh,insr) were significantly upregulated and some are downregulated(dgat1b, hb9, Apoa1) (Cui et al. 2017).

Chronic PFOS exposure to low levels of PFOS on lipid metabolism, this study shows a serious hepatic steatosis in liver of male zebrafish. Quantitative PCR indicated that PFOS significantly increased the transcriptional expression of nuclear receptors and the genes associated with fatty acid oxidation. Chronic PFOS exposure significantly decreased liver ATP content and serum level of VLDL/LDL lipoprotein in males (Cheng et al. 2016).

The main effect of PFOS in zebrafish larvae is an un-inflated swim bladder. The exact mechanisms leading to this effect are currently unknown. The results demonstrate that PFOS does not affect the budding phase, and does not cause deflation of already inflated swim bladders. (Hagenaars et al. 2014)

**PFOA**

PFOA studied in zebrafish (Danio rerio) embryos were exposed to PFOA and GenX. estimated kinetic bioconcentration factors (BCFkin). BCFkin for GenX was lower than PFOA at equimolar concentrations (Satbhai et al. 2022)

LC-MS lethal concentration analysis proved that PFOA can accumulate in the kidney of zebrafish. Hydropic endoplasmic reticulum, swelling of mitochondria and vacuolization were observed in kidney immune cells of zebrafish. PFOA could affect antibodies by increasing the concentrations of proinflammatory cytokines. Changes in antibody levels further influenced the expression of other cytokines, which eventually caused disorders in the zebrafish immune system (Zhang et al. 2021)

Male and female zebrafish (Danio rerio) were continuously exposed to radiolabeled perfluorooctanoic acid ((14)C-PFOA). Fish were sampled for liquid scintillation counting and whole body autoradiography to profile the bioconcentration and tissue distribution of PFOA. The highest labeling of PFOA in bile and intestines, which implies enterohepatic circulation of PFOA. in maturing vitellogenic oocytes, suggesting chemical accumulation via yolk proteins into oocytes with plausible risk for adverse effects on early embryonic development and offspring health (Ulhaq et al. 2015)

PFOA exposed fish were significantly smaller in total weight and length. Gene expression analysis found a significant decrease of transporters slco2b1, slco4a1, slco3a1 and tgfb1a, and a significant increase of slco1d1 expression. PFOA exposed fish produced significantly fewer eggs with reduced viability and developmental stage delay in F1 (Jantzen et al. 2017)

In the zebrafish chemotaxis assay, this study showed that wounding induced significant neutrophil migration to the site of injury, and that neutrophil number in the wound region was significantly reduced in response to 48-h PFOA exposure (Pecquet et al. 2020)

Zebrafish fertilized eggs were exposed to different concentrations of PFOA. Dopaminergic neuron development might be one of the targets of early-life PFOA exposure, the locomotor activity of zebrafish was decreased, the mRNA levels of nuclear receptor subfamily 4 group a member 2b (nr4a2b), paired box 2 and 5 (pax2, pax5), tyrosine hydroxylase 1/2 (th1/th2) and dopamine transporter (dat) were increased (Yu et al. 2021).

Zebrafish (Danio rerio) were exposed to nominal concentrations of PFOA. After exposure, each fish was decapitated, and the spleen was removed to detect the expression patterns of P65 transcription factor, myeloid differentiation 88, relative interleukins (ILs), and antibody genes. Myd88/NF-κB pathway is mediate inflammatory cytokine (IL-1β and IL-21) in zebrafish spleen. IL secretion disorder is possibly closely related to PFOA-induced TLR2 damage in zebrafish spleen (Zhang et al. 2014)

HPLC-MS analysis proved that PFOA can accumulate in the spleen of zebrafish. Morphological observations revealed that PFOA can damage immune cells in zebrafish spleen by inducing vacuolization, lipofuscin granule production, and mitochondrial swelling.
The Ig-mediated pathway can be affected by PFOA (Zhong et al. 2020).

PFOA distribution in tissues and estimate the exposure stages. Zebrafish were continuously exposed to 25 mg/L PFOA for 30 days to simulate the environmental process. Three levels (mild, moderate, and deep pollution stage) of PFOA pollution in zebrafish can be evaluated (Bian et al. 2022).

**GenX**

Apoptotic capacity of GenX in human liver cells was investigated. Shows human-derived liver cells (HepG2 cells) were treated with GenX for 12 h, cell viability was reduced, and apoptosis was greatly increased (Yoo et al. 2021)

Explored the underlying mechanisms of PFOA and GenX induced hepatocellular damage. Liver hepatocellular carcinoma cell line HepG2 was used as a model to study induced liver inflammation in vitro at the cellular, genetic, and epigenetic levels. With GenX, the global methylation level decreased and then increased (Wen et al. 2020).

Rat brain capillaries were isolated to measure transporter activities. Resulting nanomolar levels of GenX inhibited P-gp and BCRP but not MRP2 transport activities in male and female rat brain capillaries, and GenX reduced P-gp and BCRP transport activity in human cells.
(Cannon et al. 2020).

Investigated the dose-dependent effects of GenX on primary human hepatocytes (PHH). At lower doses GenX can interfere with metabolic pathways and at higher doses can induce fibroinflammatory changes in human hepatocytes (Robarts et al. 2022).

**ADONA**

ADONA was detected only in a few samples slightly above the limit of quantification in the drinking water by collecting plasma samples of German blood donors living in South Germany at different time points (Fromme et al. 2017).

PFASs can enter thyroids. Studied thyroid disrupting effects of PFOA, GenX and ADONA in vitro with both rat thyroid cell line FRTL5 and primary normal human thyroid (NHT) cells. PFOA and GenX reduced thyroid cell viability. Thyroid disrupting effects are increased in the order of GenX > PFOA > ADONA. (Zhang et al. 2021).

**PFBS**

Acute zebrafish larvae in the PFBS until 168hpf with probiotic Lactobacillus rhamnosus bacteria. PFBS exposure significantly decreased the larval body weight, weight gain and specific growth rate, while probiotic supplementation efficiently inhibited the growth retardation caused by PFBS (Sun et al. 2021). PFBS exposures repress hatchability while increasing malformation and mortality in zebrafish embryos, cause reproductive toxicity and hepatotoxicity, disrupt thyroid functions, and damage embryonic development. PFNA exerts more severe cardiotoxic effects in zebrafish when compared with PFBS (Gong et al. 2022).

An acutely exposed zebrafish larvae along with probiotics (Lactobacillus rhamnosus).
Probiotics supplements can reduce the toxicity of PFBS. Acute exposure to PFBS significantly increased the cortisol concentration in zebrafish larvae, subsequently inducing stress response and hyperactive behavior (Hu et al. 2022).

Probiotic supplements are able to mitigate the growth retardation defects of PFBS. PFBS single exposure significantly increased the cortisol concentrations, suggesting the induction of stress

response, while probiotic supplementation effectively decreased the cortisol levels in co-exposed larvae in an attempt to relieve the stress of PFBS toxicant (Sun et al. 2021).

Adult zebrafish were exposed to PFBS. After PFBS or/and probiotic exposures, PFBS alone increases blood glucose level. Liver of male fish from the coexposure group functioned appropriately, which immediately increased insulin levels (Liu et al. 2021)

PFBS exposed to adult zebrafish with or without probiotic. In female fish, the PFBS and probiotic combination enhanced fatty acid synthesis and β-oxidation, but mitigated the accumulation of cholesterol in the blood compared with PFBS single exposure. Co-exposure to PFBS and probiotics caused significant accumulation of triglyceride in male livers (Chen et al. 2020).

Exposed adult zebrafish in PFBS. Probiotics inhibited the dysbiosis of PFBS and shaped the skin microbiome in the combined exposure group. PFBS single exposure also promoted the production of mucus on the skin of male zebrafish (Hu et al. 2021).

Aged zebrafish exposed to PFBS at environmentally relevant concentrations. PFBS exposure significantly inhibited the enzymatic activity of α-amylase in the gut, but increased the alanine aminotransferase (ALT) activity in the blood of the aged zebrafish. Benefit young fecal transplantation to protect the aged from the glucose metabolism toxicity of PFBS (Liu et al. 2022). Maternal preconception PFBS exposures were found to alter egg and embryo development of zebrafish offspring, which is mediated by direct toxicant loading in the eggs, nutrient loading into eggs, and the function of Nrf2a (Annuziato et al. 2022).

Dechorionated zebrafish embryos from two different transgenic fish lines (Tg[insulin:GFP], Tg[ptf1a:GFP]) exposed in PFBS. Embryos had significantly increased caudal fin deformities, delayed swim bladder inflation, and impaired yolk utilization. PFBS exposed disturb embryonic development, energy homeostasis, and pancreatic organogenesis (Sant et al. 2019).

**PFPeS**

perfluoropentanesulfonic acid (PFHpS), perfluorononanesulfonic acid (PFNS)] were exclusively or significantly more frequently detected at higher levels in firefighters compared to controls (Rotander et al 2015)

**PFESA1**

ADONA, PFESA1, or PFOA exposure resulted in detectable levels of parent compound in larval tissue but yielded negative toxicity results. (Gaballah et al. 2020).

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