Toxicity of shorter-chained perfluoroalkyl substances (PFAS) during early life stages of zebrafish (*Danio rerio*);

assessment of morphological effects and activation of the peroxisome proliferator-activated (Ppar) signalling pathway

Mari Eline K. Dybwad

Master of science in biology in Environmental Toxicology

Department of Biological Sciences

University of Bergen

June 2023

Supervisors: Nadja R. Brun, Ståle Ellingsen, Anders Goksøyr and Odd A. Karlsen

Acknowledgments

This master thesis has been funded by “Felleslegat til fordel for biologisk forskning” fund at the Faculty of Mathematics and Natural Sciences, University of Bergen. Additionally, NRB is supported by a Marie Skłodowska-Curie Actions (MSCA) individual fellowship, grant agreement Number 101023171.

First and foremost, I would like to express my gratitude to all my supervisors Odd André Karlsen, Ståle Ellingsen, Nadja Rebecca Brun and Anders Goksøyr. I really appreciate all the help, wisdom and support you have given me throughout this year, as well as your enthusiasm and interest in my work, both in the laboratory and during the writing process. To Odd André, Ståle and Nadja, I also wish to say a big thank you for all the Friday meetings, answers to my many questions and for the guidance through my master thesis, I really appreciate it. Furthermore, I would like to express my gratitude for the opportunity to attend the NSFT winter meeting in Beitostølen, January 2023.

I would like to extend my gratitude to Nadja for teaching and helping me in the laboratory, with the setup of the project, help with calculations and support through this year. I also want to thank all the people in the environmental toxicology group, including fellow students, for providing a supportive environment, for all your help and for sharing your knowledge with me.

Finally, I would like to thank my family and friends for all their support during my education, and the interest you have shown in my thesis. To my friends from BIO, I really appreciate all of you and our friendships. I also want to give an extra big thanks to Kjetil for all the encouragement, and for always supporting and believing in me.

Bergen, May 2023

Mari Eline K. Dybwad

Abstract

Per- and polyfluoroalkyl substances (PFAS) are synthetic fluorinated compounds that are persistent in the environment and possess bioaccumulative and biomagnifiable properties. Exposure to PFAS has been associated with developmental and reproductive toxicity, liver dysfunction, and potential carcinogenicity in both humans and wildlife. These characteristics, combined with their ubiquitous presence in nature and biota, have raised global concerns. The long-chained perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been extensively used for many decades and are included in the Stockholm Convention on Persistent Organic Pollutants. Due to the restrictions and limitations on their use, new shorter-chained and branched PFAS congeners have emerged as replacement compounds. Perfluorobutanoic acid (PFBA) and perfluorobutane sulfonate (PFBS) are two short-chained homologues of PFOA and PFOS, possessing similar physico-chemical properties as their long-chained predecessors. So far, most toxicological studies have focused on the long-chained PFAS, with far fewer focusing on the newer shorter-chained congeners. Previous research has demonstrated the activation of the peroxisome proliferator-activated receptor alpha (Ppara) by certain PFAS congeners, such as PFOS and PFOA. In this thesis, zebrafish (*Danio rerio*) development were exposed in vivo to four PFAS congeners, i.e., PFOA, PFOS, PFBA and PFBS, as well as a Ppara selective agonist (WY14643), during the early stages of development. Morphological changes in addition to lethal and sub-lethal endpoints were monitored, while effects on the Ppara signalling pathway were assessed by gene expression analyses of the *pparaa, pparab, cyp1a* and *acox1* genes using quantitative polymerase chain reaction (qPCR). All PFAS congeners were found to influence the hatching rate or hatching success. PFBS exposure affected survival, while PFOS exposure caused severe spine deformities. Additionally, zebrafish larvae exposed to PFOS, PFBS, and WY14643 showed reductions in body length, eye size, and yolk sac area at 96 hours post fertilization. In line with previous studies, WY14643 upregulated most genes in the Ppara signalling pathway. Notably, also all PFAS congeners had an effect on the Ppara signalling pathway, although the specific genes that were impacted varied among the different compounds. Our findings suggest that carbon backbone length of the PFAS congeners played a more prominent role in modulating the Ppara signalling pathway in comparison to the functional group. The findings indicate that exposure to shorter-chained PFAS congeners can cause adverse effects during early life stages of zebrafish. displaying both distinct toxicity profiles and Ppara modulation patterns.

Abbreviations

Acox1	Acyl-coenzyme A oxidase 1
ADONA	Dodecafluoro-3H-4,8-dioxanonanoate
AF-1	Activation function 1
AF-2	Activation function 2
ANOVA	Analysis of variance
AR	Androgen receptor
cDNA	Complementary DNA
Cpt1a	Carnitine palmitoyltransferase 1A
Cq	Quantification cycle
DMSO	Dimethyl sulfoxide
DBD	DNA binding domain
DDT	Dichlorodiphenyltrichloroethane
Dpf	Days post fertilisation
ECHA	European Chemicals Agency
EDCs	Endocrine disruptive compounds
ER	Estrogen receptor
F-53B	6:2 chlorinated polyfluoroalkyl ether sulfonate
FA	Fatty acids
FAO	Fatty acid oxidation
HCB	Hexachlorobenzene
HFPO-DA	Perfluoro(2-methyl-3-oxahexanoic
Hpf	Hours post fertilization
ISH	*In situ* hybridisation
LBD	Ligand binding domain
MOPS	3-Morpholinopropane-1-sulfonic acid
MQ	Milli-q
NaOH	Sodium hydroxide
NF	Nuclease free water
NR	Nuclear receptor
PCBs	Polychlorinated biphenyls

PCR	Polymerase chain reaction
PFAS	Per- and polyfluoroalkyl substances
PFBA	Perfluorobutanoic acid
PFBS	Perfluorobutanesulfonic acid
PFHxA	Perfluorohexanesulphonic acid
PFHxS	Perfluorohexanesulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFPeA	Perfluoropentanoic acid
POPs	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptors
PPRE	Peroxisome proliferator responsive elements
Rpm	Rounds per minute
PXR	pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RT-PCR	Real-time PCR
RXR	Retinoid X receptor
TAB	Tübingen AB
TAE	Tris-acetate-EDTA
Zf	Zebrafish

Table of content

1. Introduction

1.1 Perspective

The rapid growth of industrialisation, urbanisation and modern agricultural development has led to an increase in environmental pollutants in nature at an alarming rate. The pollutants are introduced to the environment by anthropogenic activities such as shipping, oil drilling, use of pesticides, mining, waste discharges, and industries. Once introduced into the environment, these chemicals can further spread by ocean currents, rivers, and atmospheric transport. After transportation, they tend to reappear in food, feed and drinking water, potentially harming both animals and humans (FHI, 2021). The ability to accumulate in organisms and be resistant towards degradation are common traits for many environmental pollutants (Rasheed *et al*., 2019). Potential adverse effects resulting from exposure to such chemicals are many and include disruption of reproductive abilities, altered behaviour, and developmental effects (ibid.).

One important group of environmental contaminants are the persistent organic pollutants (POPs). This group of compounds consists of products and byproducts from industrial processes, including chemicals such as polychlorinated biphenyls (PCBs), per- and polyfluoroalkyl substances (PFAS), dioxins and furans, among several others (Alharbi *et al.,* 2018). POPs possess physicochemical properties that make them persistent and stable in the environment, as well as being lipophilic, allowing their ability to bioaccumulate in fat-rich tissue in animals and biomagnify through the food chains (Figure 1.1.1) (ibid.). As many POPs have the potential for long-range transportation, the release from one location can affect nature and wildlife far from where it originates, including remote areas such as the Arctic and polar regions. In wildlife certain mammals, birds, and fish have experienced a decline in population levels, and abnormalities and diseases in many animals have been linked to POPs exposure (EPA, 2023). Furthermore, studies have linked POPs to a variety of human diseases, including diabetes, obesity, cancer, and endocrine disruption (Alharbi *et al*., 2018). These associations have brought significant attention to environmental and human health issues. As a result, global concerns regarding POPs have escalated since the early 2000s. Today, POPs are recognised as global challenges that must be addressed by internationally coordinated efforts. As an initial approach, the Stockholm convention on persistent organic pollutants was signed in 2001 as the

first global treaty to reduce and/or eliminate the production, release, and use of 12 abundant POPs, known as the "dirty dozen". These POPs were recognised for their potential adverse effects on both human health and wildlife, and included pesticides such as aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, mirex and heptachlor, as well as industrial chemicals and biproducts such as hexachlorobenzene (HCB), PCBs, furans and dioxins (Stockholm convention, 2019). Additional pollutants have since been added to this list, including chlordecone, hexachlorobutadiene, pentachlorobenzene, lindane, as well as the PFAS congeners perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Tokuç A. 2013; Stockholm convention, 2019; Menger *et al*., 2020).

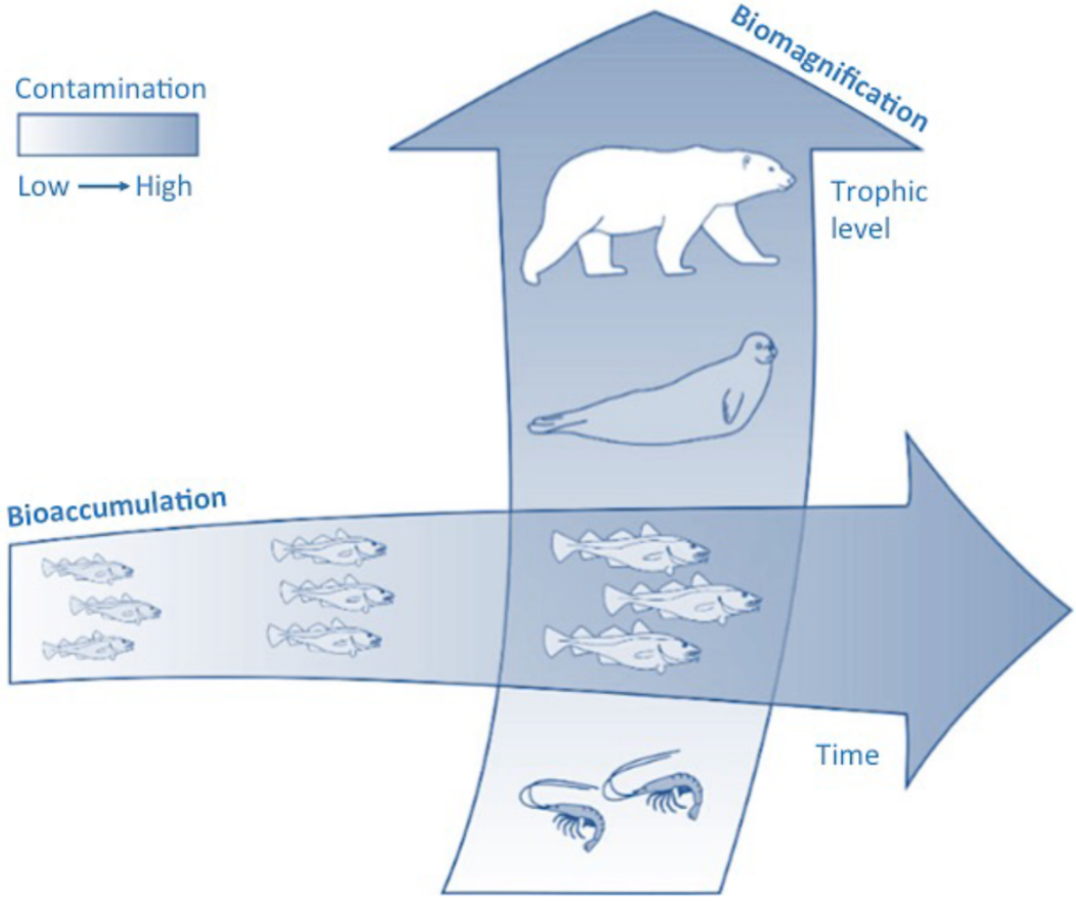


Figure 1.1.1: Illustration of bioaccumulation and biomagnification of POPs in a marine food web. Illustration source: Søderstrøm, S. 2017, based on an illustration from Madsen, A. K. 2016.

1.2 Per- and polyfluoroalkyl substances

The group of organic, fluorinated compounds known as PFAS have been used both in industrial and commercial products since the late 1940s (Glüge *et al*., 2020). It is assumed that over 4700 PFAS congeners (Glüge *et al*., 2020; Liu *et al*., 2019) are, or have been, available on the commercial market and are present in a variety of industrial and everyday products, including non-stick cookware, pesticides, personal care products, paint, fire-fighting foams and more (Mahoney *et al*., 2022; Gebreab *et al*., 2020; Buck *et al*., 2011). The carbon-fluorine bonds in these molecules give them a high thermal stability and an increased chemical and biological resistance, which in turn makes them extraordinary stable and persistent in the environment. Hence, they are often referred to as "forever chemicals" (Hagenaars *et al*., 2011; Mahoney *et al*., 2022). The extensive use of PFAS in a variety of products led PFAS to being considered as global environmental contaminants (Menger *et al*., 2020). As for other environmental contaminants, they are introduced into the environment through anthropogenic activities, and further transported with rivers, ocean currents and wind, resulting in a ubiquitous global distribution (Blake and Fenton, 2020). PFAS accumulate in biota, with especially high levels of PFAS found in marine top predators and mammals, like polar bears, killer whales, seals, and birds (Galatius *et al*., 2013; Sciancalepore *et al*., 2021). This accumulation is assumed to be due to PFAS's high affinity towards plasma proteins, allowing them to bind to blood proteins and accumulate in tissues with high protein content, such as the kidneys and liver (Tartu *et al.,* 2017).

The widespread human exposure to PFAS, combined with their high biological half-life and environmental persistence, have also resulted in measurable levels of PFAS in the blood of the general human population (reviewed by Fenton *et al.,* 2021). In addition to being detected in human blood, PFAS has also been measured in urine, breastmilk, hair and nails (reviewed by Jian *et al*., 2018). Exposure to PFAS has the potential of causing a variety of adverse health effects depending on the exposure conditions, such as route of exposure, magnitude, and duration, as well as factors related to the exposed individuals, e.g., sex, age, ethnicity, genetic predisposition, and health status (reviewed by Fenton *et al.,* 2021).

So far, most toxicological studies on PFAS have been conducted on the long-chained PFAS congeners, the carboxylic acid PFOA and the sulfonic acid PFOS (Figure 1.2.1). Studies have

linked exposure to PFOA and PFOS to a range of adverse health effects, with both compounds causing developmental and reproductive toxicity in animals. Furthermore, some studies using laboratory animals have tied PFOA exposure to altered thyroid function and reduced birthweight, as well as inducing tumours in testicles, liver, and pancreas (Steenland, Fletcher and Savitz, 2010). Other studies have shown that exposure to PFOS may cause hepatotoxicity, delayed development, neurotoxicity, immunotoxicity, reproductive toxicity, thyroid disruption, behavioural effects, pulmonary toxicity, renal toxicity, and cardiovascular toxicity in both laboratory animals and in *in vitro* human systems (Zeng *et al*., 2019; Mylroie *et al*., 2021).

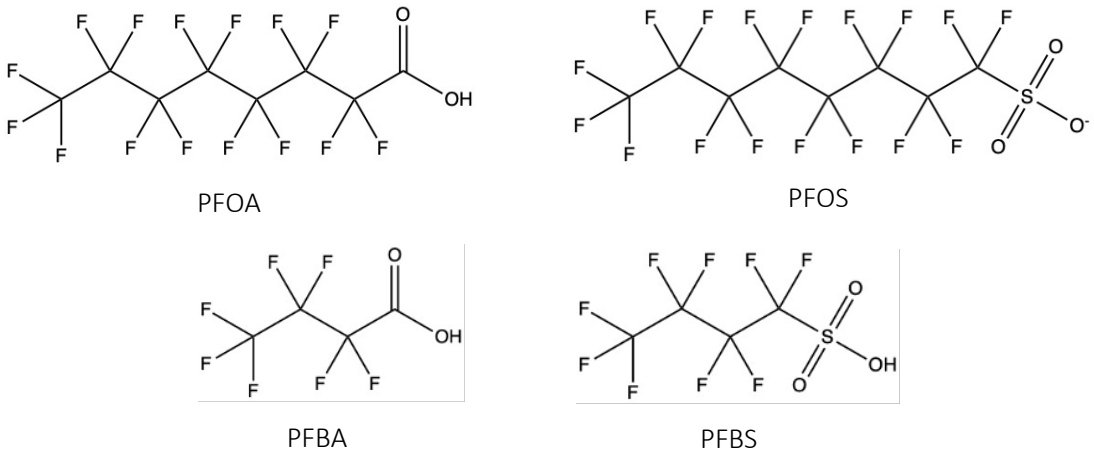


Figure 1.2.1: Chemical structures of four PFAS congeners. Chemical structures of two long-chained and two short-chained PFAS congeners. PFOA ($CF_3(CF_2)_6COOH$) and PFOS ($CF_3(CF_2)_7SO_3^-$) possess a fluorinated seven and eight-carbon chain, respectively, whilst the shorter-chained congeners, PFBA ($CF_3(CF_2)_2COOH$) and PFBS ($CF_3(CF_2)3SO_2OH$) possess a fluorinated three and four-carbon chain, respectively. PFOA and PFBA exhibit a carboxyl functional group and PFOS and PFBS containing a sulfonated functional group. The structures are drawn in ChemDraw 20.0.

Upon recognising the ubiquitous distribution and undesirable impacts of long-chained PFAS exposure on both wildlife and humans, several PFAS manufacturers initiated a phase-out of the production of these compounds. Instead, they substituted long-chained PFAS with their shorter-chained homologues possessing similar physico-chemical properties (Wang *et al*., 2013; Hagenaars *et al*., 2011; Brendel *et al*., 2018). Perfluorobutanoic acid (PFBA) and perfluorobutanesulfonoic acid (PFBS) (Figure 1.2.1) are short-chained PFAS that have emerged and are generally considered as safer alternatives for PFOA and PFOS, especially due to the shorter half-lives observed in animal models (Weatherly *et al.*, 2021). However, in recent research, similar adverse effects as their predecessors have been suggested (Gomis *et al*.,

2018). Moreover, a study found that PFBA induces liver toxicity and alterations of peroxisome proliferator-activated receptor (PPAR) target genes in mice (ibid.), whilst a study by Pérez *et al.*, (2013) utilising human tissue found a high accumulation of PFBA in lungs and kidneys in comparison to PFOA. For PFBS, some studies have reported that exposure can affect fertility, reproduction, size, and body weight of rats in two-generation studies (Chen *et al.*, 2018; Lieder *et al.*, 2009; Luebker *et al.*, 2005). PFBS exposure have also been found to disturb gut microbiota, retinoid metabolism and visual signalling in teleosts (Hu *et al.*, 2020). As there is a limited number of PFAS compounds with well characterised health effects, as well as hundreds of other PFAS lacking toxicity data, it is important to study and characterise the toxicity and potential adverse effects associated with the exposure to these emerging chemicals.

1.3 Nuclear receptors

Nuclear receptors (NR) are a superfamily of ligand-activated transcription factors that play an essential role in regulation of a wide range of physiological processes within all vertebrate species, including development, metabolism, cell proliferation, reproduction, enzymatic activities, and immune responses (Mitchell *et al.*, 2018; Schaaf, 2017; Weikum and Ortlund, 2018). All NRs share a structurally similar modular domain structure and functional domains, i.e., an N-terminal domain that harbours the transcriptional activation function 1 (AF-1), a DNA binding domain (DBD) containing a zinc-twist structure, a hinge region, and a C-terminal ligand-binding domain (LBD) that hosts the activation function 2 (AF-2) (Figure 1.3.1) (Schaaf, 2017; Weikum and Ortlund, 2018; Kersten, 2014). The amino acid sequence variations in the DBD and LBD allow different members of the NR superfamily to bind various ligands and regulate different cellular processes in order to preserve normal cellular functioning (Schaaf, 2017; Weikum and Ortlund, 2018).

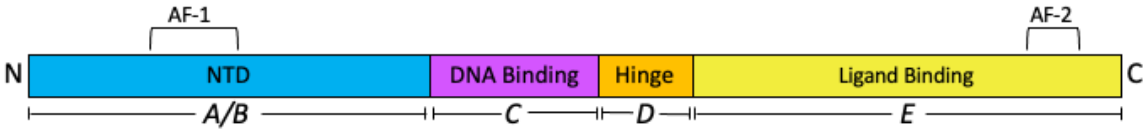


Figure 1.3.1: Modular domain structure of nuclear receptors (NR). Functional domains in NRs constituting of *A/B*: an N-terminal domain containing the activation function 1 (AF-1), *C*: a DNA binding domain, *D*: a hinge region, *E*: and a C-terminal ligand-binding domain containing the activation function 2 (AF-2). Adapted figure from Weikum and Ortlund (2018).

It has been demonstrated that many POPs can act as agonists, or antagonists, and thus activate or inhibit, respectively, the transcriptional activity of several NRs, such as the estrogen receptor (ER) (NR3A2), androgen receptor (AR) (NR3C4), pregnane X receptor (PXR) (NR1I2), and the peroxisome proliferator-activated receptors (Delfosse *et al.,* 2015). Such POPs are often denoted as endocrine disruptive compounds (EDCs), having the ability to activate or inhibit NRs through several mechanisms, and consequently modifying cellular processes and signalling pathways. One mechanism is by binding to the nuclear receptors directly through the LBD, thus altering the structural conformation of the NR. EDCs can also interfere with the synthesis, transport and metabolism of endogenous hormones, leading to altered levels of hormones that can bind to and activate or inactivate NRs. Additionally, EDCs can bind to co-regulators, which are proteins that interact with the NRs and either enhance or inhibit their activity (Balaguer, Delfosse and Bourguet, 2019; Delfosse *et al*., 2015). The conformational changes in NRs from interactions with EDCs may lead to adverse health effects, including reproductive and developmental problems, metabolic dysfunction, insulin resistance, immunotoxicity, and cancer (Behr *et al*., 2020; Blake and Fenton 2020; Margolis and Sant 2021; Ehrlick *et al*., 2023).

1.4 Peroxisome proliferator-activated receptors

At the molecular level, PFAS-mediated toxicities are recurrently associated with activation of NR transcription factors, and particularly the peroxisome proliferator-activated receptors (Takacs and Abbott, 2007). PPARs belong to the NR1C subfamily within the superfamily of NRs (Tyagi *et al*., 2011). Three PPAR subtypes have been described in vertebrates, PPARa (NR1Cx), PPARb (NR1Cy) and PPARg (NR1Cz), which are encoded by different genes (Schaaf, 2017). The three PPAR subtypes differ in target gene repertoire, tissue distribution and ligand specificity. PPARa is highly expressed in the liver, where it regulates fatty acid catabolism, but is also expressed in other tissues such as the heart, kidneys and skeletal muscle (Kersten, 2014). PPARg is among the most frequently studied NR and is known for its expression in adipose tissue and its role in lipid accumulation and adipogenesis, in addition to glucose homeostasis and insulin sensitisation (Schaaf, 2017; Urbatzka *et al.,* 2013; Venezia *et al*., 2021). PPARb is not as widely researched as the two other subtypes. It is ubiquitously expressed at lower levels and suspected to be involved in the control of lipid homeostasis, glucose uptake, and in organogenesis, as it is expressed in early embryonic development (Kersten, 2014; Schaaf, 2017; Urbatzka *et al.,* 2013).

In some teleost, two orthologs of Ppara[1] is present, denoted as Pparaa and Pparab. It has been shown that the two orthologs of mammalian PPARa have a similar role during neural development, with regulating the formation of neurons and glial cells (Hsieh *et al.*, 2018; Li *et al*., 2020). Research on *pparaa* and *pparab* using zebrafish have shown that *pparab* is expressed more in tissues with a high oxidative activity than *pparaa* (Li *et al*., 2020). Following binding of a ligand to PPAR, it forms a heterodimer with the nuclear receptor retinoid X receptor (RXR) (NR2B). This heterodimer (PPAR:RXR) then binds to peroxisome proliferator responsive elements (PPRE) in the promotor regions of the target genes. This binding leads to a recruitment of co-activators and co-repressors, which in turn regulate gene transcription (Figure 1.4.1) (Huang, 2008; Schaaf, 2017; Gebreab *et al*., 2020; Hsieh *et al*., 2018). Target genes regulated by PPARa include those involved in lipid and glucose metabolism, such as *acox1* (acyl-coenzyme A oxidase 1) and *cpt1a* (carnitine palmitoyltransferase 1A) (Rakhshandehroo *et al*., 2010). Both proteins are key regulatory enzymes for beta-oxidation, with *acox1* catalysing the first step of straight-chained fatty acids, while *cpt1a* is a mitochondrial enzyme that catalyses the transfer of long-chained fatty acids across the inner mitochondrial membrane (Rakhshandehroo *et al*., 2010; Gobin *et al.*, 2003).

[1] In this thesis, the nomenclature used follows the "HGNC" Guidelines for naming proteins. Proteins from mammals are written in all capital letters, whilst short names of fish proteins are written with only the first letter capitalised, as described by Dunn *et al.,* (2019).

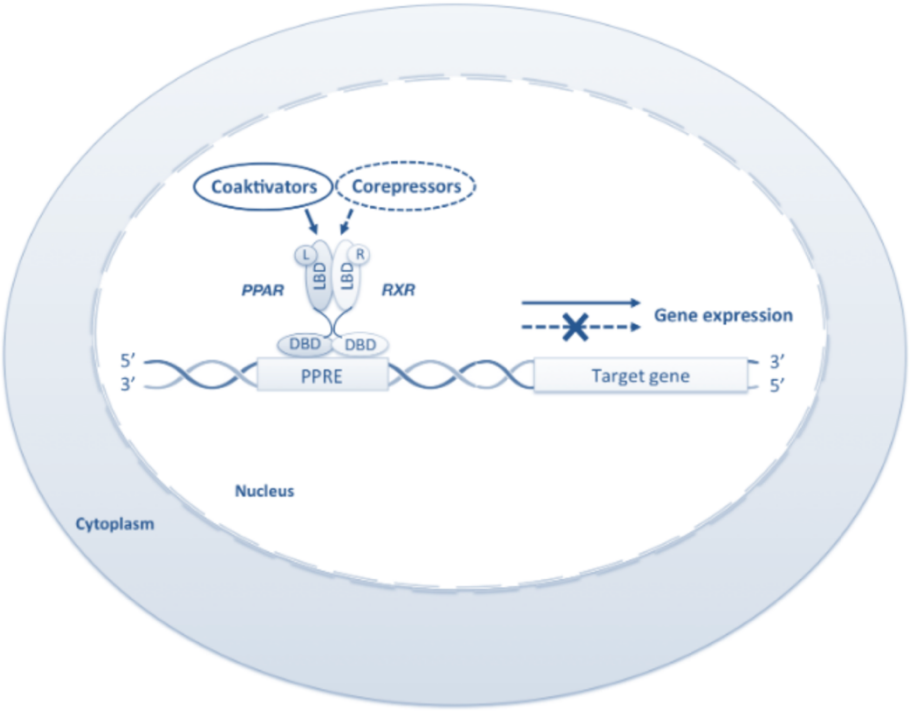


Figure 1.4.1: Mechanism of ligand activation of peroxisome proliferator-activated receptors (PPARs). PPAR recognise and bind to ligand, the receptor then heterodimerise with retinoid X receptor (RXR) and form the heterodimer PPAR:RXR. This heterodimer binds to peroxisome proliferator response elements (PPREs) located in the promotor region of target genes. In response to this binding a recruitment of co-activators and co-repressors are facilitated, allowing the transcription of target genes. Illustration source: Sofie Søderstrøm 2017.

PFAS share structural similarities to fatty acids (FA), which also consists of a hydrophobic carbon backbone of varying length and a hydrophilic carboxylated functional group at the end (Shabalina *et al*., 2016). Being structurally similar to FA, several carboxylated PFAS congeners, including PFOA, perfluorononanoic acid (PFNA) and perfluorohexanoic acid (PFHxA), as well as sulfonated PFAS congeners such as PFOS and perfluorohexanesulfonic acid (PFHxS), have previously been demonstrated to activate PPARa and PPARg pathways in both fish and mammals (Behr *et al.,* 2020; reviewed by Fenton *et al*., 2021; Almeida, Eken and Wilson, 2021; Rosen *et al*., 2017; Søderstrøm *et al*., 2022, Ehrlic *et al*., 2023). Activation of PPARa and PPARg pathways by PFAS has been associated with a range of physiological effects, including developmental and reproductive effects, such as decreased foetal growth, altered sexual development, and decreased fertility in animals (reviewed by Fenton *et al*., 2021). In addition, PPARg activation by PFAS has been shown to affect multiple aspects of the immune system, due to acting as a key regulator for mast cells and the presence of PPARg in β cells (Ehrlic *et al*., 2023). It has also been shown that PFAS-induced PPARa activation can lead to an upregulation

in genes involved in fatty acid oxidation and lipid transport (Rosen *et al*., 2017). A study by Søderstøm *et al*., (2022) demonstrated an activation of gmPpara1 in Atlantic cod (*Gadus morhua*) by three carboxylated PFAS congeners, including PFOA, PFNA and PFHxA, as well as the sulfonic congener PFHxS. In addition, it was reported a potentiating interaction effect when gmPpara1 was treated with both PFOA and PFOS simultaneously. Notably, no activation of gmPpara2 was observed with any of the PFAS congeners tested.

1.5 Zebrafish as a model species

Zebrafish (*Danio rerio*) is an increasingly popular model organism used for *in vivo* studies in many contexts, e.g., biomedicine, developmental biology, and gene regulation. Its genome was sequenced and annotated in 2001, revealing many genetic and physiological similarities to mammals (Howe *et al*., 2013). Zebrafish has a particularly high value in toxicological and biomedical studies concerning embryonic development and morphogenesis (Schaaf, 2017), taking advantage of their small size, easy maintenance, short generation time, rapid embryogenesis, and high fecundity, in addition to their embryonic and larval transparency (Sri Ranjani *et al.,* 2020; Schaaf, 2017). One important property is that most compounds can easily penetrate their skin, particularly during early embryonic larval stages. Thus, exposure to toxicological compounds is simply done by adding the substance to the surrounding water (Schaaf, 2017). Due to the advantages with zebrafish as a model, it has been used to study potential adverse effects of many environmental contaminants, including legacy PFAS such as PFOA and PFOS. Some studies have shown that exposure to these compounds can lead to developmental abnormalities, neurotoxicity, and liver damage in zebrafish embryos and larvae (Gaballah *et al*., 2020; Menger *et al*., 2020; Yang *et al*., 2023). Today, zebrafish are increasingly used to study the potential toxic effects of the new emerging PFAS congeners (Gaballah *et al*., 2020).

1.6 Aims and objectives

Currently, the majority of the available toxicity data for PFAS are primarily obtained for the long-chained legacy congeners. Due to limited information on toxicity of the new generation of PFAS molecules, as well as an increase in their use, the need for knowledge on their potential toxicity is higher than ever before.

The aim of this master thesis was to characterise the toxicity of the two shorter-chained PFAS congeners, PFBA and PFBS, during early developmental stages of zebrafish, and to compare their potential toxic effects and morphological changes with the two legacy, long-chained PFAS, i.e., PFOA and PFOS. Additionally, the aim was to obtain insights about the molecular mechanisms underlying the toxicity of the shorter-chained PFAS congeners by investigating changes in the expression of Ppara target genes. Analyses of PFAS-mediated modulation of the zebrafish Ppar signalling pathways were to be supported by exposing zebrafish to the Ppara-selective agonist, WY14643.

The specific objectives for this thesis are listed below.

I. Expose developing zebrafish from 2 to 96 hours post fertilisation (hpf) to five different concentrations of PFBA, PFBS, PFOS, PFOA, and the Ppar-agonist WY 14643, and assess selected lethal and sublethal endpoints until 96 hpf, including coagulation, lack of heartbeat, lack of movements, pigmentations, malformations, edema, developmental retardation, and lack of detachment of the tail bud from the yolk sac.
II. Record morphological changes, including length, area of yolk sac, and eye size, on exposed zebrafish larvae at 96 hpf.
III. Examine activation of the Ppara signalling pathway by assessing changes in gene expression of selected Ppara target genes (*cpt1a* and *acox1*) as well as the receptor encoding genes (*pparaa* and *pparab*) by qPCR analysis.

2. Material

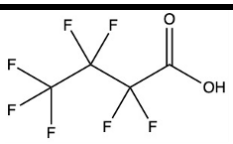
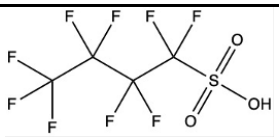
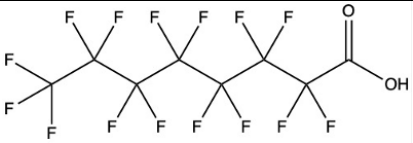
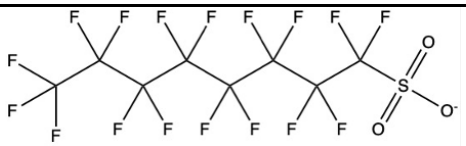
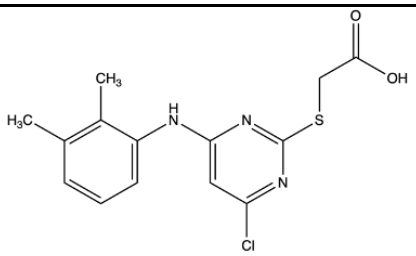
2.1 Test species

Wild type zebrafish of the Tübingen AB (TAB) strain were used for all experiments and kept under a 14:10 light/dark photoperiod at 28±1°C at the zebrafish facility at BIO, University of Bergen. The zebrafish facility at BIO is approved by Mattilsynet (The Norwegian Food Safety Authority) (Ref 21/112624).

2.2 Test chemicals

The chemicals presented in Table 2.2.1 were used during the exposures of zebrafish embryos and larvae.

Table 2.2.1: List of chemicals used for exposure studies in this thesis, including supplier and chemical structure.

Ligand	Supplier	Structure
Perfluorobutanoic acid (PFBA)	Sigma-Aldrich	
Perfluorobutanesulfonic acid (PFBS)	Sigma-Aldrich	
Perfluorooctanoic acid (PFOA)	Sigma-Aldrich	
Perfluorooctanesulfonic acid (PFOS)	Sigma-Aldrich	
WY14643 (pirinixic acid)	Sigma-Aldrich	

2.3 Chemicals and reagents

Table 2.3.1: List of chemicals and reagents used in this thesis

Name	CAS # / Cat #	Supplier
6x loading dye	-	Promega
2Log DNA ladder	-	New England Biolabs
Agarose	9012-36-6	Sigma-Aldrich
$CaCl_2 \bullet 2H_2O$	-	MERK
Chloroform	67-66-3	Sigma-Aldrich
DMSO	67-68-5	Sigma-Aldrich
Ethanol	64-17-5	VWR chemicals
Formamide	75-12-7	Sigma-Aldrich
GelRed	41003 *	Biotium
IScript cDNA Synthesis Kit	170-8891 *	BioRad
Isopropanol	-	Antibac
$MgCl_2 \bullet 6H_2O$	7791-18-6	Sigma-Aldrich
Milli-Q	-	Lab-tec
MOPS (10 mM)	1132-61-2	Sigma-Aldrich
NaCl	7647-14-5	Sigma/MERK
Nuclease free water	7732-18-5	VWR chemicals
PFBA	375-22-4	Sigma-Aldrich
PFBS	375-73-5	Sigma-Aldrich
PFOA	335-67-1	Sigma-Aldrich
PFOS	1763-23-1	Sigma-Aldrich
LightCycler 480 SYBR Green I Master	-	BioRad
SsoAdvanced Universal SYBR® Green Supermix	1725274 *	BioRad
WY14643	50892-23-4	MERK/Sigma
Tricaine (powder)	5704-04-1	Sigma-Aldrich
TriReagent (Trizol)	-	Sigma-Aldrich
Tris base (1,5M)	161-0798 *	BioRad

* Cat nr.

2.4 List of solutions and media

60x stock of E3 medium was made with the components and amounts shown in Table 2.4.1. For 1x E3 medium, 16.5 mL 60x stock was diluted in 1000 mL Milli-Q (MQ).

Table 2.4.1: E3 medium (60x), adjusted to pH 7.2

Component	Mass (g)	Volume (mL)
NaCl	34.8	-
$CaCl_2 \bullet 2H_2O$	1.6	-
$MgCl_2 \bullet 6H_2O$	5.8	-
MQ	-	2000

Table 2.4.2: 10 mM 3-Morpholinopropane-1-sulfonic acid (MOPS), adjusted to pH 7.2

Component	Amount
MOPS	523.15 mg
E3 medium	250 mL

Tricaine stock/MS222 used for anaesthesia of zebrafish larvae was made with tricaine powder and MQ water, the pH was adjusted to 7.2 with Tris base (Table 2.4.3).

Table 2.4.3: Tricane stock (20x), adjusted to pH 7.2 with Tris base

Component	Amount
Tricane powder	200 mg
MQ	48 mL
1,5 M Tris base	Variable

Table 2.4.4: Tris-acetate-EDTA (TAE) buffer (1x)

Component	Concentration (mM)
Tris	40
Acetic acid	20
EDTA	1

Agarose gel used for gel electrophoresis were made with low-melt agarose and TAE. The agarose gel used for embedding of zebrafish larvae and taking pictures were made with low-melt agarose and E3 medium (Table 2.4.5).

Table 2.4.5: 1.5% low melt agarose

Component	Amount
Low-melt agarose	0.75 g
E3 medium/ 1x TAE	50 mL

Table 2.4.6: Components of stock solutions

Stock compound	Solvent
PFOA	DMSO and E3 medium
PFOS	DMSO and E3 medium
PFBA	MOPS and E3 medium
PFBS	E3 medium
WY 14643	DMSO and E3 medium

2.5 Primers

Table 2.5: Primers used to examine gene expression in exposed zebrafish larvae.

Primer name	Sequence
acox1	Fwd: GGGTGTATTTCAAGAGCCC Rev: GAGCACGGATGTGTGTACCG
zf_ctp1a	Fwd: GCAGATGTACAGACTGGCGA Rev: GGCTCTGACAGCACCTCTTT
pparaa	Fwd: TGCTGGACTACCAGAACTGTGACA Rev: TGCTGGCTGAGAACACTTCTGAG
pparab	Fwd: TCAGGATACCACTATGGCGTTCAT Rev: AGCGGCGTTCACACTTATCGTA
tuba1c	Fwd: GGTGCCCTCAATGTGGATCT Rev: GCCACAGAGAGCTGCTCATG

2.6 Equipment and software

Table 2.6.1: Equipment, their applications, and suppliers used during this master thesis.

Instrument	Application	Supplier
Analog Vortex Mixer	PFAS mixtures	VWR
ChemiDoc XRS+ with Image Lab software	Gel electrophoresis picture	Bio Rad
CFX96™ Real-Time System, C1000 Thermal Cycler	qPCR	Bio Rad
Heraeus Multifuge X3R centrifuge	qPCR	Thermo Scientific
Himac CT 15RE	RNA extraction	VWR
Leica DMIL LED	Microscope	Leica
LeicaM420	Pictures of 96 hpf zebrafish larvae	Leica
MIR-154_PE Cooled Incubator	Incubator	Panasonic
NanoDrop One	RNA integrity	Thermo Scientific
PowerPac™ HC Power Supply	Gel electrophoresis	Bio Rad
T100 Thermal Cycler	cDNA synthesis	Bio Rad
Tissue homogeniser	RNA extraction	Xenox
VWR Mini Star silverline	cDNA synthesis	VWR
VWR Mini Star silverline, Mini vortex	RNA extraction	VWR

Tabel 2.6.2: Software used during this thesis

Software	Application	Provider
BioRender 2023	Figures	BioRender®
ChemDraw 20.0	Compound structures	PerkinElmer Informatics
Excel	Data treatment	Microsoft
Fiji/ImageJ	Measurements	ImageJ2
GraphPad PRISM v.9.5.1	Figures and statistics	GraphPad Software
Imagelab	Agarose gel visualisation	BioRad
Image-Pro® Primer 9.1.4	Pictures of 96 hpf zebrafish larvae	MediaCybernetics
PowerPoint	Figures	Microsoft
Word	Thesis writing	Microsoft

3. Methods

3.1 Embryo production

The evening before egg collection, adult males and females were placed in breeding tanks with a divider separating the sexes. Three to five tanks were used for breeding. On the following morning, the separator was removed to mix males and females (approx. 09:00), and eggs were collected after 1-2 h. Synchronised and fertilised eggs were selected for further use. The eggs were maintained in E3 medium until the time of exposure. Fertilised eggs were then randomly distributed by adding one egg to each well of a 48 well plate.

3.2 Experimental design

The synchronised and fertilised zebrafish eggs were exposed to five different concentration of the test PFAS and the PPAR model agonist. For each compound, two 48-well plates were used with a total of 16 eggs per concentration, as well as 16 eggs in the control group for each compound. The 48-well plates were placed in an incubation chamber (Panasonic MIR-154-PE) at 28°C. The exposure solution was refreshed every 24 hours to maintain the concentration of the exposure solutions (Figure 3.2.1). The embryos were exposed until 96 hpf. Visual observations and monitoring of selected endpoints were made at 24, 48, 72 and 96 hpf using a Leica DMIL LED microscope, including both lethal endpoints such as coagulation, as well as sublethal endpoints such as heartbeat, tail detachment, spontaneous movements, pigments, developmental retardation, hatched eggs, and presence of edema and malformations.

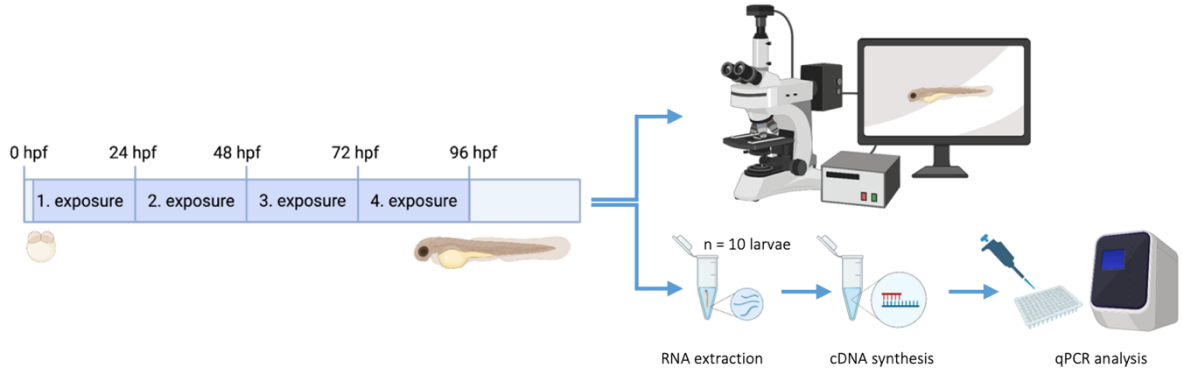


Figure 3.2.1: Experimental design. Figure showing the experimental design of this thesis. Step 1: the exposures of early life stages of zebrafish development, step 2: pictures taken of exposed 96 hpf zebrafish larvae, and step 3: RNA extraction, cDNA synthesis, and qPCR analysis. Figure made with BioRender.com

Stock solutions (10 000x) of PFOS, PFOA and WY14643 were prepared in dimethyl sulfoxide (DMSO), while stock solutions of PFBS and PFBA were prepared with MQ. Due to the acidity of PFBA, an addition of 10 mM MOPS was added to the PFBA exposure solution and adjusted to pH 7.2 with sodium hydroxide (NaOH). All compounds used in the exposure assays were dissolved in E3 medium. 0.01% DMSO was used as a control group for the exposure with PFOS, PFOA, and WY 14643, this concentration was selected based on previous observations that demonstrated its lack of inducing any malformations or behaviour changes in zebrafish larvae (Chen, Wang and Wu, 2011). 10 mM MOPS was used as a control for the exposure with PFBA, and E3 medium was used as a control group for the exposure of PFBS. Stock solutions for each compound were diluted to the exposure concentrations as shown in Table 3.2.1. The highest exposure concentrations in this study were selected based on the LC_{50} values reported in previous articles (Satbhai, Vogs and Crago, 2022; Gebreab *et al*., 2020; Hagenaars *et al*., 2011) aiming to omit concentrations that induce coagulation rates of more than 35%. Depending on the LC_{50} values and previously reported effect concentrations, 2- or 3-fold dilutions were selected. A test exposure of the concentrations for each compound was performed. Subsequently, the highest concentration that did not induce a coagulation rate of more than 20% within 24 hpf was determined as the maximum exposure concentration for the experiment. For the exposure of WY14643, a concentration of 10 µM (equivalent to 3.24 mg/L) was utilised as the highest concentration, employing a 2-fold dilution (Rizvi, Mehta and Oyekan, 2013). It should be noted that in this thesis, the calculation of LC_{50} values for each substance was not performed as I did not test concentrations that induce 100% mortality.

Table 3.2.1: Exposure concentrations from diluted stock solutions.

PFOS (mg/L)	PFOA (mg/L)	PFBS (mg/L)	PFBA (mg/L)	WY14643 (mg/L)
50	40	25	750	3.24
15.7	20	12.5	375	1.62
5.5	10	6.2	187.5	0.81
1.8	5	3.1	93.7	0.4
0.6	2.5	1.5	46.9	0.2

3.3 Selected endpoints

Zebrafish larvae was examined using toxicological endpoints based on lethal, sublethal and teratogenic effects (Table 3.3.1). These endpoints were recorded at 24, 48, 72 and 96 hpf. Embryos were checked for coagulation after 24 hours, as well as a detached tail-bud, heartbeat, spontaneous movements, and developmental retardation, including embryonic shape and epiboly condition. Heartbeat and movement were checked by looking at each embryo/larvae for 2 minutes or until heartbeat and movement was detected. All endpoints were recorded at 48, 72 and 96 hpf. At 96 hpf the larvae were photographed (Leica M420) and images were analysed using ImageJ/FIJI to determine body length, area of yolk sac and eye size. Dead embryos and larvae were removed consecutively. Body length was measured as the distance from snout to tail-end, without the caudal fin. Area of yolk sac was measured as the circumference of the yolk sac, including the yolk sac extension. The eye size was measured as the length of the eye, as well as the hight, with these values the area of an ellipse was calculated and used as the eye size.

Table 3.3.1: Toxicological endpoints used to assess the lethal, sublethal and teratogenic effects of exposure chemicals, adapted from Hagenaars *et al*., 2011.

Toxicological endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Coagulation	●	●	●	●
Undetached tail	●	●	●	●
No heartbeat	●	●	●	●
No pigmentation	-	o	o	o
No movement	o	o	o	o
Developmental retardation	o	o	o	o
Hatch	-	o	o	o
Malformation	o	o	o	o
Edema	o	o	o	o

(●) - Lethal criterion used to determine mortality rate.

(○) - Documented but not evaluated as lethal criterion.

(-) - Not documented.

3.4 RNA extraction

From the three highest concentrations where no morphological changes on the larvae were observed for each of the compounds tested, 10 individuals of 4 days post fertilisation (dpf) zebrafish larvae were placed in an Eppendorf tube with all liquid removed and directly submerged in liquid nitrogen. These concentrations were selected since gene expression can be altered at low levels of exposure to toxicants, even before morphological changes are visible. Moreover, higher doses tend to induce more nonspecific toxicological responses. The frozen zebrafish larvae were stored in a -80°C freezer until use.

250 µL trizol (TriReagent Sigma-Aldrich) was added before homogenisation of each sample, using an automated homogenisation tool. The tip (pestle) of the homogenisation tool was rinsed with 75% ethanol between each sample, and thoroughly washed with 75% ethanol and MQ water when switching between compounds. When all samples were homogenised, they were incubated in room temperature for 5 minutes. To phase-separate the samples, 50 µL chloroform was added to each Eppendorf tube and shaken vigorously for 15 seconds and incubated in room temperature for another 2-15 minutes. The samples were centrifuged using a cold centrifuge (Himac CT 15RE) at 12 000 x *g* for 15 minutes at 4°C, separating the liquid into an aqueous and organic phase. For the RNA to precipitate, the same amount of volume (110 µL) from the upper aqueous phase, was transferred to a new Eppendorf tube using a pipette before an equal amount of 100% isopropanol was added, mixed, and incubated at room temperature for 5-10 minutes. The samples were then centrifuged at 12 000 x *g* for 10 minutes at 4°C. The supernatant was removed using a pipette until only the RNA-pellet was left in the tube. The pellet was then washed by adding 1 ml of 75% ethanol, vortexed and centrifuged at 7500 x *g* for 5 minutes at 4°C. The 75% ethanol was removed, before washing once more with 1 ml 75% ethanol and removed again. The samples were centrifuged for 1 minute and the remaining residues of ethanol were removed with a pipette. The RNA pellet was left to dry with the lid open for about 20 minutes in room temperature until the ethanol had completely evaporated, and the RNA pellet had changed colour from white to transparent. 15 µL RNAse-

free water was added to each tube to dissolve the RNA, then vortexed and incubated at 60°C for 20 min. The samples were kept on ice while measuring the concentration (ng/µL) and purity ($A_{260/280}$, $A_{260/230}$) of the RNA using a Nanodrop One instrument (Nanodrop-One Thermo Scientific).

3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to assess the integrity of the RNA samples. 1% agarose gel was prepared by adding 50 mL TAE with 0.5 g low melt agarose, this mixture was heated and mixed, before chilled to ~60°C and 0.5 mL gel red was added to the mixture. Gel red was used to stain the RNA and visualise the nucleic acids. The agarose gel was poured into a cast chamber and left to polymerise for 1h. An RNA loading buffer was made by adding 20 mL 6x loading buffer and 80 mL formamide to a PCR tube. Formamide was used to remove any secondary structures in the RNA. The RNA samples were prepared by adding 9 µL of the RNA loading buffer and 1 µL of 100 ng RNA. To denaturate the RNA, samples were incubated at 60°C for 5-10 minutes before immediately placed on ice. 0.5 µL of a DNA ladder (2Log) was loaded into one of the wells as a size marker and 10 µL of RNA sample was loaded into the remaining wells in the gel. The electrophoresis was run at 80V for 1 hour, before visualisation of the gel using the ChemiDocTM XRS+ system (BioRad).

3.6 Preparation of complementary DNA

Complementary DNA (cDNA) was synthesised by using the iScript™ cDNA Synthesis Kit (BioRad). This kit includes a reverse transcriptase, a reaction mix (dNTPs, primer and buffer) and RNase-inhibitor (Table 3.6.1). RNA isolated from zebrafish larvae was used to synthesise cDNA with the RNA samples thawed over ice.

Nuclease free (NF) water was added to the bottom of each PCR-tube, before 1000 ng RNA was added to the NF-water. A master mixture containing four parts 5x iScript Reaction Mix and one-part iScript Reverse Transcriptase was made, and 5 µL master mixture was added to the side of each tube. The samples were centrifuged and mixed before polymerase chain reaction (PCR) was performed following the program in Table 3.6.2. A “no reverse transcriptase” sample was made from a mixture of RNA-samples to monitor potential DNA contamination in the master mixtures. In this case, the same procedure as for the other RNA samples was followed, but with

NF-water as a substitute for RNA template during the cDNA synthesis. Synthesised cDNA was stored at -20°C until used.

Table 3.6.1: Table of reagents, volume and concentration

Component	Volume per reaction (µL)
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template (1000 µL)	Variable
Total volume	20

Table 3.6.2: Table of PCR program/reaction protocol

Reaction	Temperature (°C)	Time (min)
Priming	25	5
Reverse transcription	46	20
RT inactivation	95	1
Optional step	4	Hold

3.7 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) (often also denoted as real-time PCR (RT-PCR)) was used to study changes in gene expression of selected genes in zebrafish larvae after exposure of PFOS, PFOA, PFBS, PFBA, and WY14643. Primer efficiencies were tested for each primer before use. The specific primer sequences used for the qPCR analysis can be found in Table 2.5. Primer mixtures was made by adding 180 µL NF-water, 10 µL forward and 10 µL reverse primer to an Eppendorf tube and mixing well. The primer mixtures were stored in -20°C freezer until use.

In a 96-well plate, 2 µL of cDNA was added to the bottom of each well, before a mixture containing 5 µL SYBR Green, 0.8 µL primer mix and 2.2 µL NF-water was added to the side wall of each well. A plastic seal was added to the top of the plate, before it was centrifuged at 500 rounds per minute (rpm) for 1 minute to make sure the contents were mixed and placed at the

bottom of the wells. The well plate was then transferred to the RT-PCR machine (Bio Rad CFX96^{TM} Real-Time System) using the program found in Table 3.7.1. A melting curve analysis was performed to ensure purity of the PCR product and specificity of the tested primers.

To normalise the qPCR data, quantification cycle (Cq) values retrieved after qPCR analysis were processed using the comparative C(T) method, also referred to as the 2DDCT method (Schmittgen and Livak, 2008) in Microsoft Excel (2023). To determine the relative gene expression the Cq-values of target genes (*pparaa, pparab, acox1* and *cpt1a*) and housekeeping gene (*tuba1c*) were obtained from duplicate measurements and the average Cq-values were calculated. ΔCq was calculated by subtracting Cq of the target gene from Cq of the housekeeping gene. The average ΔCq was then calculated for the control samples. For each sample $\Delta\Delta$Cq was calculated by subtracting the average of Cq of the control samples to the ΔCq of the samples. Fold-change was then determined by calculating 2 to the power of $-\Delta\Delta$Cq (Eq 1.). The average fold change to the control samples were then calculated. Lastly, the control-normalised fold-change was obtained by dividing each fold-change by the average fold-change of the control samples.

$$\text{Fold change} = 2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ Internal control})\text{treated} - (C_T \text{ gene of interest} - C_T \text{ internal control})\text{control}] \quad \text{[Eq 1.]}$$

Table 3.7.1: qPCR analysis reaction protocol using SsoAdvanced Universal SYBR® Green Supermix.

Reaction	Temperature (°C)	Time (min)
Enzyme activation	95	10
Amplification (39 rounds)	95	0.25
	60	1
Melting curve	65°C to 95°C	0.5

3.8 Morphological measurements and statistical analyses

3.8.1 Morphological analysis in zebrafish exposed to PFAS and WY14643

Morphological data were measured using FIJI/ImageJ. The universal scale was set by measuring a picture taken of Stage Micrometer Grouped Graduations (Graticules optics) at the same magnification as for the rest of the pictures of the zebrafish larvae. Body length, area of yolk sac and eye size of 96 hpf zebrafish larvae were measured, before values were transferred to Microsoft Excel (2023). GraphPad PRISM v.9.5.1 was used to produce illustrations of morphological values. Grubbs outlier test (alpha = 0.05) was performed, and outliers were removed. To examine significant differences between control group and exposure group, a One-way analysis of variance (ANOVA) test followed by Dunnett´s post-hoc test with significance level of 0.05 was used.

3.8.2 Morphological endpoints during embryonic development

Microsoft Excel (2023) was initially used to process the morphological endpoint data. The categorial values of each endpoint (yes/no) was gathered and placed in contingency tables, before GraphPad PRISM v.9.5.1 was used to produce illustrations and statistical analyses. The values of morphological endpoints were compared between the exposure groups and their respective control groups using Fisher´s Exact Test with significance level of 0.05. The percentage of individuals that were affected for each endpoint was calculated and then used to make illustrations.

3.8.3 Target gene expression

To calculate the significant fold change in gene expression, GraphPad PRISM was used to transform the gene expression data (y=log2(y)) before a One-way ANOVA test and Dunnett´s post-hoc test with significance level of 0.05 was performed. After statistical analysis, illustrations were made for each exposure group.

4. Results

4.1 Morphological analysis in zebrafish larvae exposed to PFAS and WY14643

Zebrafish embryos (2 hpf) were exposed *in vivo* to increasing concentrations of PFOS, PFOA, PFBS, PFBA, and WY14643. The exposures were terminated at 96 hpf and morphological changes in the zebrafish larvae, including body length, eye size, and area of yolk sac were assessed as described in section 3.3 (Figure 4.1.1). Changes in body length, as well as jaw deformation were prevalent in the three highest concentrations of PFOS. Impaired yolk sac absorption (yolk retention) was observed in larvae exposed to the highest concentrations of both sulfonic acids (PFOS and PFBS), as well as WY14634. In addition, spine deformation was frequently seen in the two highest concentrations of zebrafish larvae exposed to PFOS (16.7 and 50 mg/L).

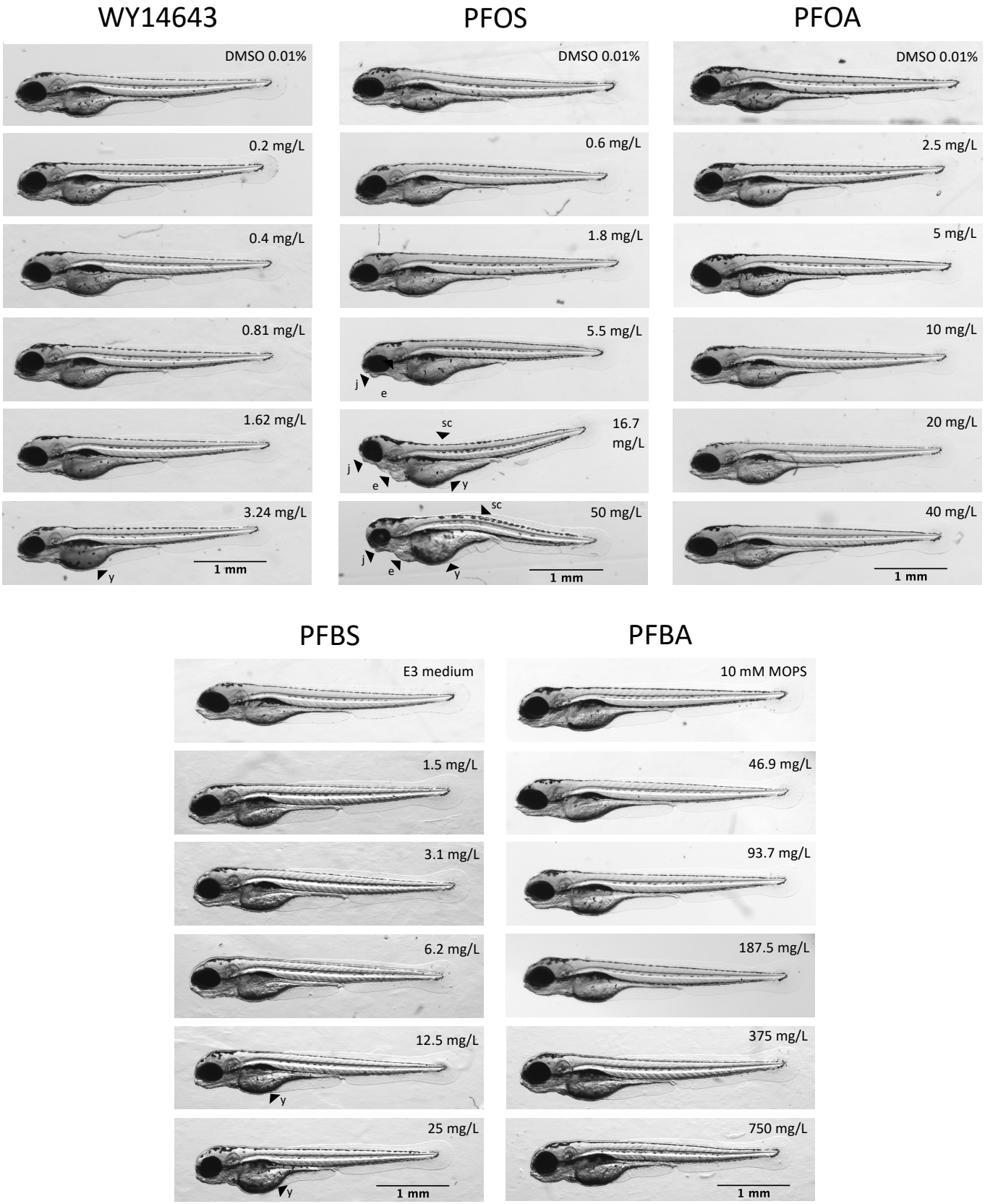


Figure 4.1.1: Morphology of exposed zebrafish larvae at 96 hpf. Microscope pictures of zebrafish larvae at 96 hpf exposed to solvent controls (DMSO 0.01%, E3 medium and 10 mM MOPS), WY14643 (0.2, 0.4, 0.81, 1.62 and 3.24 mg/L), PFOS (0.62, 1.87, 5.55, 16.7 and 50 mg/L), PFOA (2.5, 5, 10, 20 and 40 mg/L), PFBS (1.56, 3.13, 6.25, 12.5 and 25 mg/L) and PFBA (46.9, 93.75, 187.5, 375 and 750 mg/L) as indicated. Black arrowheads indicate a change in y = yolk retention, j = jaw, sc = spine curvature and e = edema, compared with the control to the exposed larvae.

Furthermore, significant reductions in the length (mm) of the exposed zebrafish larvae were observed in a dose-dependent manner for both PFOS and PFBS, as well as in the highest concentration of WY14643 (3.24 mg/L) (Figure 4.1.2). No significant changes in the body length of zebrafish larvae exposed to the carboxylic acids PFOA and PFBA were detected.

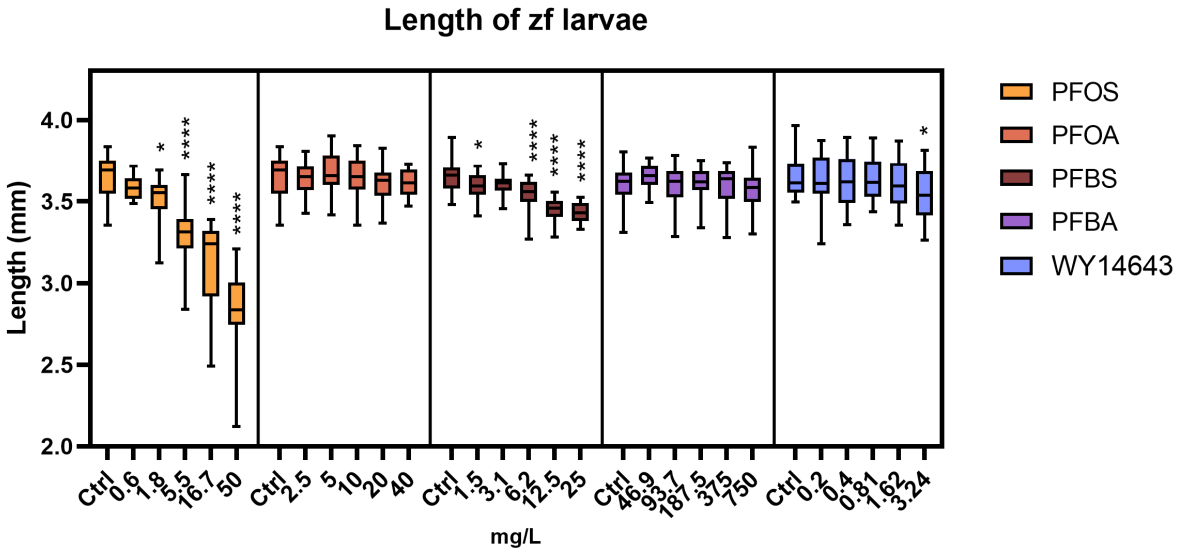


Figure 4.1.2: Body length of exposed zebrafish (zf) larvae (96 hpf). Body length of 96 hpf zebrafish larvae (mm) exposed to PFOS (0.6, 1.8, 5.5, 16.7 and 50 mg/L), PFOA (2.5, 5, 10, 20 and 40 mg/L), PFBS (1.5, 3.1, 6.2, 12.5 and 25 mg/L), PFBA (46.9, 93.7, 187.5, 375 and 750 mg/L) and WY14643 (0.2, 0.4, 0.81, 1.62 and 3.24 mg/L). Bars indicate min and max values, while black line shows the median. Statistically significant changes are indicated (*$p < 0.05$; ****$p < 0.0001$) and based on a comparison with their respective solvent control (section 3.2).

A significant sustained yolk sac volume was evident in larvae exposed to the two highest concentrations of the sulfonic acids, PFOS and PFBS, as well as WY14643 (Figure 4.1.3). No significant changes in the yolk sac volume were detected in larvae exposed to the carboxylic acids, PFOA and PFBA.

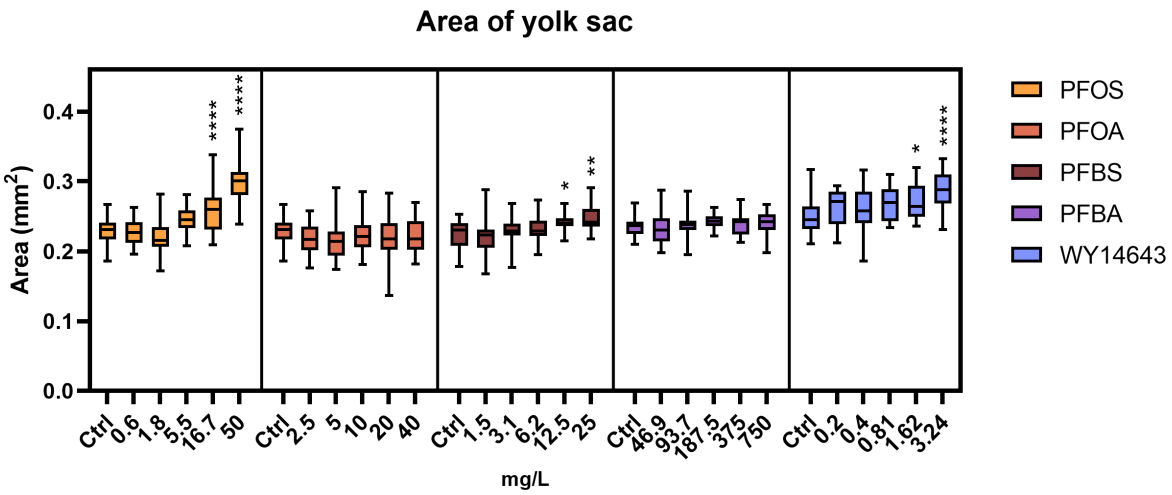


Figure 4.1.3: Area (mm^2) of yolk sac in exposed zebrafish larvae at 96 hpf. Area of yolk sac in 96 hpf zebrafish larvae (mm^2) exposed to increasing concentrations of PFOS (0.6, 1.8, 5.5, 16.7 and 50 mg/L), PFOA (2.5, 5, 10, 20 and 40 mg/L), PFBS (1.5, 3.1, 6.2, 12.5 and 25 mg/L), PFBA (46.9, 93.7, 187.5, 375 and 750 mg/L) and WY14643 (0.2, 0.4, 0.81, 1.62 and 3.24 mg/L). Bars indicate min and max values, while black line shows the median. Significant changes in area (*p < 0.05; **p < 0.01; ****p < 0.0001) indicate a comparison with their respective solvent control (section 3.2).

Moreover, the morphological analyses also revealed significant dose-dependent reductions in the area (mm^2) of the eyes in zebrafish larvae exposed to the sulfonic acids PFOS and PFBS (Figure 4.2.4). Additionally, a trend towards a reduced eye size was also observed with increasing concentrations of PFBA and WY14643, where some of the concentrations used demonstrated statistically significant changes compared to the controls. No significant changes were detected in the eye size of zebrafish larvae exposed to PFOA.

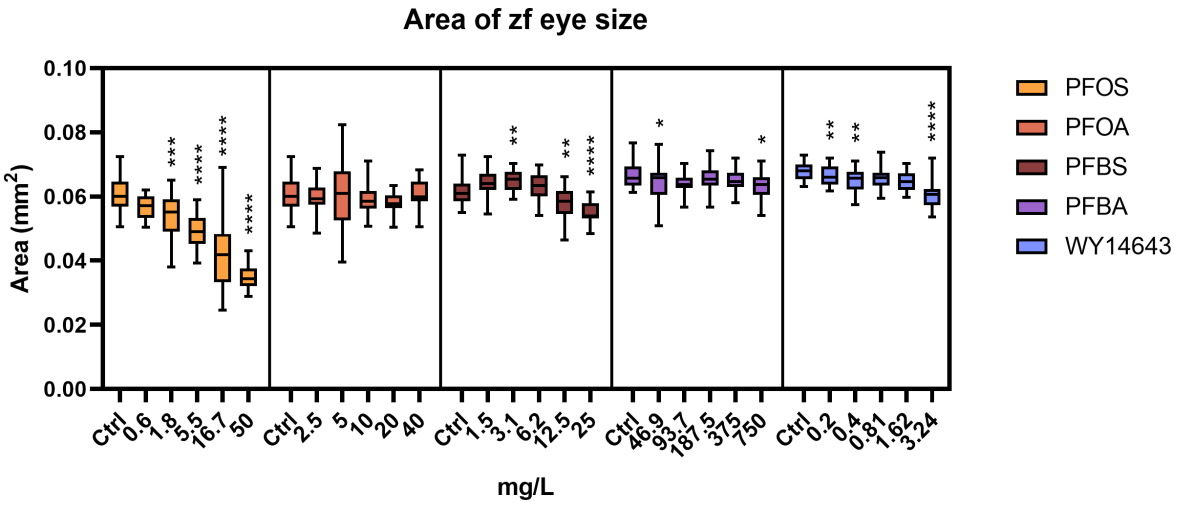


Figure 4.1.4: Eye size of exposed zebrafish larvae at 96 hpf. The areas of the eye size in 96 hpf zebrafish larvae (mm^2) exposed to increasing concentrations of PFOS (0.6, 1.8, 5.5, 16.7 and 50 mg/L), PFOA (2.5, 5, 10, 20 and 40 mg/L), PFBS (1.5, 3.1, 6.2, 12.5 and 25 mg/L), PFBA (46.9, 93.7, 187.5, 375 and 750 mg/L) and WY14643 (0.2, 0.4, 0.81, 1.62 and 3.24 mg/L) are shown (bars indicate min and max values, while black line shows the median). Significant changes are indicated (*p < 0.05; **p < 0.01; ***p< 0.001; ****p < 0.0001) and based on a comparison with their respective solvent control (section 3.2).

4.2 Morphological endpoints during embryonic development

Morphological endpoints, including coagulation, heartbeat, spontaneous movements, pigment, hatching, malformations, developmental retardation, and edema were recorded during early embryonic development at 24, 48, 72 and 96 hpf (Figure 4.2.1). Zebrafish embryos subjected to continuous PFBS exposure exhibited significant effects on survival success. The two highest concentrations of PFBS resulted in significant coagulation in comparison to the solvent control. However, no significant coagulation was observed in embryos exposed to either PFOS, PFOA, PFBA, or WY14643.

Significant changes in hatching success were detected during the exposure of all PFAS congeners. However, no changes were observed in the hatching success during the exposure of WY14643. Embryos exposed to PFOA and PFOS resulted in delayed hatching at 72 hpf compared to the solvent control. On the contrary, exposure to the three lowest concentrations of PFBS resulted in earlier hatching at 72 hpf. Exposure to PFBA was the only compound resulting in a lower hatching success after 96 hpf.

No difference in larval movement were recorded in exposures of PFOA, PFBA, PFBS and WY14643. The two highest concentrations of PFOS resulted in significantly less larval movement at both 48 and 72 hpf. Furthermore, at 96 hpf the three highest concentrations of PFOS caused significantly less larval movement than the solvent control.

Malformations became apparent in larvae exposed to the three highest concentrations of PFOS at 96 hpf. However, the exposure of 50 mg/L PFOS revealed malformations in larvae after 72 hpf (Figure 4.2.1). Malformations in spine curvature were observed, as well as jaw deformation (jaw deformations were not quantified in this study), as shown in zebrafish larvae at 96 hpf in Figure 4.1.1. No statistically significant malformations were observed in zebrafish embryos exposed to PFOA, PFBS, PFBA and WY14643. For other visualisations of affected endpoints, see Appendix I.

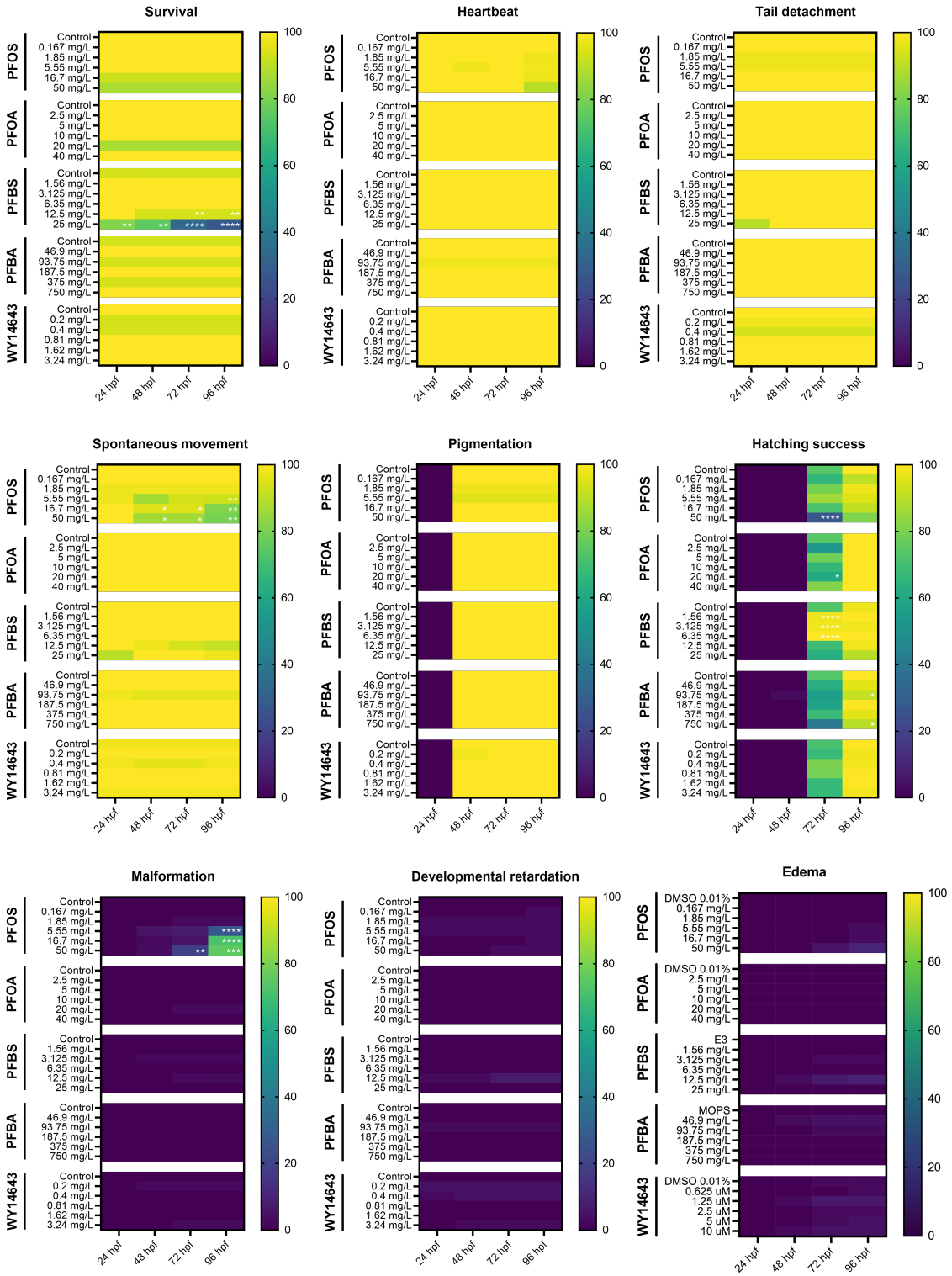


Figure 4.2.1: Morphological endpoints observed in exposed zf larvae during the first 96 hpf. Heatmaps displaying morphological endpoints on zebrafish larvae during the first 96 hpf after exposures to PFOS, PFOA, PFBS, PFBA and WY14643. Colour intensity of each square represent the magnitude of change from 0-100% in a specific endpoint compared to the control group. Statistically significant changes between control and test compound are indicated with an asterisk (*p < 0.05; **p < 0.01; ***p< 0.001; ****p < 0.0001) and determined using Fishers Exact test.

4.3 PFAS-mediated changes in the expression of Ppar-related genes

The three highest concentrations that did not produce any observable malformations during exposures with PFOS, PFOA, PFBS, PFBA and WY14643, were used to assess changes in the expression of four genes belonging to the Ppar-signalling pathway with qPCR. The integrity of RNA extracted from exposed 96 hpf zebrafish larvae was confirmed with agarose gel electrophoresis (Appendix II) and the RNA was used as templates for cDNA synthesis. qPCR analysis was thereafter used to examine any transcriptional changes of *pparaa* and *pparab*, *acox1* and *cpt1a.* Fold changes in expression levels are presented in Figure 4.3.1.

When assessing the expression pattern of these genes, an initial downregulation in *pparaa*, *pparab* and *acox1* was observed in response to exposure to both PFOS and PFOA at the lower concentrations. A downregulation was also observed in *cpt1a* expression at the lowest concentration of PFOA. At higher concentrations of PFOS and PFOA, the expression levels are similar to that of the solvent controls. The same trend is not observed after exposure to the shorter-chained PFBA and PFBS, and neither in the expression of *cpt1a* after exposure to PFOS. On the other hand, the expression levels in response to PFBA and WY14643 exposure gave a significant upregulation for all the genes assessed, except for *cpt1a* after WY14643 exposure, which showed no significant changes in expression levels. Notably, *pparaa* was the only gene that showed a significant increase in fold change in response to PFBS exposure.

Transcription of *pparaa* was upregulated by all compounds except from PFOA and the lowest concentration used for PFOS, which both caused a downregulation, with only PFOA exhibiting statistically significant effects. The *pparab* and *acox1* expression pattern was similar to that of *pparaa*, but with no significant change from PFBS, and a significant downregulation from PFOS. The expression of *cpt1a* was significantly downregulated by PFOA and PFOS, and a slight upregulation in *cpt1a* in the higher concentrations of PFOA was observed. Exposure to PFBA produced a significant upregulation for *cpt1a*, especially in the higher concentrations used. No significant changes in *cpt1a* expression were observed after exposure to PFBS and WY14643 (Figure 4.3.1).

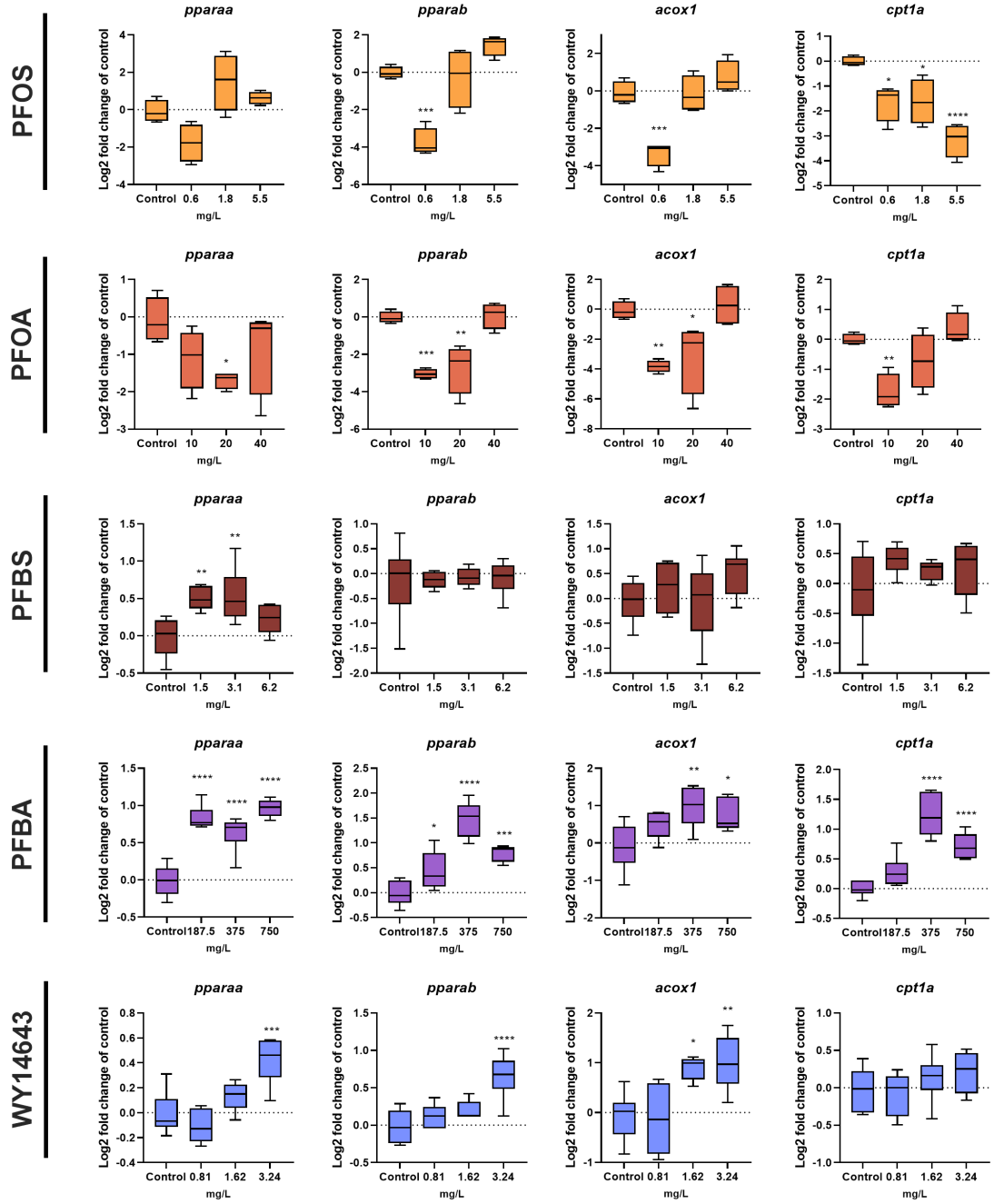


Figure 4.3.1: Gene expression of Ppar-related genes in exposed zf larvae (96 hpf). Gene expression analyses of *pparaa, pparab, acox1* and *cpt1a* in zebrafish larvae (*n* = 4-6, with a pool of 10 larvae per *n*) exposed to PFOS (0.6, 1.8 and 5.5 mg/L), PFOA (10, 20 and 40 mg/L), PFBS (1.5, 3.1 and 6.2 mg/L), PFBA (0.187.5, 375 and 750 mg/L) and the PPAR agonist WY14643 (2.5, 5 and 10 µM) at 96 hpf. The box plot shows fold change in expression levels relative to solvent controls (bars indicate min and max values, while the black line shows the median). Statistical significance was determined using a One-way ANOVA test and Dunnett´s post-hoc test. Statistically significant changes in gene expression compared to the control groups are indicated as: *p < 0.05; **p < 0.01; ***p< 0.001; ****p < 0.0001.

5. Discussion

PFAS have been widely used for several decades, resulting in their ubiquitous distribution throughout the environment. Exposure to PFAS has been associated with endocrine disruption, developmental and reproductive toxicity, and potential carcinogenicity in both humans and wildlife. However, the majority of toxicological data available for PFAS is based on studies of PFOA and PFOS, while only limited information exists for the emerging shorter-chained and branched congeners. Obtaining knowledge on the toxicity of these emerging PFAS is important for understanding their environmental and human risks, especially considering their increasing presence in biota and the environment.

The focus of this master's thesis was to examine the toxicity of shorter-chained PFAS congeners, specifically through the examination of morphological changes in developing zebrafish. Additionally, the study aimed to explore how such shorter-chained PFAS congeners can modulate the Ppara signalling pathway, and thus potentially provide insights into the molecular mechanisms underlying their toxicity. For this, the carboxylic acid PFBA and the sulfonic acid PFBS, were selected as two representatives of shorter-chained PFAS congeners, and their toxic effects were compared to that of their longer-chained legacy PFAS variants, i.e., PFOA and PFOS.

The decision to use the shorter-chained PFBA and PFBS congeners in this thesis was based on these compounds being equivalents to PFOA and PFOS. Furthermore, PFBA and PFBS have been proposed as potential alternatives/substitutes for long-chained PFAS (Gomis *et al*., 2018). To support the analyses of PFAS-mediated modulation of the Ppara signalling pathway, WY14643 was included as a selective Ppara agonist. WY14643 is a well-known model agonist for PPARa across various species, including different fish species such as turbot (*Scophthalmus maximus*), brown trout (*Salmo trutta* f. *fario*), Atlantic cod, and zebrafish (Urbatzka *et al.,* 2015; Madureira *et al.,* 2019; Søderstrøm *et al*., 2022; Rizvi, Mehta and Oyekan, 2013).

5.1 PFAS-mediated effects on mortality

Throughout the exposure period, the two highest concentrations of PFBS (12.5 mg/L and 25 mg/L) resulted in coagulation among 2% to 58% of exposed eggs and larvae between 24 and 96 hpf (Figure 4.2.1). Exposure to the other PFAS congeners did not cause any significant changes in mortality, nor did WY14643.

As for the increased coagulation in zebrafish larvae following PFBS exposure, the absence of chorion after hatching allows direct skin absorption of PFBS, possibly leading to an increase in mortality after hatching has occurred. A study by Huang *et al.,* (2010) regarding PFOS exposure, indicated that the presence of chorion affects the PFOS uptake in zebrafish embryo due to the limited permeability through the chorion. These observations suggest that PFOS and PFBS might have a higher potential for absorption or exhibiting a greater impact on survival compared to the other compounds tested, although this was not observed with PFOS exposure in this thesis. A previous study reported reduced embryo survival following maternal PFBS exposure in zebrafish (Annunziato *et al*., 2022), supporting the severe effects PFBS may have on zebrafish survival and development.

5.2 Sulfonic acids and WY14643 cause a reduction in body length and area of yolk sac

Hagenaars *et al*., (2011) reported a reduction in body length of zebrafish larvae at 120 hpf after exposure to PFOS (1 mg/L) and PFOA (100 mg/L and higher), while no reduction in length was observed following exposures to PFAS congeners with backbones containing less than eight carbons. In this thesis, a significant dose-dependent reduction in body length was observed after the exposure to both the sulfonic acids, PFOS and PFBS. Notably, exposure to PFBS have previously not been associated with a decreasing body length in zebrafish larvae (Sun *et al*., 2021). Exposure of zebrafish embryos to the carboxylic acids, PFOA and PFBA, did not cause any statistically significant changes in body length, suggesting that the sulphonate functional group present in the PFAS congeners may be important for the reduction in body length. Jantzen *et al.*, (2016) as well as Liu *et al.*, (2022) reported a decrease in zebrafish body length at PFOA concentrations of 1 µg/L, but at time points beyond the 96 hpf timeframe used in this thesis. There is therefore some discrepancy between their results, and those as reported by Hagenaars *et al.*, (2011), which reported a reduction in zebrafish length at PFOA concentrations of 100 mg/L and higher. A reduction in body length after PFBA exposure (4 and 400 mg/L) in

zebrafish embryos after 72 hpf has previously been reported (Wasel, Thompson and Freeman, 2022), although as mentioned above, a PFBA-mediated reduction in length was not observed in this thesis.

The highest concentration used for WY14643 also caused a significant reduction in body length. Physiological effects of WY14643 exposure have previously been described in only a few other fish species, such as juvenile brown trout and turbot (Madureira *et al*., 2019; Urbatzka *et al*., 2015), and none of these studies have assessed effects on body length during early development. WY14643-mediated morphological changes in zebrafish are to my knowledge not documented in earlier research.

The reduced body length after PFAS exposure might be related to yolk retention. At the developmental stage (96 hpf) the zebrafish was measured in my experiment, the larvae have not yet started feeding and all nutrients originate from the yolk sac. The yolk sac serves as a lipid-rich structure that gradually provides essential nutrients to support the embryos development into a free-feeding larva (reviewed by Quinlivan and Farber, 2017). The difference in yolk sac size could indicate a disruption in processes related to lipid transport, nutrient storage, and utilisation (Jantzen *et al*., 2016). This disruption may be linked to disturbances in the Ppar signalling pathway, which plays a crucial role in regulating lipid metabolism and nutrient mobilisation. This is also supported by the observed yolk retention upon exposure to the Ppara agonist WY14643 in this thesis. Consequently, a disruption in these processes could lead to the inadequate delivery of important nutrients, resulting in the phenotype of reduced body length.

5.3 Reduction in eye size

An apparent dose-dependent reduction in eye size was observed after exposure to WY14643, PFOS and PFBS. In addition, the exposure of PFBA resulted in noticeable differences in eye size compared to the control group (10 mM MOPS), with statistically significant effects at the lowest (46.9 mg/L) and highest (750 mg/L) exposure concentration. However, there was no clear dose-dependent relationship in the changes observed in eye size after PFBA exposure. Previous studies have demonstrated a link between eye malformation, such as reduced eye size (microphthalmia), and the suppression or activation of certain genes, including *sox2, otx2,*

pax6a, and *pax6b* (Guerrero-Limón *et al*., 2023). However, these genes are not included in the Ppara signalling pathway and were not examined in this thesis. The observed effects of WY14643 exposure on eye size in zebrafish larvae may indicate the involvement of other genes within the Ppara signalling pathway. Given that PPARa is known for its regulatory role in retinal neuroprotection and angiogenesis in human eyes, it is important to further explore the potential connections between WY14643 exposure, PPARa signalling, and the changes in eye size observed in this study.

5.4 PFOS induced spine deformations

The appearance of spine deformities, such as bent spine and scoliosis, observed after PFOS exposure in this thesis, are in agreement with previous reports of PFOS-induced malformations in zebrafish larvae (Hagenaars *et al*., 2014; Martínez *et al*., 2019; Huang *et al*., 2010). However, the specific mechanisms underlying these effects are not yet fully understood, but several reports present factors that may contribute to the development of spine deformities. For instance, a study by Cheng *et al*., (2000) suggests that spinal deformities observed in zebrafish larvae following PFOS exposure are attributed to a reduction in myosin and myotome formation, which are essential for proper musculoskeletal system development. Another suggestion put forward by Zhang *et al.,* (2011) is that PFOS-mediated toxicological effects to fish primarily involve interference with microtubules of the cytoskeleton, as well as altered expression patterns of axons and the functions of motor neurons. The effects leading to alterations in motor neuron differentiation may ultimately contribute to the spine deformations after PFOS exposure. The spine malformations observed in this thesis were specific to PFOS exposure and were not observed after exposure to the other compounds examined. This observation is consistent with findings from other studies that exposed zebrafish larvae to PFOS, PFOA, PFBS and PFBA, as reported by Hagenaars *et al*., (2011).

5.5 Hatching success

Significant delayed hatching was detected at different timepoints in PFOS, PFOA and PFBA-exposed zebrafish larvae. On the other hand, PFBS exposure demonstrated a statistically significant increase in early hatching success. Hatching is a critical stage in embryogenesis that relies on processes such as chorion digestion by hatching gland enzymes and embryo

movement to open the chorion structure (Hagenaars *et al*., 2011). These processes can be influenced by toxicants, leading to delayed or decreased hatching success. A significant delay in hatching success was observed at 72 hpf after exposure to 40 mg/L PFOA, as well as 50 mg/L PFOS. Previous studies have reported varying results regarding hatching after PFOA and PFOS exposure. One study measuring the hatching success after 54 hpf did not observe any significant differences between PFOA-exposed zebrafish larvae and controls (Satbhai, Vogs and Crago, 2022), while another study reported a delayed hatching success for embryos exposed to concentrations of 50 mg/L or higher (Hagenaars *et al*., 2011). Regarding PFOS exposure, Shi *et al*., (2008) reported a delayed hatching success after exposure of 1, 3 and 5 mg/L PFOS in zebrafish larvae, but a delayed hatching was not observed in zebrafish larvae exposed to these concentrations in this study.

PFBA was the only PFAS congener influencing hatching success at 96 hpf, delaying the hatching after exposure of 93.75 mg/L and 750 mg/L PFBA. In contrast, PFBS exposure was the only compound that demonstrated a statistically significant increase in early hatching success in zebrafish larvae, occurring already at low concentrations. Previous studies have reported different observations regarding hatching success in zebrafish exposed to PFBS, including a delayed hatching at 72 hpf (Tang *et al*., 2020; Gong *et al*., 2022), whilst Hagenaars *et al*., (2011) reported no significant changes in zebrafish hatching after 50 - 3000 mg/L of PFBS and PFBA exposure.

5.6 Impaired movement after PFOS exposure

PFOS exposure resulted in a significant decrease in zebrafish movement between 48 and 96 hpf (Figure 4.2.1). The first rhythmic spontaneous tail bends (“twitching”), observed after 24 hpf, are independent of higher brain inputs and originate from spinal neuron innervation (Huang *et al*., 2010). The observed abnormal behaviour in movement after 24 hpf is suggested to occur from potential damage to the muscle, nervous system, and skeletal system. Exposure to PFOS have been suggested to cause damage to the cardiovascular system, resulting in abnormal movements (ibid.). Previous studies have also observed a decrease in swimming speed and distance in zebrafish larvae following exposure to PFOS, PFOA, and PFNA in concentrations between 0.001 mg/L and 1 mg/L (Hagenaars *et al*., 2014; Huang *et al*., 2010; Jantzen *et al*., 2016). These results suggest that PFAS exposure have an impact on the behaviour

of the zebrafish larvae, and support the observed impaired movement detected in the larvae exposed to PFOS. However, no observed change in movement were detected after exposure to the other PFAS congeners.

5.7 Additional sublethal endpoint measured

No significant changes were observed in endpoints such as lack of heartbeat, tailbud detachment, pigmentation, edema, or developmental retardation in zebrafish larvae exposed to PFAS and WY14643. These results diverge from the observations reported by Hagenaars *et al.,* (2011) and Ulhaq *et al.,* (2013), where edemas was detected in zebrafish larvae exposed to long-chained PFOS and PFOA, as well as PFBS and PFBA. Furthermore, the study by Hagenaars *et al.,* (2011) reported an increase in heartbeat at 48 hpf following exposure to sulfonic acids PFBS and PFOS, while PFOA exposure led to a decrease in heart rate. However, the assessment of heart rate was not conducted in this thesis.

5.8 Modulation of the Ppara-signalling pathway by WY14643 and PFAS

Toxicant-mediated changes in gene expression can occur at low exposure levels before toxic effects manifest in visible morphological changes. Therefore, gene expression analyses of the Ppara-signalling pathway were done with larvae exposed to the three highest PFAS concentrations where no malformations were observed. Previously, PPARs have been demonstrated to be molecular targets for several PFAS congeners in several species (Gebreab *et al*., 2020; Behr *et al.,* 2020; Rosen *et al*., 2017). As Ppars are involved in the regulation of energy homeostasis, modulation of the Ppar-signalling pathway can lead to alterations in metabolic pathways, including gluconeogenesis, ketogenesis, bile synthesis and secretion, as well as fatty acid uptake, activation and oxidation (Kersten, 2014).

All the genes examined in this thesis, namely *pparaa, pparab, cpt1a*, and *acox1*, are known to be involved in the Ppara signalling pathway, which has an important role in lipid metabolism and energy homeostasis (*ZFIN Gene: pparaa,* 2022). The gene *pparaa* is involved in regulation of fatty acid metabolism, cholesterol storage, and transcriptional regulation, whilst *pparab* is responsible for responding to activity and cellular responses to cold temperatures (*ZFIN Gene: pparab,* 2022). Additionally, *acox1* contributes to fatty acid beta-oxidation through acyl-CoA

oxidase (*ZFIN Gene: acox1,* 2022), and *cpt1a* is involved in FAO (*ZFIN Gene: cpt1aa,* 2022). An upregulation of these genes can enhance FAO and increase energy production. Conversely, a downregulation can result in a decrease in FAO, disrupted lipid homeostasis, and impaired energy production in zebrafish larvae.

In agreement with other studies, the exposure of WY14643 resulted in a significant upregulation for *pparaa, pparab,* and *acox1.* This activation is consistent with *acox1* being a recognised Ppara target gene and WY14643 a selective Ppara agonist (Xu, Denning and Lu, 2022). The activation of these genes by WY14643 has previously been described in other species, including Atlantic cod and mice (Eide *et al*., 2023; Xu, Denning and Lu, 2022). Notably, in contrast to WY14643 exposure, the gene expression analysis following PFOS and PFOA exposure revealed low-dose effects, where the lowest doses caused a downregulation in the expression of *pparaa, pparab*, and *acox1*. The same low dose effect was also observed in the expression of *cpt1a* after PFOA exposure. At higher doses, the expression of these genes was similar to the control for both PFOS and PFOA. The striking similarities in the expression patterns, which were different from those of PFBA and PFBS, suggest that the carbon backbone length of these compound may be responsible for this specific modulation of gene expression of Ppara target genes. Previously, a significant downregulation in *pparaa* has also been observed in zebrafish larvae after exposure of 16 mg/L PFOS (Sant *et al*., 2021), supporting that PFOS can have inhibitory effects on Ppara gene expression. Furthermore, the expression of *cpt1a* after PFOS exposure consistently showed downregulations for all concentrations. Notably, this downregulation contradicts previous studies that reported an upregulation of *cpt1a* in PFOS-exposed mice and in zebrafish chronically exposed to PFOS from embryo to 150 dpf (Wang *et al*., 2014; Cheng *et al.*, 2016). This difference suggests that the response of *cpt1a* expression to PFOS exposure may vary over time and also across different species. c*pt1a* is involved in FAO, and primarily expressed in the liver. A downregulation in *cpt1a* could possibly lead to a decreased FAO, resulting in reduced energy production in zebrafish larvae (*ZFIN Gene: cpt1aa,* 2022).

The shorter-chained congeners, PFBA and PFBS, exhibited similar patterns of gene expression as WY14643, but separated themselves from the long-chained PFAS congeners. Specifically, both PFBS and PFBA exposure led to a significant upregulation of *pparaa* (Figure 4.3.1). However, both PFBS and PFBA acted differently for the other genes. PFBS did not affect the expression levels of the other genes tested, while PFBA significantly upregulated all the genes examined, similar to the effects observed after WY14643 exposure. Interestingly, in accordance with this thesis, a study by Weatherly *et al*., (2021) examining PFBA exposure in mice found this compound to induce an upregulation of both *ppara* and *acox1* expression. PFBA exposure resulted in liver dysfunction and increased liver weight in mice, which has previously also been observed after PFOA exposure (Das *et al*., 2017).

It is important to notice that the observed differences in morphology, phenotypic endpoints, and gene expression among the PFAS congeners in this study are influenced by the different concentration-ranges used for the different PFAS. Specifically, PFBA was tested across a concentration range of 187.5 to 750 mg/L, while PFBS was tested at concentrations ranging from 1.5 to 6.2 mg/L. The selection of these concentrations was based on the LC_{50} values from other articles, in addition to preliminary testing (section 3.2). Both PFBS and PFBA were initially tested at a starting concentration of 3000 mg/L, followed by a 3-fold dilution. However, coagulation was observed at the three highest concentrations of PFBS, leading to a lower exposure range compared to PFBA. As for the long-chained PFAS, PFOS did not coagulate based on the LC_{50} obtained from (Hagenaars *et al*., 2011), and it underwent a 3-fold dilution. On the other hand, PFOA started at a concentration of 200 mg/L but was subsequently diluted to 40 mg/L during the exposures due to coagulation in the highest concentrations.

5.9 Summary

Exposure to PFBS and PFOS resulted in significant morphological effects, such as reduction in body length, eye size, and nutrient absorption from the yolk sac. These results suggest that the sulfonic functional group in PFOS and PFBS might be related to these morphological effects. PFBS also produced a higher mortality rate at lower concentrations in comparison to the other compounds. In contrast, PFBA and PFOA did not show significant changes in morphology except for PFBA-exposure resulting in a slight reduction in eye size. Interestingly, the exposure of WY14643, induced similar changes in morphological endpoints as observed with PFOS and PFBS

exposure. This similarity suggests that genes in the Ppara signalling pathway may play a contributing role in the morphological changes seen in zebrafish larvae exposed to these compounds.

Gene expression analyses revealed a statistically significant upregulation of all genes assessed in the Ppara signalling pathway in response to PFBA exposure. In the case of PFBS, a slight, but significant upregulation of *pparaa* expression was only observed for the two lowest concentrations used. In comparison, the longer-chained PFAS congeners, PFOA and PFOS, exhibited low-dose effects by decreasing the expression of *pparaa, pparab* and *axoc1* at the lowest concentrations used, which was also observed for *cpt1a* after PFOA exposure. Subsequently, the expression levels of the higher concentrations remained relatively stable around the control levels. In line with expectations, WY14643 induced an upregulation of all genes in the Ppara signalling pathway, except for *cpt1a*. This finding aligns with previous studies by Urbatzka *et al*., (2015) and Eide *et al.,* (2014), which reported no significant difference in *cpt1* expression in turbot juveniles and in zebrafish liver cells following exposure to WY14643. It should be noted that higher exposure concentrations were used for PFBA exposure than for the other compounds, this could indicate that higher exposure concentrations of PFBA is needed to produce changes in gene expression.

A study by Li *et al*., (2020) revealed that zebrafish with *pparab* deficiency exhibited reduced fatty acid β-oxidation, metabolic reprogramming, and inhibition of amino acid breakdown. Interestingly, this deficiency led to an upregulation of *pparaa* and *cpt1a* expression, supporting a potential role of *pparaa* in regulating fatty acid oxidation (FAO) in the liver of zebrafish. This may indicate that *pparaa* primarily regulate hepatic FAO through *cpt1a* activity, while *pparab* primarily controls the expression of *cpt1b*. Considering the results from Li *et al*., it is reasonable to propose that exposure to PFAS could disrupt the normal function of Ppar signalling pathways. Such disruption may have consequences for FAO and metabolic regulation, potentially resulting in adverse effects on the health and development of zebrafish. The results from this thesis show an induction of *pparaa* after exposure to all compounds, except after PFOS exposure, whilst an induction of *cpt1a* was observed after exposure to PFOS, PFOA and PFBA. These findings and those of Li *et al*., suggest that PFAS exposure may interfere with Ppar signalling, affecting FAO and other metabolic processes in zebrafish.

During the morphological analyses, the sulfonic acids and WY14643 shared a similar pattern, affecting some of the same morphological endpoints. This suggest that these compounds might share some common mechanisms of action in influencing morphology. However, the gene expression analysis did not show a similar pattern among these compounds at the transcriptional level. This suggests that the morphological effects may be influenced by different genes or regulatory pathways than those assessed in the gene expression analysis in this thesis.

Conclusion

This thesis examined the toxicity of the two shorter-chained PFAS congeners PFBA and PFBS during early development of zebrafish and compared these effects to those induced by the long-chained PFOA and PFOS, as well as the Ppara agonist WY14643. My findings indicate that both the length of the carbon backbone and the functional group are related to specific toxicities of PFAS in zebrafish development. It is worth noticing that in this study, the zebrafish larvae were not exposed to the same concentrations of each substance. As it was desired to examine the toxicity of the shorter-chain PFAS, the concentrations were selected based on the LC_{50} values reported in other research papers (section 3.2). The variations in exposure concentrations between compounds makes it challenging to make direct comparisons of toxicity across all substances. Nevertheless, it is noteworthy that among the tested compounds, PFOS, with its fluorinated eight-carbon chain and sulfonic functional group, exhibited the highest number of significantly altered endpoints, indicating its greater teratogenicity during zebrafish development. Interestingly, similar morphological effects were observed in zebrafish larvae exposed to shorter-chained PFBS. This suggests that the sulfonic functional group may play a role in mediating the phenotypic effects observed, in contrast to the congeners containing a carboxylic functional group. This increased toxicity could be attributed to the higher hydrophobicity of sulphonic PFAS compounds, resulting in a greater potential for absorption and bioaccumulation (Ulhaq *et al*., 2013).

Furthermore, all tested compounds modulated the Ppara signalling pathway, but in different ways. The two long-chained PFAS congeners exhibited similar patterns of gene expression, in contrast to the two shorter-chained congeners that showed more similar effects on gene expression as the Ppara agonist WY14643, with an upregulation in *pparaa, pparab* and *acox1*. Notably, there was no apparent correlation between the sulfonic acids and the carboxylic acids in terms of affecting the Ppara signalling pathway. These findings suggest that the functional group did not appear to have determinative effects on the activation of the Ppara signalling pathway, but that the carbon backbone length played a more prominent role.

Future perspectives

This study showed that the shorter-chained PFAS congeners, PFBS and PFBA, exhibited varying degrees of toxic effects. Interestingly, their toxic effects differ not only from each other, but also from the long-chained PFOS and PFOA, as well as the PPAR-agonist WY14643. Nonetheless, some similarities were observed between the different compounds. PFBS, PFOS, and WY14643 affected some of the same morphological endpoints, while PFBA, PFBS, shared similar gene expression patterns regarding Ppara target genes. Further, it would be valuable to extend the observation of toxic effects on zebrafish development beyond the 96 hpf timeframe set in this thesis. This extension could help determine if certain congeners exhibit delayed or long-term toxicity, which may become apparent in later developmental stages, including the adult stage. Further investigation of genes involved in pathways related to the morphological changes observed in zebrafish larvae exposed to the sulfonic acids and WY14643 should be explored to understand the relationship between the sulfonic acids and WY14643, morphological changes, and gene expression.

Exploring additional endpoints can provide a more comprehensive understanding of the effects of PFAS exposure on zebrafish larvae. This could include behaviour and neurotoxic endpoints, including swimming speed and distance, as well as gene expression related to neuron and eye development and other morphological endpoints such as otolith deformation, heart rate, and jaw deformations. Jaw deformation was observed in this thesis after PFOS exposure but was not thoroughly examined. Additionally, investigating the endpoints at different time points than those in this thesis, to observe how the different stages of the developing zebrafish is affected,

particularly around the time of hatching can offer insights that may provide an extended understanding of the effects of PFAS exposure on zebrafish larvae.

To gain a broader understanding of the toxicity of different PFAS congeners, additional methods can be employed to investigate various aspects of their effects. RNA sequencing is a highly sensitive and accurate tool for measuring gene expression across the transcriptome of cells and organisms (Kukurba and Montgomery, 2015) and can provide a global view of changes in gene expression in response to PFAS exposure, possibly revealing effects on multiple signalling and metabolic pathways. Another potential method is using reporter gene assays for specifically study activation of the zebrafish Ppara receptors by different PFAS congeners *in vitro*. This can aid in assessing which PFAS that can act as Ppara agonists or antagonists and serve as a screening tool before more extensive *in vivo* experiments are conducted. Another interesting method that could be employed is *in situ* hybridisation (ISH). This technique can provide information on the precise location where changes in gene expression of Ppar-related genes are taking place, thus giving insight into the specific tissues and organs affected by PFAS exposure.

In this study, the assessment of toxicities of emerging PFAS was limited to PFBA and PFBS due to practical constraints such as time limitations and availability issues. Further studies focusing on the toxicological properties of other shorter-chained and branched PFAS congeners, such as perfluoro(2-methyl-3-oxahexanoic (HFPO-DA, also known as GenX), dodecafluoro-3H-4,8-dioxanonanoate (ADONA), perfluoropentanoic acid (PFPeA) and 6:2 chlorinated polyfluoroalkyl ether sulfonate (F-53B) should be done, as this will provide valuable insights into the potential risks associated with these emerging congeners.

To further understand the potential toxic effects of PFBA and PFBS, environmentally relevant doses of these substances should be tested in a similar way as described this thesis. This would provide insight into the specific risks associated with exposure to these shorter-chained PFAS congeners in real-world exposure scenarios. Additionally, investigating the mixture effects of PFBA and PFBS with other PFAS compounds could contribute to an increased comprehensive understanding of the combined impact of multiple PFAS congeners on toxicity. Moreover, incorporating studies that involve human or mammalian receptors in similar exposure

scenarios alongside zebrafish studies would contribute to expand our understanding of the risks associated with PFAS congeners for both humans and the environment.

As an initiative to reduce the use of PFAS, the European Chemicals Agency (ECHA) published a PFAS restriction proposal on the use of PFAS. The proposal was prepared by authorities in Denmark, Germany, the Netherlands, Norway and Sweden, and its aim is to reduce further PFAS contamination and emission in the environment in the EU (ECHA, 2023; European commission, 2023). However, the extensive use and their persistence in the environment means that PFAS continues to be an environmental pollutant. A global collaboration will be needed to continue the efforts to reduce their impact on the environment and biota.

References

Alharbi, O. M. L., Basheer, A. A., Khattab, R. A. and Ali, I. (2018) 'Health and environmental effects of persistent organic pollutants', *Journal of Molecular Liquids*. Elsevier, 263, pp. 442–453. doi: 10.1016/J.MOLLIQ.2018.05.029.

Almeida, N. M. S., Eken, Y. and Wilson, A. K. (2021) 'Binding of Per- And Polyfluoro-alkyl Substances to Peroxisome Proliferator-Activated Receptor Gamma', *ACS Omega*. American Chemical Society, 6(23), pp. 15103–15114. doi: 10.1021/ACSOMEGA.1C01304/SUPPL_FILE/AO1C01304_SI_001.PDF.

Annunziato, K. M., Marin, M., Liang, W., Coblin, S. M., Qi, W., Doherty, J., Lee, J., Clark, J. M., Park, Y. and Timme-Laragy, A. R. (2022) 'The Nrf2a pathway impacts zebrafish offspring development with maternal preconception exposure to perfluorobutanesulfonic acid', *Chemosphere*. Pergamon, 287, p. 132121. doi: 10.1016/J.CHEMOSPHERE.2021.132121.

Balaguer, P., Delfosse, V. and Bourguet, W. (2019) 'Mechanisms of endocrine disruption through nuclear receptors and related pathways', *Current Opinion in Endocrine and Metabolic Research*. Elsevier, 7, pp. 1–8. doi: 10.1016/J.COEMR.2019.04.008.

Behr, A. C., Plinsch, C., Braeuning, A. and Buhrke, T. (2020) 'Activation of human nuclear receptors by perfluoroalkylated substances (PFAS)', *Toxicology in Vitro*. Pergamon, 62, p. 104700. doi: 10.1016/J.TIV.2019.104700.

Blake, B. E. and Fenton, S. E. (2020) 'Early life exposure to per- and polyfluoroalkyl substances (PFAS) and latent health outcomes: A review including the placenta as a target tissue and possible driver of peri- and postnatal effects', *Toxicology*. NIH Public Access, 443, p. 152565. doi: 10.1016/J.TOX.2020.152565.

Brendel, S., Fetter, É., Staude, C., Vierke, L. and Biegel-Engler, A. (2018) 'Short-chain perfluoroalkyl acids: environmental concerns and a regulatory strategy under REACH', *Environmental Sciences Europe*. Springer Verlag, 30(1), pp. 1–11. doi: 10.1186/S12302-018-0134-4/FIGURES/2.

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., de Voogt, P., Jensen, A. A., Kannan, K., Mabury, S. A. and van Leeuwen, S. P. (2011) 'Perfluoroalkyl and polyfluoroalkyl substances in the environment: Terminology, classification, and origins', *Integrated Environmental Assessment and Management*. John Wiley & Sons, Ltd, 7(4), pp. 513–541. doi: 10.1002/IEAM.258.

Chen, F., Wei, C., Chen, Q., Zhang, J., Wang, L., Zhou, Z., Chen, M and Liang, Y. (2018) 'Internal concentrations of perfluorobutane sulfonate (PFBS) comparable to those of perfluorooctane sulfonate (PFOS) induce reproductive toxicity in Caenorhabditis elegans', *Ecotoxicology and Environmental Safety*. Academic Press, 158, pp. 223–229. doi: 10.1016/J.ECOENV.2018.04.032.

Chen, T.-H., Wang, Y.-H. and Wu, Y.-H. (2011) 'Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity

bioassays', *Aquatic toxicology (Amsterdam, Netherlands)*. Aquat Toxicol, 102(3–4), pp. 162–166. doi: 10.1016/J.AQUATOX.2011.01.010.

Cheng, J., Lv, S., Nie, S., Liu, S., Tong, S., Kang, N., Xiao, Y., Dong, Q., Huang, C. and Yang, D. (2016) 'Chronic perfluorooctane sulfonate (PFOS) exposure induces hepatic steatosis in zebrafish', *Aquatic Toxicology*. Elsevier, 176, pp. 45–52. doi: 10.1016/J.AQUATOX.2016.04.013.

Cheng, S. H., Wai, A. W. K., So, C. H. and Wu, R. S. S. (2000) 'Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 19(12), pp. 3024–3031. doi: 10.1002/ETC.5620191223.

Das, K. P., Wood, C. R., Lin, M. T., Starkov, A. A., Lau, C., Wallace, K. B., Corton, J. C. and Abbott, B. D. (2017) 'Perfluoroalkyl acids-induced liver steatosis: Effects on genes controlling lipid homeostasis', *Toxicology*. Elsevier, 378, pp. 37–52. doi: 10.1016/J.TOX.2016.12.007.

Delfosse, V., le Maire, A., Balaguer, P. and Bourguet, W. (2015) 'A structural perspective on nuclear receptors as targets of environmental compounds', *Acta Pharmacologica Sinica*. Nature Publishing Group, 36(1), p. 88. doi: 10.1038/APS.2014.133.

Dunn, N., Unni, D., Munoz-Torres, M., Harris, N., Yao, E., Rasche, H., Holmes, I., Elsik, C. and Lewis, S. (2019). Apollo: Democratizing genome annotation. 10.1101/512376.

ECHA (2023). Available at: https://echa.europa.eu/-/echa-publishes-pfas-restriction-proposal (Accessed: 26 May 2023).

Ehrlich, V., Bil, W., Vandebriel, R., Granum, B., Luijten, M., Lindeman, B., Grandjean, P., Kaiser, A-M., Hauzenberger, I., Hartmann, C., Gundacker, C. and Uhl, M. (2023) 'Consideration of pathways for immunotoxicity of per- and polyfluoroalkyl substances (PFAS)', *Environmental Health 2023 22:1*. BioMed Central, 22(1), pp. 1–47. doi: 10.1186/S12940-022-00958-5.

Eide, M., Goksøyr, A., Yadetie, F., Gilabert, A., Bartosova, Z., Frøysa, H. G., Fallahi, S., Zhang, X., Blaser, N., Jonassen, I., Bruheim, P., Alendal, G., Brun, M., Porte, C. and Karlsen, O. A. (2023) 'Integrative omics-analysis of lipid metabolism regulation by peroxisome proliferator-activated receptor a and b agonists in male Atlantic cod', *Frontiers in Physiology*. Frontiers Media S.A., 14. doi: 10.3389/FPHYS.2023.1129089/FULL.

Eide, M., Rusten, M., Male, R., Jensen, K. H. and Goksøyr, A (2014) 'A characterization of the ZFL cell line and primary hepatocytes as in vitro liver cell models for the zebrafish (*Danio rerio*)', Aquatic Toxicology. Elsevier, 147, pp. 7–17. doi: 10.1016/J.AQUATOX.2013.11.023.

European Commission (2023). Available at: https://environment.ec.europa.eu/strategy/chemicals-strategy/implementation_en (Accessed: 26 May 2023).

EPA (2023) 'EPA's Per- and Polyfluoroalkyl Substances (PFAS) Action Plan'. Available at: www.epa.gov/pfas (Accessed: 26 May 2023).

Fenton, S. E., Ducatman, A., Boobis, A., DeWitt, J. C., Lau, C., Ng, C., Smith, J. S. and Roberts, S. M. (2021) 'Per- and Polyfluoroalkyl Substance Toxicity and Human Health Review: Current State of Knowledge and Strategies for Informing Future Research', *Environmental toxicology and chemistry*. NIH Public Access, 40(3), p. 606. doi: 10.1002/ETC.4890.

FHI (2023) '*Environmental contaminants*'. Available at: https://www.fhi.no/en/el/environment-and-health/environmental-contaminants/ (Accessed: 2 May 2023).

Gaballah, S., Swank, A., Sobus, J. R., Howey, X. M., Schmid, J., Catron, T., McCord, J., Hines, E., Strynar, M. and Tal, T. (2020) 'Evaluation of Developmental Toxicity, Developmental Neurotoxicity, and Tissue Dose in Zebrafish Exposed to GenX and Other PFAS', *Environmental Health Perspectives*. Environmental Health Perspectives, 128(4). doi: 10.1289/EHP5843.

Galatius, A., Bossi, R., Sonne, C. and Rigét, F. F. (2013) 'PFAS profiles in three North Sea top predators: Metabolic differences among species?', *Environmental Science and Pollution Research*. Springer, 20(11), pp. 8013–8020. doi: 10.1007/S11356-013-1633-X/TABLES/2.

Gebreab, K. Y., Eeza, M. N. H., Bai, T., Zuberi, Z., Matysik, J., O´shea, K. E., Alia, A. and Berry, J. P. (2020) 'Comparative toxicometabolomics of perfluorooctanoic acid (PFOA) and next-generation perfluoroalkyl substances', *Environmental Pollution*. Elsevier, 265, p. 114928. doi: 10.1016/J.ENVPOL.2020.114928.

Glüge, J., Scheringer, M., Cousins, I. T., DeWitt, J. C., Goldenman, G., Herzke, D., Lohmann, R., Ng, C. A., Trier, X. and Wang, Z. (2020) 'An overview of the uses of per- and polyfluoroalkyl substances (PFAS)', *Environmental Science: Processes & Impacts*. The Royal Society of Chemistry, 22(12), pp. 2345–2373. doi: 10.1039/D0EM00291G.

Gobin, S., Thuillier, L., Jogl, G., Faye, A., Tong, L., Chi, M., Bonnefont, J-P., Girard, J. and Prip-Buus, C. (2003) 'Functional and Structural Basis of Carnitine Palmitoyltransferase 1A Deficiency', *Journal of Biological Chemistry*. Elsevier, 278(50), pp. 50428–50434. doi: 10.1074/jbc.M310130200.

Gomis, M. I., Vestergren, R., Borg, D. and Cousins, I. T. (2018) 'Comparing the toxic potency in vivo of long-chain perfluoroalkyl acids and fluorinated alternatives', *Environment International*. Pergamon, 113, pp. 1–9. doi: 10.1016/J.ENVINT.2018.01.011.

Gong, H., Du, J., Xu, J., Yang, Y., Lu, H. and Xiao, H. (2022) 'Perfluorononanoate and Perfluorobutane Sulfonate Induce Cardiotoxic Effects in Zebrafish', *Environmental Toxicology and Chemistry*. John Wiley & Sons, Ltd, 41(10), pp. 2527–2536. doi: 10.1002/ETC.5447.

Guerrero-Limón, G., Nivelle, R., Bich-Ngoc, N., Duy-Thanh, D. and Muller, M. (2023) 'A Realistic Mixture of Persistent Organic Pollutants Affects Zebrafish Development, Behavior, and Specifically Eye Formation by Inhibiting the Condensin I Complex', *Toxics*. MDPI, 11(4), p. 357. doi: 10.3390/TOXICS11040357/S1.

Hagenaars, A., Vergauwen, L., De Coen, W. and Knapen, D. (2011) 'Structure–activity relationship assessment of four perfluorinated chemicals using a prolonged zebrafish early life stage test', *Chemosphere*. Pergamon, 82(5), pp. 764–772. doi: 10.1016/J.CHEMOSPHERE.2010.10.076.

Hagenaars, A., Stinckens, E., Vergauwen, L., Bervoets, L. and Knapen, D. (2014) 'PFOS affects posterior swim bladder chamber inflation and swimming performance of zebrafish larvae', *Aquatic Toxicology*. Elsevier, 157, pp. 225–235. doi: 10.1016/J.AQUATOX.2014.10.017.

Howe, K., Clark, M., Torroja, C., Torrance, J. *et al.,* (2013) 'The zebrafish reference genome sequence and its relationship to the human genome', *Nature 2013 496:7446*. Nature Publishing Group, 496(7446), pp. 498–503. doi: 10.1038/nature12111.

Hsieh, Y. C., Chiang, M-C., Huang, Y-C., Yeh, T-H., Shih, H-Y., Liu, H-F., Chen, H-Y., Wang, C-P. and Cheng, Y-C. (2018) 'Pparα deficiency inhibits the proliferation of neuronal and glial precursors in the zebrafish central nervous system', *Developmental Dynamics*. John Wiley & Sons, Ltd, 247(12), pp. 1264–1275. doi: 10.1002/DVDY.24683.

Hu, C., Tang, L., Liu, M., Lam, P. K. S., Lam, J. C. W. and Chen, L. (2020) 'Probiotic modulation of perfluorobutanesulfonate toxicity in zebrafish: Disturbances in retinoid metabolism and visual physiology', *Chemosphere*. Pergamon, 258, p. 127409. doi: 10.1016/J.CHEMOSPHERE.2020.127409.

Huang, J. C. (2008) 'The role of peroxisome proliferator-activated receptors in the development and physiology of gametes and preimplantation embryos', *PPAR Research*. doi: 10.1155/2008/732303.

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M. T., Tanguay, R. L. and Dong, Q. (2010) 'Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS)', *Aquatic Toxicology*, Volume 98, Issue 2, p. 139-147. doi: 10.1016/j.aquatox.2010.02.003.

Jantzen, C. E., Annunziato, K. A., Bugel, S. M. and Cooper, K. R. (2016) 'PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression', *Aquatic Toxicology*. Elsevier, 175, pp. 160–170. doi: 10.1016/J.AQUATOX.2016.03.026.

Jian, J. M., Chen, D., Han, F-J., Gou, Y., Zeng, L., Lu, X. and Wang, F. (2018) 'A short review on human exposure to and tissue distribution of per- and polyfluoroalkyl substances (PFASs)', *Science of The Total Environment*. Elsevier, 636, pp. 1058–1069. doi: 10.1016/J.SCITOTENV.2018.04.380.

Kersten, S. (2014) 'Integrated physiology and systems biology of PPARα', *Molecular Metabolism.* Elsevier, 3(4), pp. 354-371. Doi: 10.1016/j.molmet.2014.02.002

Kukurba, K. R. and Montgomery, S. B. (2015) 'RNA Sequencing and Analysis', *Cold Spring Harbor protocols*. NIH Public Access, 2015(11), p. 951. doi: 10.1101/PDB.TOP084970.

Li, L. Y., Lv, H-B., Jiang, Z-Y., Chen, L-Q., Zhang, M-L. and Du, Z-Y. (2020) 'Peroxisomal proliferator-activated receptor α-b deficiency induces the reprogramming of nutrient metabolism in zebrafish', *The Journal of Physiology*. John Wiley & Sons, Ltd, 598(20), pp. 4537–4553. doi: 10.1113/JP279814.

Lieder, P. H., York, R. G., Hakes, D. C., Chang, S-C. and Butenhoff, J. L. (2009) 'A two-generation oral gavage reproduction study with potassium perfluorobutanesulfonate (K+PFBS) in Sprague Dawley rats', *Toxicology*. Elsevier, 259(1–2), pp. 33–45. doi: 10.1016/J.TOX.2009.01.027.

Liu, Y., D´Agostino, L. A., Qu, G., Jiang, G. and Martin, J. W. (2019) 'High-resolution mass spectrometry (HRMS) methods for nontarget discovery and characterization of poly- and per-fluoroalkyl substances (PFASs) in environmental and human samples', *TrAC Trends in Analytical Chemistry*. Elsevier, 121, p. 115420. doi: 10.1016/J.TRAC.2019.02.021.

Liu, X., Liu, S., Qiu, W., Magnuson, J. T., Liu, Z., Yang, G., Chen, H., Li, Y., Xu, X. and Zheng, C. (2022) 'Cardiotoxicity of PFOA, PFOS, and PFOSA in Early Life Stage Zebrafish: Molecular Changes to Behavioral-level Response', *Sustainable Horizons*. Elsevier, 3, p. 100027. doi: 10.1016/j.horiz.2022.100027

Luebker, D. J., Case, M. T., York, R. G., Moore, J. A., Hansen, K. J., & Butenhoff, J. L. (2005) 'Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats', *Toxicology*. Elsevier, 215(1–2), pp. 126–148. doi: 10.1016/J.TOX.2005.07.018.

Madsen, A. K. (2016). 'Kloning, karakterisering og ligandaktivering av aryl hydrokarbonreseptor 2 (AHR2) fra Atlanterhavstorsk (Gadus morhua)', Master thesis, University of Bergen, Bergen.

Madureira, T. V., Pinheiro, I., Malhão, F., Castro, L. F. C., Rocha, E and Urbatza, R. (2019) 'Silencing of PPARαBb mRNA in brown trout primary hepatocytes: effects on molecular and morphological targets under the influence of an estrogen and a PPARα agonist', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. Pergamon, 229, pp. 1–9. doi: 10.1016/J.CBPB.2018.12.001.

Mahoney, H., Xie, Y., Brinkmann, M. and Giesy, J. P. (2022) 'Next generation per- and poly-fluoroalkyl substances: Status and trends, aquatic toxicity, and risk assessment', *Eco-Environment & Health*. Elsevier, 1(2), pp. 117–131. doi: 10.1016/J.EEHL.2022.05.002.

Margolis, R. and Sant, K. E. (2021) 'Associations between exposures to perfluoroalkyl substances and diabetes, hyperglycemia, or insulin resistance: a scoping review', *Journal of Xenobiotics*. MDPI, 11(3), pp. 115–129. doi: 10.3390/JOX11030008/S1.

Martínez, R., Navarro-Martín, L., Luccarelli, C., Codina, A. E., Raldúa, D., Barata, C., Tauler, R. and Piña, B. (2019) 'Unravelling the mechanisms of PFOS toxicity by combining morphological and transcriptomic analyses in zebrafish embryos', *Science of The Total Environment*. Elsevier, 674, pp. 462–471. doi: 10.1016/J.SCITOTENV.2019.04.200.

Menger, F., Pohl, J., Ahrens, L., Carlsson, G. and Örn, S. (2020) 'Behavioural effects and bioconcentration of per- and polyfluoroalkyl substances (PFASs) in zebrafish (*Danio rerio*) embryos', *Chemosphere*. Pergamon, 245, p. 125573. doi: 10.1016/J.CHEMOSPHERE.2019.125573.

Mitchell, C. A., Dasgupta, S., Zhang, S., Stapleton, H. M. and Volz, D. C. (2018) 'Disruption of Nuclear Receptor Signaling Alters Triphenyl Phosphate-Induced Cardiotoxicity in Zebrafish Embryos', *Toxicological Sciences*. Oxford Academic, 163(1), pp. 307–318. doi: 10.1093/TOXSCI/KFY037.

Mylroie, J. E., Wilbanks, M. S., Kimble, A. N., To, K. T., Cox, C. S., McLeod, S. J., Gust, K. A., Moore, D. W., Perkins, E. J. and Garcia-Reyero, N. (2021) 'Perfluorooctanesulfonic Acid–Induced Toxicity on Zebrafish Embryos in the Presence or Absence of the Chorion', Environmental Toxicology and Chemistry. John Wiley & Sons, Ltd, 40(3), pp. 780–791. doi: 10.1002/ETC.4899.

Pérez, F., Nadal, M., Navarro-Ortega, A., Fàbrega, F., Domingo, J. L., Barceló, D. and Farré, M. (2013) 'Accumulation of perfluoroalkyl substances in human tissues', *Environment International*. Pergamon, 59, pp. 354–362. doi: 10.1016/J.ENVINT.2013.06.004.

Quinlivan, V. H. and Farber, S. A. (2017) 'Lipid uptake, metabolism, and transport in the larval zebrafish', *Frontiers in Endocrinology*. Frontiers Media S.A., 8(NOV), p. 319. doi: 10.3389/FENDO.2017.00319/BIBTEX.

Rakhshandehroo, M., Knoch, B., Müller, M and Kersten, S. (2010) 'Peroxisome Proliferator-Activated Receptor Alpha Target Genes', *PPAR Research*. Hindawi Limited, 2010. doi: 10.1155/2010/612089.

Rasheed, T., Bilal, M., Nabeel, F., Adeel, M. and Iqbal, H. M. N. (2019) 'Environmentally-related contaminants of high concern: Potential sources and analytical modalities for detection, quantification, and treatment', *Environment International*. Pergamon, 122, pp. 52–66. doi: 10.1016/J.ENVINT.2018.11.038.

Rizvi, Y. Q., Mehta, C. S. and Oyekan, A. (2013) 'Interactions of PPAR-alpha and adenosine receptors in hypoxia-induced angiogenesis', *Vascular Pharmacology*. Elsevier, 59(5–6), pp. 144–151. doi: 10.1016/J.VPH.2013.09.001.

Rosen, M. B., Das, K. P., Rooney, J., Abbott, B., Lau, C. and Corton, J. C. (2017) 'PPARα-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling', *Toxicology*. NIH Public Access, 387, p. 95. doi: 10.1016/J.TOX.2017.05.013.

Sant, K. E., Annunziato, K., Conlin, S., Teicher, G., Chen, P., Venezia, O., Downes, G. B., Park, Y. and Timme-Laragy, A. R. (2021) 'Developmental exposures to perfluorooctanesulfonic acid (PFOS) impact embryonic nutrition, pancreatic morphology, and adiposity in the zebrafish, *Danio rerio*', *Environmental Pollution*. Elsevier, 275, p. 116644. doi: 10.1016/J.ENVPOL.2021.116644.

Satbhai, K., Vogs, C. and Crago, J. (2022) 'Comparative toxicokinetics and toxicity of PFOA and its replacement GenX in the early stages of zebrafish', *Chemosphere*. Pergamon, 308, p. 136131. doi: 10.1016/J.CHEMOSPHERE.2022.136131.

Schaaf, M. J. M. (2017) 'Nuclear receptor research in zebrafish', *Journal of molecular endocrinology*. J Mol Endocrinol, 59(1), pp. R65–R76. doi: 10.1530/JME-17-0031.

Schmittgen, T. D. and Livak, K. J. (2008) 'Analyzing real-time PCR data by the comparative CT method', *Nature Protocols*, 3(6), pp. 1101–1108. doi: 10.1038/NPROT.2008.73.

Sciancalepore, G., Pietroluongo, G., Centelleghe, C., Milan, M., Bonato, M., Corazzola, G. and Mazzariol, S. (2021) 'Evaluation of per- and poly-fluorinated alkyl substances (PFAS) in livers of bottlenose dolphins (Tursiops truncatus) found stranded along the northern Adriatic Sea.', *Environmental Pollution*. Elsevier, 291, p. 118186. doi: 10.1016/J.ENVPOL.2021.118186.

Shabalina, I. G., Kalinovich, A. V., Cannon, B. and Nedergaard, J. (2016) 'Metabolically inert perfluorinated fatty acids directly activate uncoupling protein 1 in brown-fat mitochondria', *Archives of Toxicology*. Springer, 90(5), p. 1117. doi: 10.1007/S00204-015-1535-4.

Shi, X., Du, Y., Lam, P. K., Wu, R. S. and Zhou, B. (2008) 'Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS', *Toxicology and Applied Pharmacology*. Academic Press, 230(1), pp. 23–32. doi: 10.1016/J.TAAP.2008.01.043.

Sri Ranjani, T., Pitchika, G. K., Yedukondalu, K., Gunavathi, Y., Daveedu, T., Sainath, S. B., Philip, G. H. and Pradeepkiran, J. A. (2020) 'Phenotypic and transcriptomic changes in zebrafish (*Danio rerio*) embryos/larvae following cypermethrin exposure', *Chemosphere*. Pergamon, 249, p. 126148. doi: 10.1016/J.CHEMOSPHERE.2020.126148.

Steenland, K., Fletcher, T. and Savitz, D. A. (2010) 'Epidemiologic Evidence on the Health Effects of Perfluorooctanoic Acid (PFOA)', *Environmental Health Perspectives*. National Institute of Environmental Health Sciences, 118(8), p. 1100. doi: 10.1289/EHP.0901827.

Stockholm Convention (2019). Available at: http://chm.pops.int/ (Accessed: 26 May 2023).

Sun, B., Liu, M., Tang, L., Hu, C., Huang, Z., Zhou, X. and Chen, L. (2021) 'Probiotic supplementation mitigates the developmental toxicity of perfluorobutanesulfonate in zebrafish larvae', *Science of The Total Environment*. Elsevier, 799, p. 149458. doi: 10.1016/J.SCITOTENV.2021.149458.

Søderstrøm, S. (2017). 'Peroxisome Proliferator-Activated Receptors (PPARs) As Tools For Studying Effects Of Contaminants On The Lipid Metabolism In Atlantic Cod (*Gadus morhua*)', Master thesis, University of Bergen, Bergen.

Søderstrøm, S., Lille-Langøy, R., Yadetie, F., Rauch, M., Milinski, A., Dejaegere, A., Stote, R. H., Goksøyr, A. and Karlsen, O. A. (2022) 'Agonistic and potentiating effects of perfluoroalkyl substances (PFAS) on the Atlantic cod (Gadus morhua) peroxisome proliferator-activated receptors (Ppars)', *Environment International*. Pergamon, 163, p. 107203. doi: 10.1016/J.ENVINT.2022.107203.

Takacs, M. L., & Abbott, B. D. (2007). Activation of mouse and human peroxisome proliferator-activated receptors (alpha, beta/delta, gamma) by perfluorooctanoic acid and perfluorooctane sulfonate. Toxicological Sciences: An Official Journal of the Society of Toxicology, 95(1), 108–117. https://doi.org/10.1093/toxsci/kfl135

Tang, L., Song, S., Hu, C., Liu, M., Lam, P. K. S., Zhou, B., Lam, J. C. W. and Chen, L. (2020) 'Parental exposure to perfluorobutane sulfonate disturbs the transfer of maternal transcripts and offspring embryonic development in zebrafish', *Chemosphere*. Pergamon, 256, p. 127169. doi: 10.1016/J.CHEMOSPHERE.2020.127169.

Tartu, S., Bourgeon, S., Aars, J., Andersen, M., Lone, K., Jenssen, B. M., Polder, A., Thiemann, G. W., Torget, V., Welker, J. M. and Routti, H. (2017) 'Diet and metabolic state are the main factors determining concentrations of perfluoroalkyl substances in female polar bears from Svalbard', *Environmental Pollution*. Elsevier, 229, pp. 146–158. doi: 10.1016/J.ENVPOL.2017.04.100.

Tokuç, A. (2013) 'Stockholm Convention (2001)', BT - Encyclopedia of Corporate Social Responsibility, in: S.O. Idowu, N. Capaldi, L. Zu, A. Das Gupta (Eds.), Springer Berlin Heidelberg, Berlin, Heidelberg: pp. 2329-2336. https://doi.org/10.1007/978-3-642-28036-8_15

Tyagi, S., Gupta, P., Saini, A. S., Kaushak, C. and Sharma, S. (2011) 'The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases', *Journal of Advanced Pharmaceutical Technology & Research*. Wolters Kluwer -- Medknow Publications, 2(4), p. 236. doi: 10.4103/2231-4040.90879.

Ulhaq, M., Orn. S., Carlsson, G., Morrison, D. A. and Norrgren, L. (2013) 'Locomotor behavior in zebrafish (*Danio rerio*) larvae exposed to perfluoroalkyl acids', *Aquatic Toxicology*. Elsevier, 144–145, pp. 332–340. doi: 10.1016/J.AQUATOX.2013.10.021.

Urbatzka, R., Galante-Oliveira, S., Rocha, E., Castro, L. F. C. and Cunha, I. (2013) 'Tissue expression of PPAR-alpha isoforms in Scophthalmus maximus and transcriptional response of target genes in the heart after exposure to WY-14643', *Fish Physiology and Biochemistry*. Springer, 39(4), pp. 1043–1055. doi: 10.1007/S10695-012-9761-7/FIGURES/4.

Urbatzka, R., Galante-Oliveira, S., Rocha, E., Lobo-da-Cunha, A., Castro, L. F. C. and Cunha, I. (2015) 'Effects of the PPARα agonist WY-14,643 on plasma lipids, enzymatic activities and mRNA expression of lipid metabolism genes in a marine flatfish, Scophthalmus maximus', *Aquatic Toxicology*. Elsevier, 164, pp. 155–162. doi: 10.1016/J.AQUATOX.2015.05.004.

Venezia, O., Islam, S., Cho, C., Timme-Laragy, A. R. and Sant, K. E. (2021) 'Modulation of PPAR signaling disrupts pancreas development in the zebrafish, *Danio rerio'*, *Toxicology and applied pharmacology*. NIH Public Access, 426, p. 115653. doi: 10.1016/J.TAAP.2021.115653.

Wang, L., Wang, Y., Liang, Y., Li, J., Liu, Y., Zhang, J., Zhang, A., Fu, J. and Jiang, G. (2014) 'PFOS induced lipid metabolism disturbances in BALB/c mice through inhibition of low density lipoproteins excretion'. doi: 10.1038/srep04582.

Wang, Z., Cousins, I. T., Scheringer, M. and Hungerbühler, K. (2013) 'Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and their potential

precursors', *Environment International*. Pergamon, 60, pp. 242–248. doi: 10.1016/J.ENVINT.2013.08.021.

Wasel, O., Thompson, K. M. and Freeman, J. L. (2022) 'Assessment of unique behavioral, morphological, and molecular alterations in the comparative developmental toxicity profiles of PFOA, PFHxA, and PFBA using the zebrafish model system', *Environment International*. Pergamon, 170, p. 107642. doi: 10.1016/J.ENVINT.2022.107642.

Weatherly, L. M., Shane, H. L., Lukomska, E., Baur, R. and Anderson, S. E. (2021) 'Systemic toxicity induced by topical application of heptafluorobutyric acid (PFBA) in a murine model', *Food and Chemical Toxicology*. Pergamon, 156, p. 112528. doi: 10.1016/J.FCT.2021.112528.

Weikum, E. R., Liu, X. and Ortlund, E. A. (2018) 'The nuclear receptor superfamily: A structural perspective', *Protein Science*. John Wiley & Sons, Ltd, 27(11), pp. 1876–1892. doi: 10.1002/PRO.3496.

Xu, Y., Denning, K. L. and Lu, Y. (2022) 'PPARα agonist WY-14,643 induces the PLA2/COX-2/ACOX1 pathway to enhance peroxisomal lipid metabolism and ameliorate alcoholic fatty liver in mice', *Biochemical and Biophysical Research Communications*. Academic Press, 613, pp. 47–52. doi: 10.1016/J.BBRC.2022.04.132.

Yang, Z., Fu. L., Cao, M., Li, F., Li, J., Chen, Z., Guo, A., Zhong, H., Li, W., Liang, Y. and Luo, Q. (2023) 'PFAS-induced lipidomic dysregulations and their associations with developmental toxicity in zebrafish embryos', *Science of The Total Environment*. Elsevier, 861, p. 160691. doi: 10.1016/J.SCITOTENV.2022.160691.

Zeng, Z., Song, B., Xiao, R., Zeng, G., Gong, J., Chen, M., Xu, P., Zhang, P., Shen, M. and Yi, H. (2019) 'Assessing the human health risks of perfluorooctane sulfonate by in vivo and in vitro studies', *Environment International*. Pergamon, 126, pp. 598–610. doi: 10.1016/J.ENVINT.2019.03.002.

ZFIN Gene: *acox1* (2022). Available at: https://zfin.org/ZDB-GENE-041010-219 (Accessed: 30 May 2023).

ZFIN Gene: *cpt1aa* (2022). Available at: https://zfin.org/ZDB-GENE-060503-925 (Accessed: 30 May 2023).

ZFIN Gene: *pparaa* (2022). Available at: https://zfin.org/ZDB-GENE-041210-169 (Accessed: 30 May 2023).

ZFIN Gene: *pparab* (2022). Available at: https://zfin.org/ZDB-GENE-990415-211#summary (Accessed: 30 May 2023).

Zhang, L., Li, Yy., Chen, T., Xia, W., Zhou, Y., Wan, Y. J., Lv, Z. Q,. Li, G. Q. and Xu, S. Q. (2011) 'Abnormal development of motor neurons in perfluorooctane sulphonate exposed zebrafish embryos'. *Ecotoxicology* 20, p. 643–652. doi: 10.1007/s10646-011-0604-6

Appendix I

PFOS exposed zebrafish larvae had significant changes in spontaneous movement during the exposure period (Figure 1).

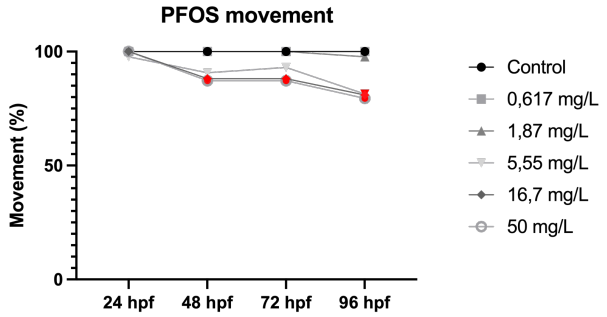


Figure 1: Spontaneous movements (%) recorded in zebrafish larvae exposed to PFOS during the first 96 hours after fertilisation. Red points indicate statistical significance (*p<0.05) compared to control group.

Spine malformations in exposed zebrafish larvae were only observed after PFOS exposure (Figure 2).

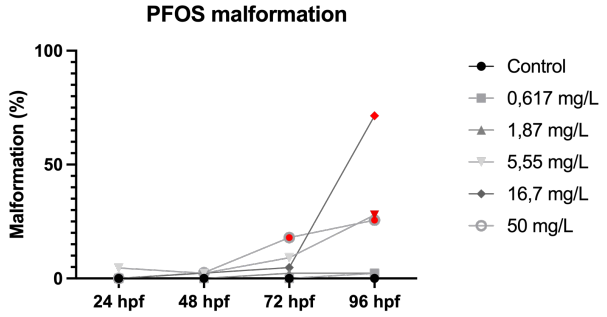


Figure 2: Percentage of malformations in zebrafish larvae exposed to PFOS in increasing concentrations over a 96-hour period. DMSO (0.01%) was used as a solvent control. Red points indicate statistical significance (*p<0.05) compared to control group.

Hatching success were significantly altered after exposure to all PFAS congeners tested in this thesis (Figure 3).

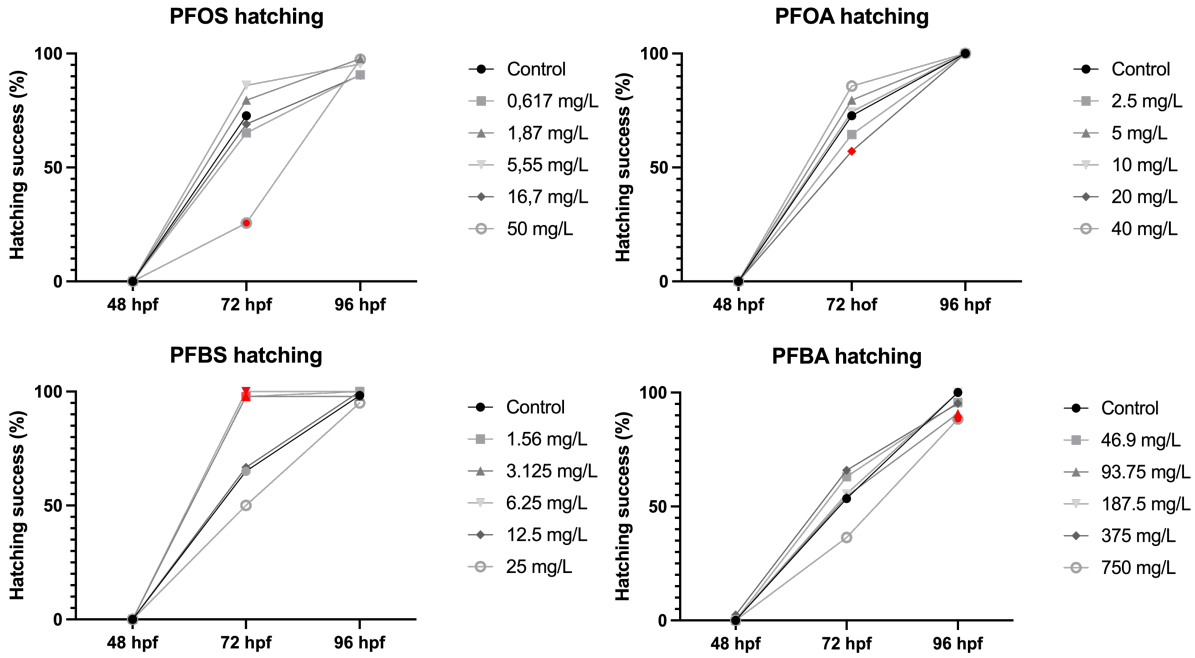


Figure 3: Hatching success (%) in a) PFOS, b) PFOA, c) PFBS and d) PFBA in the first 96 hpf. DMSO (0.01%) was used as a solvent control for PFOS and PFOA, 10mM MOPS as solvent control for PFBA and E3 medium as solvent control for PFBS. Red points indicate statistical significance (*p<0.05) compared to control group.

A significant change in the survival success in zebrafish larvae exposed to PFBS were observed during the exposure period.

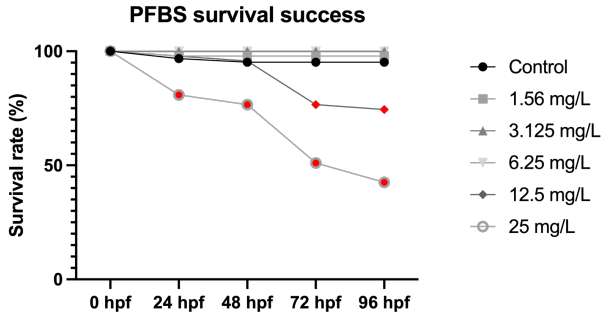


Figure 4: Survival success (%) in zebrafish larvae exposed to increasing concentrations of PFBS during the first 96-hour period. E3 medium was used as solvent control. Red points indicate statistical significance (*p<0.05) compared to control group.

Appendix II

The integrity of RNA extracted from exposed 96 hpf zebrafish larvae were assessed with agarose gel electrophoresis, shown in Figure 1-4.

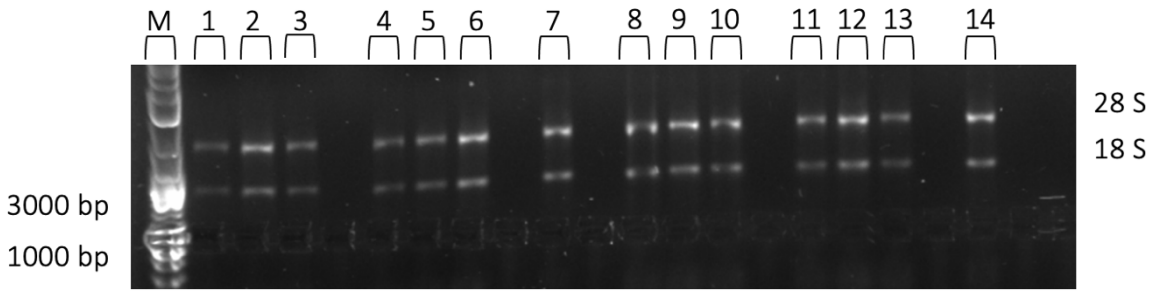


Figure 1: Control of RNA integrity after PFOS and PFOA exposure. Samples of RNA isolated from PFOA and PFOS exposed zebrafish larvae (n = 10) analysed with agarose gel electrophoresis. Zebrafish larvae were exposed to 40 mg/L (sample 1 and 8), 20 mg/L (sample 2 and 9) and 10 mg/L (Sample 3 and 10) PFOA, 5.5 mg/L (sample 4 and 11), 1.8 mg/L (sample 5 and 12) and 0.6 mg/L (sample 6 and 13) PFOS and solvent control (0.01%) DMSO (sample 7 and 14) for 96 hpf. In each well 10 μL RNA sample was added. 0.5 μL 2log DNA ladder (M) was added as size marker.

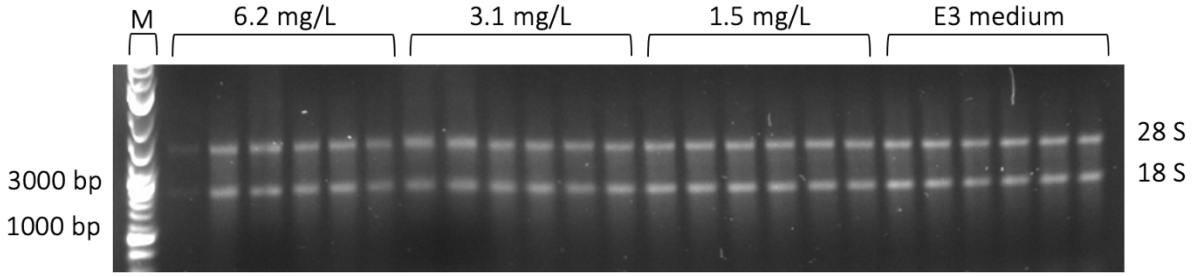


Figure 2: Control of RNA integrity after PFBS exposure. Samples of RNA isolated from PFBA exposed zebrafish larvae (n = 10) analysed with agarose gel electrophoresis. Zebrafish larvae were exposed to 6.2 mg/L, 3.1 mg/L and 1.5 mg/L PFBS and solvent control E3 medium for 96 hpf. In each well 10 μL RNA sample was added. 0.5 μL 2log DNA ladder (M) was added as size marker.

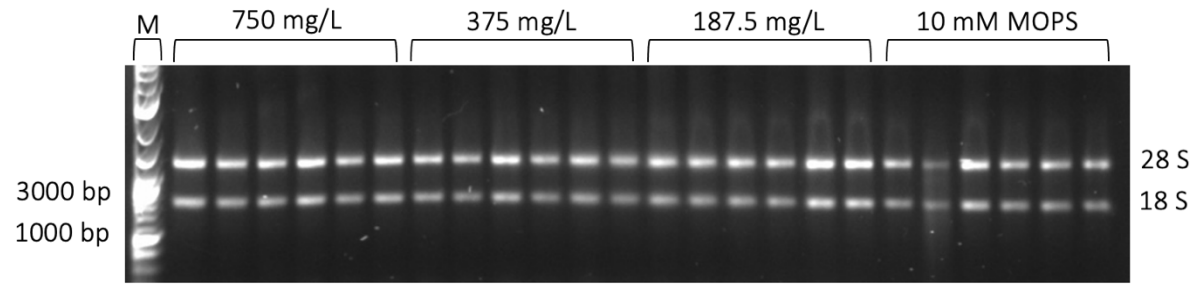


Figure 3: Control of RNA integrity after PFBA exposure. Samples of RNA isolated fromPFBA exposed zebrafish larvae (n = 10) analysed with agarose gel electrophoresis. Zebrafish larvae were exposed to 750 mg/L, 1375 mg/L and 187.5 mg/L PFBA and solvent control 10 mM MOPS for 96 hpf. In each well 10 μL RNA sample was added. 0.5 μL 2log DNA ladder (M) was added as size marker.

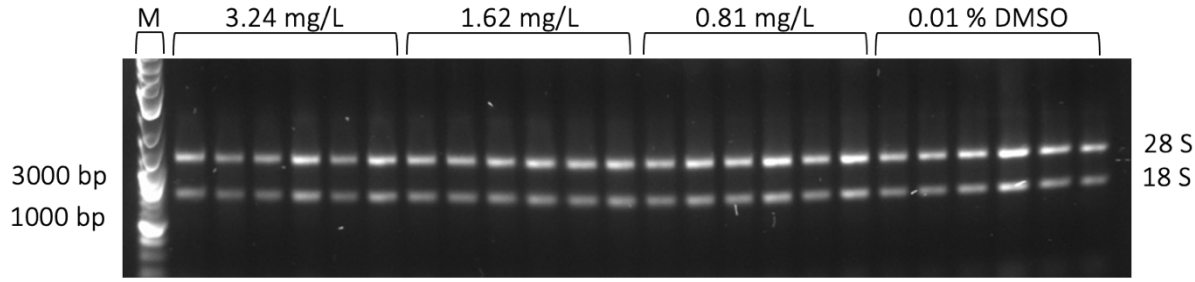


Figure 4: Control of RNA integrity after WY14643 exposure. Samples of RNA isolated from WY14643 exposed zebrafish larvae (n = 10) analysed with agarose gel electrophoresis. Zebrafish larvae were exposed to 3.24 mg/L, 1.62 mg/L and 0.81 mg/L WY14643 and solvent control (0.01%) DMSO for 96 hpf. In each well 10 μL RNA sample was added. 0.5 μL 2log DNA ladder (M) was added as size marker.