The Toxicity of Perfluoroalkyl Acids in Zebrafish (*Danio rerio*)

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Cover: A whole-body autoradiogram of female zebrafish (*Danio rerio*) 10 day after exposure to ^{14}C-PFOA (above) and the corresponding section of the same fish (below)
(photo: S. Örn)

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Abstract

Perfluoroalkyl acids (PFAAs) are globally distributed synthetic chemicals. Their unique properties make them widely used, in industry and elsewhere. These are a group of compounds with varying carbon chain lengths and functional groups. The toxicity evaluation of PFAAs is important to better assess their organismal and environmental impact.

The developmental toxicity of a group of PFAAs was evaluated in zebrafish (*Danio rerio*), using the embryotoxicity and locomotor behavior endpoints. Structure related relationships were examined using endpoints based on lethal and sublethal effect. The EC_{50} based toxicity ranking of the PFAAs indicated that the toxicity pattern was governed by carbon chain length and attached functional group. The behavior analyses showed that the PFAAs have distinct patterns in terms of locomotor activity of larvae.

Radiolabeled ^{14}C-PFOA was used to investigate the uptake, kinetics and distribution of the chemical in zebrafish. An uptake and elimination approach to experimentation was employed for kinetic assessment. A parallel equilibrium model bioconcentration strategy for 100-fold exposure range determined the internal body burden. Whole-body autoradiography and liquid scintillation techniques revealed the tissue distribution of PFOA. Remarkable levels of radioactivity were observed in target organs. The labeling of oocytes confirmed the maternal transfer of PFOA in zebrafish.

In another study the effects of PFOA on reproduction and sexual development in zebrafish were evaluated. Reproductive traits like spawning, fecundity and fertilization were not altered in a short term experiment. The period of sexual differentiation in zebrafish was examined to determine the effects of PFOA on development, growth and gonad maturation. The development and growth of the zebrafish were not affected, but a trend in decreased survival in maternally exposed embryos was observed. The expression of Vtg mRNA level in the liver of PFOA-exposed male fish was not induced. Similarly, an inhibitory trend of PPARα mRNA expression in the liver of the highest PFOA exposed zebrafish was observed.

The developmental toxicity ranking of PFAAs and kinetic assessment of PFOA with bioconcentration in zebrafish will provide an insight into risk assessment and replacement strategies of the compounds.

Keywords: zebrafish, perfluoroalkyl acids, PFOA, toxicity, reproduction, early development, kinetic

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وَلَا تَسْتَوِي الْحَسَنَةُ وَلَا السَّيِّئَةُ ۚ ادْفَعْ بِالَّتِي هِيَ أَحْسَنُ فَإِذَا الَّذِي
بَيْنَكَ وَبَيْنَهُ عَدَاوَةٌ كَأَنَّهُ وَلِيٌّ حَمِيمٌ ﴿٣٤﴾

And not equal are the good deed and the bad. Repel (evil) by that (deed) which is better; and thereupon the one whom between you and him is enmity (will become) as though he was a devoted friend. (Al-Quran 41:34).

To memories of my late mother

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ulhaq, M., Carlsson, G., Örn, S. & Norrgren, L. 2013. Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos. *Environmental Toxicology and Pharmacology* 36, 423-426.

II Ulhaq, M., Örn, S., Carlsson, G., Morrison, D. A. & Norrgren, L. 2013. Locomotor behavior in zebrafish (*Danio rerio*) larvae exposed to perfluoroalkyl acids. *Aquatic Toxicology* 144-145C, 332-340.

III Ulhaq, M., Örn, S., Sundström, M., Larsson, P., Gabrielsson, J., Bergman, Å. & Norrgren, L. Tissue uptake, distribution and elimination of ^{14}C-PFOA in zebrafish (*Danio rerio*). (Submitted)

IV Ulhaq, M., Örn, S., Carlsson, G., Tallkvist, J., Norrgren, L., Effect of perfluorooctanoic acid (PFOA) on zebrafish (*Danio rerio*) reproduction and sexual development. (Manuscript)

Abbreviations

ANOVA	Analysis of variance
AR	Accumulation ratio
AUC	Area under curve
AUMC	Area under moment curve
BCF	Bioconcentration factor
CAT	Catalase
dpf	Days post fertilization
EC_{50}	Effective concentration (50% affected)
EDC	Endocrine disrupting chemical
FSDT	Fish sexual development test
FSTRA	Fish short term reproduction assay
GLM	General linear models
GR	Glutathione reductase
GtH	Gonadotropin hormone
HE	Haematoxylin and eosin
hpf	Hours post fertilization
LC_{50}	Lethal concentration (50% mortality)
LOEC	Lowest observed effect concentration
LSC	Liquid scintillation counting
MANOVA	Multivariate analysis of variance
MRT	Mean residence time
OECD	Organisation for economic cooperation and development
PFAS	Perfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylic acid
PFSA	Perfluoroalkyl sulfonic acid
PPAR	Peroxisome proliferating activated receptor
RDA	Redundancy Analysis
Real-time	

RT-PCR	Real time reverse transcription polymerase chain reaction
$T_{1/2}$	Half life
Vtg	Vitellogenin
WBA	Whole body autoradiography

1 Introduction

1.1 General background

Environmental pollution is a problem that is linked to the development, and expansion of industrial society, and to rising population density during the 20th century. Environmental contamination is intensified by the huge production of anthropogenic chemicals with properties favorable for specific purposes. In the past 80 years the production of chemicals has increased from 1 to >400 million tons annually (Vogelgesang, 2002). Moreover, many chemicals are often present in consumer products, which we regularly come across in daily life. An aspect of the widespread problem is the presence of endocrine active chemicals. Many such chemicals are released and enter aquatic ecosystems.

It is intuitively obvious that aquatic organisms like fish are continuously exposed to a number of waterborne toxicants from different sources. The presence of unknown toxic chemicals can be detected by measuring the effects on the physiological mechanisms in aquatic organisms (Scott & Sloman, 2004). Therefore, traditionally these have been regarded as an important component of toxicity testing strategies. A variety of potential sources and routes of toxicant exposure exist in aquatic systems, some distinctly different from those of terrestrial animals. Aquatic organisms play an important role as early warning and monitoring systems for pollutant burdens in the environment. Fish display a vast diversity represented by more than 20,000 species and spanning more than 400 million years of evolution (Marshall, 1966). Unlike terrestrial animals, fish live in an atmosphere virtually devoid of oxygen. Consequently, fish extract volumes of water weighing thousands of times of their body weight daily to satisfy their oxygen demands. The integument and gills of aquatic organisms, which differ in permeability according to species, are important sites of contaminant uptake. In fishes, the efficient circulatory system allows the rapid distribution of contaminants crossing the barriers.

Aquatic organisms may accumulate chemicals directly from the water. Bioaccumulation assessment is particularly important for understanding the potential risk of contaminants, their fate and dynamics in aquatic species. The toxicity of a chemical is dependent on the extent of its bioaccumulation in an organism and particularly the quantity reaching the target organ or tissue to stimulate a response. Hence, the magnitude of bioaccumulation is often considered as internal exposure in dose response modeling.

Early stages of development are considered to be very sensitive to chemical exposure, especially for oviparous organisms like fish and birds (Nagel, 2002; Russell *et al.,* 1999; von Westernhagen, 1988). "Developmental toxicity" is a somewhat broader term than "teratology" covering embryotoxicity, compromised growth, functional deficiency in the offspring (larval stage) and morphological abnormalities. Toxicants exert their effects particularly during the critical periods of development. Intense research has been conducted on endocrine disruption, mainly focused on disruption of sex hormones, sexual differentiation and reproductive system (Andersen *et al.,* 2006; Meucci & Arukwe, 2005; Orn *et al.,* 2003; Vinggaard *et al.,* 2000).

The nervous system is one of the most complex organs in the body, comprising several cell types, anatomies, structural characteristics and functions. These features make it a unique target for toxicants that may act on multiple sites in different ways (Moser, 2008). In the past, the screening of chemicals for potential neurotoxicity was heavily dependent on finding aversive effects on the structure of the nervous system (neuropathology). Chemical induced changes in behavior like functional endpoints are increasingly being used now in risk assessment studies. Recently, neurobehavioral and pathological evaluations of the nervous system have been complementary components of the toxicity testing of the environmental contaminants. Behavioral endpoints characterization is particularly important for neurotoxicity risk assessments where the contaminants do not result in neuropathology.

The National Academy of Sciences (of the USA) defined behavior in the broadest sense to be the net result of integrated sensory, motor and cognitive function occurring in the nervous system (National academy of sciences, 1975). The chemical induced changes in behavior are generally considered a sensitive indicator of a nervous system dysfunction (Kulig *et al.,* 1996; Tilson, 1990; Tilson & Mitchell, 1984; Norton, 1978). The complexity of the interactions of the nervous system with other organs provides a logical basis for the supposition that early toxic response of behavior at lower concentrations cause morphological or other changes. Functional measures, especially behavioral endpoints, are now routinely used to identify and

characterize the potential neurotoxic effects of environmental contaminants (Hagenaars *et al.,* 2011; Huang *et al.,* 2010; Tilson, 1987; Weiss, 1975).

The continuous exposure of contaminants to the aquatic system requires efficient methods to assess the potential risk to aquatic species. A possible decline in the population of aquatic life due to these toxic exposures is reported continually, and this makes it important to design aquatic organism test strategies for policy makers in regulatory bodies for assessment and environmental monitoring. There is a huge demand to develop reliable, robust and relevant testing strategies for aquatic toxicants by such bodies. Several testing guidelines have been developed by OECD, US-EPA, EU-REACH and other regulatory bodies. Continuous efforts are being made to refine and increase the sensitivity and specificity of these test methods, by adding additional sublethal endpoints to address the effects on development, nervous system, physiology, behavior and reproduction, all of which might be equally important in ecological relevance in the long run, but occur at earlier times and lower exposure levels (Gunnar & Quevedo, 2007; Holbech *et al.,* 2006; Hori *et al.,* 2006; Fort *et al.,* 2004a; Fort *et al.,* 2004b; Orn *et al.,* 2003; Peitsaro *et al.,* 2003; Darland & Dowling, 2001).

1.2 Perfluoroalkyl acids

The extensive use of chemicals in industry and house hold utilities because of increased human activities and the modern life style is influencing aquatic environments. The discharge of these chemicals ends up in the aquatic systems. Perfluoroalkyl acids (PFAAs) are a group of such emerging contaminants which have been detected in human, wildlife and the environment (Giesy *et al.,* 2010; Kannan et al., 2005a; Kannan *et al.,* 2005b; Giesy & Kannan, 2001).

Perfluoroalkyl acids (PFAAs) are a subdivision of the family of perfluoroalkyl substances (PFAS) which consists of perfluoroalkane carboxylic, sulfonic, sulfinic, phosphonic and phosphinic acids. Perfluoroalkyls are those chemical substances where all hydrogen atoms in the carbon chain are replaced with fluorine with the exception of the functional head group that may still contain hydrogen atoms. Perfluorooctanoic acid (PFOA) and Perfluorooctane sulfonic acid (PFOS) are two intensively studied perfluoroalkyl carboxylic acid (PFCA) and perfluoroalkyl sulfonic acid (PFSA) groups, respectively.

Perfluoroalkyl acids (PFAAs) are typically four to fifteen carbons long, fully fluorinated organic chemicals, with a functional group attached at their tail. The carbon-fluorine (C-F) bond, which is the strongest chemical bond uniquely, makes them extremely resistant towards thermal, chemical and

biological degradation (SEPA, 2006; Kissa, 2001). Additionally, the hydrophobic fluorinated alkyl chain and the lipophobic functional group (Kissa, 2001) make them useful in many industrial and ordinary applications. Both PFCAs and PFSAs are the stable end-product chemicals in the environment that are not degraded under any environmental circumstances (SEPA, 2006).

PFAAs have synthetically been produced over several decades as their surface active properties, in addition to others, offer great advantages for use in industrial and consumer products, such as firefighting foams, coatings to textile and paper products for food packaging, surfactants, adhesives, cosmetics, agrochemicals and medicines (KemI, 2006; Kissa, 2001; Renner, 2001; 3M Company, 1999). The release of these chemicals to the environment is possible during their production, use and disposition as waste. Formation of PFCAs is considered to occur also by the atmospheric degradation of the volatile precursor molecules, e.g. fluorotelomer alcohols (Dinglasan *et al.,* 2004; Ellis *et al.,* 2004; Tomy *et al.,* 2004). In the environment, PFAAs are mostly associated with aquatic ecosystems. The solubility of these chemicals in water and their ability to bioconcentrate in fish are likely the reasons of this association (Rayne & Forest, 2009). PFAAs bind to proteins (such as albumin) in liver, plasma and eggs. Intracellular binding of these chemicals to fatty acid binding proteins (FABPs) due to their analogy (Lau *et al.,* 2007) to endogenous fatty acids (Kannan *et al.,* 2005a; Kerstner-Wood, 2003; Luebker *et al.,* 2002) is also reported.

The toxicity and accumulation of these chemicals is commonly determined by the carbon chain length and the functional group attached (Buhrke *et al.,* 2013; Dai *et al.,* 2013; Reistad *et al.,* 2013; Zhao *et al.,* 2013; Inoue *et al.,* 2012; Zheng *et al.,* 2012; Hagenaars *et al.,* 2011; Liu *et al.,* 2011; Jeon *et al.,* 2010; Yeung *et al.,* 2009a; Wolf *et al.,* 2008; Kleszczynski *et al.,* 2007; Matsubara *et al.,* 2006; Martin *et al.,* 2003a; Martin *et al.,* 2003b). Long-chained PFAAs and / or sulfonated PFAAs are more toxic than those of shorter chains. The production of typical PFAAs (PFOA and PFOS) has decreased over the years. Meanwhile, short-chained PFAAs have been produced and used as alternate compounds (KemI, 2006; KemI, 2009). Studies have reported that PFBA and PFBS are becoming the predominant PFAAs in different matrices (Cai *et al.,* 2012; Glynn *et al.,* 2012; Eschauzier *et al.,* 2010; Lange *et al.,* 2007; Skutlarek *et al.,* 2006).

The binding of PFAAs with blood proteins, and their non-biodegradable nature in living organisms, make them persistent and bio-accumulative in humans and wildlife, including animals in remote locations such as polar bears (Persson *et al.,* 2013; Yamashita *et al.,* 2008; Calafat *et al.,* 2006; Giesy &

Kannan, 2001). The release of these chemicals ends up in sewage waters, and discharge from municipal sewage water treatment plants is a significant source of contamination for aquatic environments. Several studies indicate the presence of these chemicals in wastewater and their final release into sludge and natural waters (Olofsson *et al.,* 2013; Pan *et al.,* 2011; Yeung *et al.,* 2009b). In fish embryo toxicity studies, developmental malformations, physiological disturbances and impaired larval behavior have been observed after the exposure to different PFAAs (Zheng *et al.,* 2012; Hagenaars *et al.,* 2011).

Perfluorooctanoic acid (PFOA) is one of the most widely detected PFAAs in humans and environmental samples (Domingo *et al.,* 2012; Miege *et al.,* 2012; Rudel *et al.,* 2011; Kannan *et al.,* 2005a). It has been produced in large quantities and can also be formed as a metabolite from other perfluorinated chemicals. The exposure pathways of PFOA and related PFAAs to humans are being elucidated. Drinking water (Murray *et al.,* 2010), indoor dust, the consumption of the fish (Falandysz *et al.,* 2006) and farm animals' meat (Guruge, 2005) are considered to be major contributors of PFAAs to human exposure. Although, the production of PFOA has been decreased in recent years, the chemical will remain a health and risk concern due to its presence in household and commercial products manufactured before the start of stewardship program (Betts, 2007; Dupont, 2006).

Toxicological studies of PFAAs have been conducted in laboratory animals, including zebrafish (Zheng *et al.,* 2012; Hagenaars *et al.,* 2011; Andersen *et al.,* 2008; Lau *et al.,* 2007). Different studies have already demonstrated their toxicity, and their interference with lipid metabolism, the immune system, development and reproduction (DeWitt *et al.,* 2012; Lau *et al.,* 2006; Kennedy *et al.,* 2004; Lau *et al.,* 2004; Hu *et al.,* 2002; Luebker *et al.,* 2002). The mode of action of PFAAs has not been elucidated. They are considered to be fatty acids in the body, due to their structural similarity to endogenous fatty acids (Lau *et al.,* 2007), such as transporting protein albumin in blood (Bischel *et al.,* 2010) and FABPs binding in the cell (Luebker *et al.,* 2002). Like endogenous fatty acids PFAAs are the ligands of the peroxisome proliferator activated receptor alpha (PPARα) (Wolf *et al.,* 2008) a nuclear receptor and regulator of lipid metabolism (Berger & Moller, 2002).

1.3 The zebrafish

The zebrafish (*Danio rerio*) is a tropical freshwater cyprinid fish originating from South Asia, especially Bangladesh, India, Nepal and Pakistan, among others. This fish has become a model organism in ecotoxicology and

environmental sciences, with a range of prospective applications in integrative risk assessment of chemicals (Schirmer *et al.,* 2008). This is because, it possess sterling qualities such as small size, cheapness, easy husbandry and superb fertility. In captivity, it breeds all year round, at frequent intervals of 1-6 days, and eggs are not sticky and can be collected easily in large quantities. The zebrafish genome is fully sequenced, so it can be used for molecular and genetic analyses. Its life cycle is rapid under ideal rearing conditions. These traits have led to the wide-spread use of this species in standardized testing protocols for evaluation of drugs and chemicals (OECD, 2013; Langheinrich, 2003). Zebrafish are further suitable for non-invasive treatments particularly for water soluble drugs and chemicals. In addition, *ex utero* exposure further avoids maternal influence.

Zebrafish physiology and neuroanatomy are parallel to those of humans (Panula *et al.,* 2010; Panula *et al.,* 2006). In zebrafish larvae (>3dpf) the majority of the organs and locomotor responses are developed, suggesting that the functions of behavior are in place (Kimmel *et al.,* 1995). The zebrafish nervous system becomes functional within days and all major components of the brain are present at 5 dpf (Nusslein-Volhard, 2002). The rudimentary forms of locomotor activity begin to develop in early life stages in zebrafish. Thus, zebrafish have emerged as a powerful model to study the development and functions of the nervous system (Strähle & Korhz, 2004) and the behavioral, genetic and biochemical aspects of locomotion (Fetcho & McLean, 2010; Fetcho *et al.,* 2008; Fetcho, 2007; Guo, 2004; Drapeau *et al.,* 2002). The zebrafish behavioral repertoire is robust, conserved and similar to that of mammals and has high throughput validity due to the powerful video-tracking tools developed recently (Stewart *et al.,* 2012; Padilla *et al.,* 2011; Champagne *et al.,* 2010; Mathur & Guo, 2010; Spence *et al.,* 2008).

The zebrafish is a well characterized model for testing the toxicological effects on reproductive capability (Spitsbergen & Kent, 2003; Laan *et al.,* 2002). Since, the zebrafish genome is sequenced, a variety of biomarkers are available to measure. The molecular mechanisms of the zebrafish endocrine and hormonal signaling pathways are highly similar to those of other vertebrates, allowing for extrapolation of data from this model to other species (Segner, 2009; McGonnell & Fowkes, 2006; Laan *et al.,* 2002). Endocrine disrupting biomarkers are equally sensitive in zebrafish as in other species (Holbech *et al.,* 2006; Hutchinson *et al.,* 2006; Holbech *et al.,* 2001).

However, one must be careful when generalizing the responses in a model species such as zebrafish, because, inbred laboratory-reared animal populations are generally genetically less diverse than non-model or wild organisms. Consequently the response outcome might be affected (Brown *et al.,* 2012; Coe

et al., 2009). In ecotoxicology, the zebrafish as a test organism is being used for acute, chronic and early life stage toxicity testing. It is a recommended species in the European scientific community, United States Environmental Protection Agency and the Organization for Economic Co-operation and Development (OECD) guidelines (Braunbeck & Lammer, 2005; Braunbeck *et al.,* 2005; Nagel, 2002; OECD, 1992). To summarize, the zebrafish is well-established both in applied molecular biological research and in ecotoxicological testing.

1.4 Developmental toxicity

Fish traditionally have been considered a key component in chemical toxicity strategies (Braunbeck *et al.,* 2005). Fish are exposed to the toxicants throughout their life cycle, and so, fish acute toxicity testing (OECD, 1992) has been of importance. However, increased animal welfare considerations have motivated the search for alternative assays to the use of adult fish. The embryos have therefore been on tests that show sublethal effects on more sensitive endpoints in the early development, as compared to adult fish (Dahl *et al.,* 2006). The early life stages of the fish have long been recognized as very sensitive in response (Marchetti, 1965). In most cases, embryo-larval and early juvenile stage sensitivities are also comparable to those of full life cycle tests within a factor of two (Mckim, 1977). Long term toxicity can be predicted by testing the developmental stages of fish and other freshwater organism (Munley *et al.,* 2013; Mckim, 1977).The guidelines for testing the embryos and larvae of fish have been standardized (OECD, 2013; ISO, 1999), so that the traditional adult fish acute toxicity test has been replaced (OECD, 2013; Braunbeck & Lammer, 2005; Braunbeck *et al.,* 2005; ISO, 1999; OECD, 1992) with a *Danio rerio* embryo test (DarT) set-up by Nagel, (2002).

1.4.1 Zebrafish embryo toxicity test

In the zebrafish embryo test the eggs are exposed in a static or semi-static system from fertilization to the completion of embryogenesis, in a microtiter plate in a small volume of the exposure media. Various responses of the individual organisms are recorded at certain time points during the exposure (Schulte & Nagel, 1994). To monitor the effects, lethal and sublethal developmental endpoints are selected (Nagel, 2002). Coagulation of the egg, non-detachment of the tail from the yolk, and lack of somite and heart beat represent the lethal endpoints. Developmental sublethal endpoints that might indicate the mode of action of the toxic response can include completion of gastrulation, eye development, spontaneous movement, heart rate / blood

circulation, pigmentation and edema (Nagel, 2002). Teratogenic effects are investigated following any malformation in aforementioned sublethal endpoints. Implementation of sublethal effects makes the testing more compatible with ethical and animal welfare legislation (Lammer *et al.,* 2009).

Monitoring certain morphological changes or physiological responses might indicate some modes of action to identify and classify some unknown chemical compounds of the exposure media. The test can be expanded to cover other related effects by extending the time of exposure as well as other endpoints like deformations of the spine (Hollert *et al.,* 2003) and locomotor activity. This test has been standardized in Germany for routine effluent monitoring as well as validated in ISO and OECD for sewage water and chemical testing (Strecker, 2013; Braunbeck *et al.,* 2005). PFAAs have already been tested by this assay (Hagenaars *et al.,* 2011; Huang *et al.,* 2010).

1.4.2 Zebrafish larval locomotor behavior test

The developmental toxic potential of environmental chemicals and pharmaceuticals has recently been evaluated. The known model drugs acting on the central nervous system in mammals have been assessed by locomotor activity in zebrafish larvae (Irons *et al.,* 2010). Behavior is gaining more recognition due to its 10-1,000 times higher sensitivity than the conventional methods to determine LC_{50} (Hellou, 2011; Robinson, 2009; Hellou et al., 2008). Zebrafish behavior is a response indicator of sublethal toxicity and may provide information on the mode of action of the tested chemical.

Behavior is the cumulative expression of genetic, biochemical, physiological and environmental cues. Zebrafish swimming behavior is measured by monitoring the locomotor activity of the organisms. Any un-coordination in swimming behavior can help to predict deformities in embryogenesis during the development. Locomotor activity of zebrafish has been used widely to develop specific behavioral response profiles for characterization of drugs and chemicals (Ali *et al.,* 2011; Kokel *et al.,* 2010; Rihel *et al.,* 2010). The visual response test is the measure of locomotor activity of zebrafish larvae in light and dark, to detect any physical or chemical stress affecting swimming activity patterns. Lighting conditions affect the locomotor activity of zebrafish larvae; less activity is recorded during light as compared to that in darkness (Irons *et al.,* 2010; MacPhail *et al.,* 2009; Emran *et al.,* 2008).

1.5 Uptake, distribution and kinetics

Chemical kinetics seeks to understand the chemical behavior in a biological system, where the processes of chemical absorption, distribution and elimination are mathematically characterized. Through this mathematical modeling, the amounts and concentrations of the chemical in the body are quantitatively predicted as a function of time and exposure level. Thermodynamic differences in the chemical activities in the storage (animal) and the source (water) compartments are assumed to be the prime driving force behind the toxicokinetics.

Methods to estimate the steady states in the waterborne exposure kinetic models for aquatic organisms have been developed (Neely, 1979). These steady state models predict the maximum potential of toxicant accumulation in an organism. The major uptake route for waterborne chemicals in fish is directly through the gills (Streit, 1992) and direct uptake of water soluble compounds from water is probably much more important than accumulation from food (Bruggeman *et al.,* 1981).

Different types of kinetic models have been employed to describe the accumulation and distribution of the contaminants in aquatic organisms (Landrum *et al.,* 1992). Two major approaches for toxicokinetic data analysis are compartmental and non-compartmental. They estimate important kinetic parameters like total body clearance, but the approaches differ in their assumptions to estimate the distribution of chemicals within the body (DiStefano, 1982). Non-compartmental analysis assumes the chemical is absorbed into and excreted from a central compartment without requiring any peripheral compartment (DiStefano, 1982). Both methods are commonly used for analyzing clinical data. However, non-compartmental analysis is rarely applied in ecotoxicology, even though this approach has the advantage of fewer assumptions and less complicated calculations.

To study the tissue distribution of a chemical in an intact animal a technology of whole-body autoradiography (WBA) is used in which a radiolabeled chemical is employed. This technology has the advantage of providing high resolution images of the qualitative distribution of the chemical at the tissue level. This technique has previously been used to understand the kinetics and tissue distribution of PFAAs in different species (Bogdanska *et al.,* 2011; Borg *et al.,* 2010; Vanden Heuvel *et al.,* 1991). The characteristic distribution of PFOA in the tissues is considered to be partly due to its affinity to proteins as PFOA has been shown to bind to liver fatty acid binding proteins (FABP) (Luebker *et al.,* 2002).

Bioaccumulation is a fundamental process in environmental toxicology and risk assessment because it determines the internal exposure of a chemical

(Mackay & Fraser, 2000). Bioaccumulation of organic chemicals in fish is the net result of the competing processes of uptake from the ambient environment and elimination from the body. Bioconcentration is the process of accumulation of chemicals by fish and other aquatic organisms through non-dietary routes. The rate of uptake and bioconcentration of organic chemicals (concentration in an aquatic organism over the time) from water by fish and other organisms can often be described mathematically. The bioconcentration factor (BCF) is a proportionality constant relating the dissolved concentration of a chemical in the exposure water to its level in aquatic animal at steady state equilibrium (Veith *et al.,* 1979). BCFs are assessed typically in fish, as they are a human food source and many standardized testing protocols for toxicity evaluation are available. Fish bioconcentration and compound specific distribution potential are used to measure and predict the environmental effects of newly introduced chemicals, and their persistency.

1.6 PFOA exposure and toxicity

The chemical toxicity to fish is partly determined by its uptake, distribution and elimination. Understanding these processes in fish is important because they demonstrate the phenomenon direct from the environment (water) in which it occurs.

1.6.1 Fish reproduction

Reproductive success is considered one of the most ecologically relevant endpoints in fish exposure studies (Arcand-Hoy & Benson, 1998). The deleterious effects of chemicals induced during early development may subsequently express their altered effects in adulthood or even in offspring generations (Kavlock *et al.,* 1996). To assess the toxic potency of a chemical on population relevant endpoints, a study of chronic exposure is used.

In zebrafish and other vertebrate fish, reproduction is regulated by coordinated interactions along hypothalamus-pituitary-gonadal (HPG) axis, and involves a complex cascade of steroid hormones and biochemical pathways that also control growth and metabolism (Ma *et al.,* 2012; Blazquez *et al.,* 1998). The steroidogenesis of gonad tissue is equally important in the process. The chemicals can affect the aforementioned mechanism and perturb the endocrine system, with possible effects on reproduction of fish (Ji *et al.,* 2013; Liu *et al.,* 2010). Moreover, reproduction in zebrafish is strongly regulated by environmental factors such as temperature and photoperiod (De Vlaming, 1972). Sex steroids regulate different reproductive processes like gametogenesis, sexual phenotype and sexual behavior. Change in sex steroid

hormone levels interferes with the regulatory mechanism of the HPG axis, and subsequently causes reproductive dysfunction (Xi *et al.,* 2011).

1.6.2 Gonad development

Patterns of gonad development vary among fish species. Zebrafish is an undifferentiated gonochoristic species, in which all individuals develop gonads first with ovarian tissue. Zebrafish is a juvenile transitory-hermaphrodite, in which bisexual differentiation takes place later during development (Hsiao & Tsai, 2003; Takahashi, 1977). The hermaphroditism may be simultaneous or sequential. In sequential hermaphroditism, zebrafish is a protogynous developing first as female and later becomes male. Gonad development encompasses the sexual differentiation and maturation periods. Endogenous sex steroid hormones are among the regulators of gonadal sex differentiation (Sandra & Norma, 2010; Devlin & Nagahama, 2002). The fish is most sensitive also to exogenous sex steroids during these periods.

For ovarian development, somatic and germ cells proliferate and differentiate into follicles. A specific ovarian type, cytochrome P450 aromatase enzyme, which aromatizes testosterone into estrogen, does exist in zebrafish and is known to be involved in the regulation of ovarian differentiation (Chiang *et al.,* 2001; Kishida & Callard, 2001). In juvenile zebrafish it shows a dimorphic expression pattern (Traut & Winking, 2001), and modulation of its activity induces the feminization or masculinization, to develop the bisexual stage.

Growth and differentiation of oocytes involves both cytoplasmic and nuclear (meiotic) maturation. The endocrine system is a modulator and regulator of maturation. In zebrafish, the development of the oocyte has different stages from primary oocyte to maturation through vitellogenesis (Tyler & Sumpter, 1996). During vitellogenesis, a precursor lipoglycophospho-protein, vitellogenin (Vtg), produced in the liver, is accumulated as a major yolk protein in developing oocytes (Tyler *et al.,* 1991). Vitellogenin production is induced by ovarian sex steroids. Sex steroid hormones are produced in response to gonadotropin hormone (GtH) (Yousefian & Mousavi, 2011; Mylonas *et al.,* 2010). The Vtg is carried through blood to the ovary, where the oocytes take it up by receptor-mediated endocytosis (Wallace & Selman, 1990; Wallace, 1985; Ng & Idler, 1983). The processes of Vtg uptake in oocytes and their maturation and ovulation are suggested to be stimulated by GtH-I and II, respectively (Clelland & Peng, 2009; Swanson *et al.,* 1991; Tyler *et al.,* 1991; Scott & Sumpter, 1983).

2 Aims of the thesis

The overall aim of this thesis was to investigate whether waterborne exposure to perfluorinated chemicals affects embryonic development, reproduction and sexual development in zebrafish.

More specifically, the project objectives were:

- To evaluate and compare a set of structurally diverse PFAAs for their toxicity during the embryonic development of zebrafish.
- To develop a model to extend the comparison and toxicological evaluation of the PFAAs through a more sensitive locomotor behavior assay of zebrafish larvae.
- To investigate the disposition of ^{14}C-PFOA in adult zebrafish following waterborne exposure of the chemical for risk assessment particularly for extrapolation from high experimental exposure to low human/environmental relevance as well as to identify potential new target organs to clarify the mode of action for general systemic toxicity.
- To study the reproductive fitness, early life stage and sexual development after waterborne exposure of PFOA, and to study whether PFOA affects certain gene expressions.

3 Materials and methods

The work done in this thesis is based on five experiments approved by the local animal ethics committee. The sections below provide a summary of the materials and method, with a focus on experimental designs and core endpoints. Detailed descriptions of the techniques, materials and methods used are presented individually in the associated publications and manuscripts (I-IV).

3.1 Chemicals

Perfluoroalkyl acids of different carbon chain length and attached functional group have previously been used for persistency, bioaccumulation and toxicological evaluation. In the studies of embryonic development (Paper I, II), the chemicals were selected to cover different chain lengths and functional groups attached. The list of the perfluorinated chemicals (and their necessary information) used in the experimental work is presented in Table 1. All of these chemicals were purchased from Sigma-Aldrich, Germany except PFBA that was purchased from Alfa Aesar® GmbH and Co KG, Germany. For Paper III, radiolabeled Perflurooctanoic Acid (^{14}C-PFOA) with a specific activity 59 mCi /mmol was synthesized at Stockholm University. Hydrogen peroxide (30% in water) and Ethyl 3-aminobenzoate methanesulfonic acid (MS-222), an anesthetic agent, was purchased from Sigma-Aldrich (Germany). The solubilization reagent Soluene® -350 and Hionic-Flour™ Scintillation cocktail were purchased from PerkinElmer (PerkinElmer Life and Analytical Sciences, Boston, USA). All other chemicals used were of pro-analysis quality and obtained from regular commercial sources. A stock solution of each chemical was prepared in reconstituted standardized water at a concentration well below its reported solubility in water at 25 °C. Exposure solutions were freshly prepared prior to testing on zebrafish embryos.

Table 1. *Perfluoroalkyl acids (PFAAs) tested in zebrafish, their groups, acronyms, structures, CAS numbers and nominal exposure concentration ranges.*

Paper	Test compound	Acronym	Formula	CAS[a] Registry number (Purity%)[b]	Carbon chain length	Nominal exposure concentration range (mg/L)
	Perfluoroalkyl carboxylic acids	**PFCAs**	$C_nF_{2n+1}COOH$			
I& II	Trifluoroacetic acid	TFAA	CF_3COOH	76 -05-1 (>98)	2	10-3000
	Perfluorobutyric acid	PFBA	C_3F_7COOH	375-22-4 (99)	4	10-3000
	Perflurooctanoic acid	PFOA	$C_7F_{15}COOH$	335-67-1 (96)	8	3-1000
	Perflurononanoic acid	PFNA	$C_8F_{17}COOH$	375-95-1 (97)	9	0.03-10
	Perflurodecanoic acid	PFDA	$C_9F_{19}COOH$	335-76-2 (98)	10	0.1-30
	Perfluoroalkane sulfonic acids	**PFSAs**	$C_nF_{2n+1}SO_3H$			
	Perflurobutane sulfonic acid	PFBS	$C_4F_9SO_3H$	375-73-5 (>98)	4	10-3000
	Perflurooctane sulfonic acid	PFOS	$C_8F_{17}SO_3H$	1763-23-1 (98)	8	0.03-10
III	Perfluorooctanoic acid	^{14}C-PFOA	$C_7F_{15}COOH$	335-67-1 (96)	8	0.3-30 x10^{-3}
IV	Perfluorooctanoic acid	PFOA	$C_7F_{15}COOH$	335-67-1 (96)	8	0.2-20

[a]Chemical Abstracts Service
[b]Purity (%) as described by the manufacturer

3.2 Animals

Adult zebrafish (*Danio rerio*) of AB strain were used for breeding to get the fertilized eggs (Paper I, II and IV). For Paper III & IV, adult zebrafish were acquired from a local supplier in Uppsala, Sweden. Adult fish were maintained in a flow through system (Paper IV) of carbon filtered tap-water (pH 7.2 – 7.6; hardness 6.7; temperature 26 ± 1 °C ; conductivity 468 µS /cm; light cycle of

14 hours). Stock fish were fed daily with commercial flakes (SERA Vipan) as a staple food with added freeze-dried chrionomids (Naturafin), frozen chironomids and frozen *Artemia nauplii* (Akvariteknik). According to ISO (1996), reconstituted standardized water was prepared from deionized water with the addition of $CaCl_2$ x$2H_2O$ (117.6 mg /L), $MgSO_4$ x$7H_2O$ (49.3 mg/L), $NaHCO_3$ (25.9 mg/L) and KCl (2.3 mg/L), and used throughout the zebrafish embryo / larvae experiments.

3.2.1 Breeding and selection of fertilized eggs

Breeding groups of male female (3:2) adult zebrafish were placed in 10-L glass aquaria equipped with spawning nets the evening before the collection of eggs. The following morning, half an hour after onset of lights, eggs were collected from the breeding group tanks and rinsed for removal of debris. Normally developed fertilized eggs were selected under a stereomicroscope for the experimental studies.

3.3 Zebrafish embryo toxicity test (Paper I)

This test is based on an OECD guideline (OECD, 2013) and described in detail in paper I. Each PFAA was tested individually in six concentrations (spaced by a constant factor of 3.3) prepared from its stock solution. Fertilized eggs were randomly distributed into flat bottom, 48-well polystyrene plates (Costar®), one egg in each well, with 750 µl of test substance or water. For each PFAA, four 48-well plates were used, with 6 embryos /concentration / plate. The plates were covered with parafilm, and the embryos were exposed to the PFAAs until 144 hours post fertilization (hpf). The eggs were examined for malformations under the stereomicroscope at 24, 48 and 144 hpf. The hatching time of each embryo was recorded with an over-head mounted camera by photographing at 1 h intervals until 144 hpf (Paper I and II). The endpoints studied at different time points are presented in Table 2.

Table 2. *Endpoints studied in zebrafish at different time points in the studies included in this thesis.*

Paper	Endpoint	Time line (day)
I	***Lethal categorical***	1-6
	Coagulation	
	Lack of heart beats	
	Sublethal categorical	1-6
	Tail deformation	
	Edema	
	Side-lying	
	Unhatched	
	Uninflated swim bladder	
	Sublethal continuous	1-6
	Movements	
	Heart rate	
	Time to hatching	
II	***Locomotor behavior***	6
	Activity counts (n)	
	Swimming time (s)	
	Swimming distance (mm)	
	Relative swimming time (%)	
	Average swimming speed(mm/s)	
	Active swimming speed (mm/s)	
III	***Kinetics***	40
	Uptake and distribution	
	Body clearance and half-life	
	Bioconcentration	40
IV	***FSA***	21
	Spawning	
	Fecundity	
	Fertilization	
	FSDT	100
	Body Growth	
	Sex ratio	
	Gonad maturation	
	Gene analysis	

3.4 Larval locomotor behavior test (Paper II)

The larval locomotor activity test was performed using the Viewpoint Zebrabox® behavioral recording system (ViewPoint Life Sciences, Lyon, France), after evaluating the embryos for lethal and sublethal toxicity at 144 hpf (Paper I). This system monitors movements using automated video recording, with a multi-well plate holder Zebrabox equipped with internal LED lights (light recordings), infrared illumination (darkness recordings) and a mounted camera. The locomotor activity of larvae was recorded by placing each 48-well plate individually into the Zebrabox. After an acclimation phase in light, the locomotor activity was recorded during four consecutive 10-min phases of alternate dark and light.

Locomotor activity was estimated by a subtraction method used for detection of objects darker than background with a minimum object size. To remove system noise, a threshold of 0.135 mm (minimum distance moved) was used for filtering all of the data. Locomotor endpoints were designed to express the changes in the general swimming activity in response to any physical or chemical stress of exposure. The typical behavioral endpoints quantified through movement analysis were as described by Murphy *et al.* (2008) and Alvarez & Fuiman (2005), and are presented in Table 2.

3.5 Uptake, distribution and kinetics (Paper III)

3.5.1 Exposure and experimental design

In Experiment 1, the nominal concentration of non-labeled PFOA was 10 µg PFOA /L of water and 1.4 µCi ^{14}C-PFOA / L was added as a tracer. Both male and female zebrafish were exposed for 40 days, which was followed by a washout period of 80 days. The activity of ^{14}C-PFOA in the exposure tanks was regularly measured and maintained at ≥ 90% throughout the period of exposure. Fish were sampled for LSC after 5, 10, 15, 20, 30 and 40 days of exposure. At termination of exposure, the remaining fish were transferred to separate tanks with cleaned PFOA free water for a washout period. The fish were similarly sampled for LSC at 1, 3, 8, 16, 30, 55 and 80 days post-exposure. At each sampling point, 5 fish of each gender were sampled for LSC. Furthermore, fish were also sampled for WBA at 1, 5, 10, 30 and 8, 16, 30 days of exposure and post-exposure, respectively.

In Experiment 2, the nominal exposure concentrations were 0.3, 1, 3, 10 and 30 µg/L with levels of 0.046, 0.152, 0.460, 1.52 and 4.60 µCi ^{14}C-PFOA /L tracer for the respective exposure concentrations. The zebrafish were exposed similarly for 40 days, and sampled at the termination of exposure for

LSC. In addition, fish were sampled and dissected for determination of PFOA in different organs.

On day 40 of exposure, a relationship was established by plotting the exposure concentrations at steady state versus total body concentration of PFOA in fish. The bioconcentration factor (BCF) or accumulation ratio (AR) for each fish was calculated. The BCF is defined as the volume of exposure water that is depleted of PFOA by the fish within the exposure period (ml /g body weight).

3.5.2 Liquid scintillation counting

Water samples were regularly analyzed for radioactivity by liquid scintillation counting. Water samples were mixed with 10 ml Hionic-Flour™ scintillation counting cocktail and analyzed by a Tri-carb 1900 CA Liquid Scintillation Analyzer (Packard).

At each sampling point, the sampled zebrafish were rinsed in clean water, euthanized in 100 mg /L MS-222 aqueous solution and weighed. Each fish was chopped down to subsamples of maximum 40 mg, transferred into a 20 ml glass scintillation vial (VWR, Sweden), and solubilized in Soluene®- 350 at 60 °C for 24 h by agitating intermittently to ensure complete solubilization. The solubilized samples were then allowed to cool down to room temperature. Some of them were bleached with 30 % hydrogen peroxide. Ten (10) ml scintillation cocktail Hionic-Flour™ was added to each scintillation vial. Before LSC, the samples were placed in ambient light and temperature overnight to minimize background counts. The tissue contents ^{14}C-PFOA were determined by LSC, and based on these results the total concentration of PFOA was calculated from the specific activity of ^{14}C-PFOA added to the water. The activities of the individual subsamples were summed and adjusted for the weight of the fish to determine the concentration of PFOA.

3.5.3 Whole body autoradiography

For whole-body autoradiography (WBA), zebrafish were sampled, euthanized and weighed as already described for LSC. The fish were mounted in aqueous carboxymethyl cellulose (CMC) gel and frozen in a bath of hexane cooled with dry ice. The frozen blocks were processed further for WBA as described by Larsson & Ullberg (1981). A series of 20 µm thick whole-body sagittal sections were taken at different levels and collected on tape and freeze-dried. The distribution of non-extractable radioactivity was studied by successively extracting every other freeze-dried section with trichloroacetic acid (5%), ethanol (50%), 99.5% ethanol and heptane for 1 min, and then rinsed with tap water for 5 min. The extracted sections were dried and exposed to x-ray film

together with the adjacent non-extracted sections. The sections were stored at -20 °C for exposure. The exposed films were developed thereafter 2 to 9 months.

To illustrate the uptake and elimination, the radioluminographic technique was also employed. This technique is more sensitive than traditional exposure to x-ray film, and thus makes it possible to detect a wide range of radioactivity levels. Thus, sections from the exposure and wash out periods were exposed to one phosphor imaging plate (Storage Phosphorous Screen). After 5 days of exposure, radioluminographic images were then obtained by developing the plate using the Packard Cyclone® Plus Storage Phosphor System, (PerkinElmer, Inc., IL, USA).

3.5.4 Kinetic assessment

In brief, the total body exposure was assessed by means of concentration-time and amount-time data collected over the period of 120 days. A non-compartmental approach was used to assess the major determinants of exposure, namely body clearance (removal) and uptake-rate (input) based upon the assumptions that PFOA was chemically and metabolically stable during the observational period (Sundstrom *et al.,* 2012b; Vanden Heuvel *et al.,* 1991; Ylinen *et al.,* 1989; Ophaug & Singer, 1980). Since blood or plasma was not available for the estimation of plasma clearance we applied the concepts of total body clearance based on the total body tissue concentration. Body clearance Cl_b was estimated from the amount of PFOA at steady-state A_{ss} and the post-exposure (washout period) area from t* (end of tank exposure period to PFOA) to infinity $AUC_t{}^{*-\infty}$ derived by the trapezoidal method. The relationship between the amount of PFOA at steady-state, total body clearance and the post-exposure area under the exposure curve was calculated mathematically, as expressed in Paper III in detail. The kinetic parameters were determined and presented in Table 1 (Paper III).

A_{ss} is estimated from the total body concentration (whole fish) of ^{14}C-PFOA at steady-state multiplied by their body weight(s).The total body clearance (based on total body concentration rather than plasma PFOA concentration) can be related to plasma clearance provided that the plasma concentration is obtained from the specific animal model (e.g., zebrafish).The mean residence time (MRT) of ^{14}C-PFOA in zebrafish, which denotes the time an average molecule of PFOA remains in the organism, can be calculated from the areas of the first- and zero-moment curves and then corrected for the 40-day exposure period ($T_{exposure}$) (Benet, 2010; Gabrielsson & Weiner, 2010).

The effective half-life is a weighted half-life related to the turnover of the amount of PFOA in the body, rather than the terminal half-life of the test

compound commonly obtained from the terminal phase of the plasma concentration-time curve. 3-4 times the effective half-life gives an indication of the time to 90% of steady-state in the body during a constant exposure to the test compound.

The terminal half-life of PFOA in male and female zebrafish was estimated directly by means of log-linear regression of the terminal portion of the washout kinetics.

3.6 Fish reproduction and sexual development (Paper IV)

The evaluation of PFOA was performed using two different tests with similar experimental setups. Both tests were based on standardized OECD guidelines. The first test (FSTRA; Fish Short Term Reproduction Assay, TG 229) is a screening test where sexually mature male and female fish were exposed to the test substance for a short period (21-days) and evaluated concerning effects on reproduction. In the second test (FSDT; Fish Sexual Development Test, TG 234) fish are exposed from fertilization to maturity, and evaluated concerning impact on sexual development and gonad maturation.

In both of the tests zebrafish were exposed to PFOA at concentrations of 0.2, 2 and 20 mg/L. Each exposure group comprised of four replicates, and six for the water control. The stock solutions were distributed into the experimental tanks using a multichannel peristaltic pump (Ismatec®, Zurich, Switzerland) via glass capillaries connected by silicon tubing. In the FSTRA experiment, charcoal filtered tap water maintained at 26°C was also pumped into each of the 8-L experimental tanks with another peristaltic pump (Ismatec®) at a flow ratio 1000 times higher than that of the PFOA stock solution. In the FSDT experiment, the water flow was through gravity fall, and the flow rate was maintained such that the ratio of water and stock solution reaching in tanks was 100:1. With the given flow rates the total water volume was exchanged three times daily. Flow rates were checked daily, and adjusted if necessary. The flow through system was started three days before start of the exposure studies to allow chemical equilibrium. In the FSDT experiment, the chemical exposure was started at 8 dpf. During the exposures fish health was monitored daily, and any dead fish were removed.

3.6.1 Fish short term reproduction assay

After acclimatization, 5 male and 3 female sexually mature fish were held together in spawning cages in 10-L glass aquaria. The reproduction parameters, *viz* number of successful spawning, clutch size and fertilization ratio for each replicate, were monitored daily throughout the exposure period. From each

successful spawning the average number of laid eggs per female was determined. Fertilized eggs were then sorted under a stereo microscope, to determine the fertilization ratio. In the third week of exposure, a random sample of 24 fertilized eggs from a single clutch of each replicate was transferred to a 96 well plate in clean charcoal filtered water and followed for malformation and mortality for 6 days as described in Paper I. At the end of the experiment all fish were euthanized in a solution of 100 mg/L MS-222. The livers were dissected from each fish, placed in eppendorf tubes, frozen in liquid nitrogen, and stored at -80 °C until analysis of enzyme activities. The remaining body part of each fish was preserved in formalin for histological evaluation of gonads.

3.6.2 Liver enzyme activity

Zebrafish liver samples were sonicated in homogenization buffer. Glutathione reductase activity was measured as described by Cribb *et al.* (1989). Catalase activity was measured spectrophotometrically by the decay of hydrogen peroxide as described previously (Aebi, 1984; Orner *et al.,* 1995). Enzyme activity was measured at 240 nm (20 °C) and expressed as micromoles per minute per milligram of protein (specific activity). Protein content was quantified by the method described by Lowry *et al.* (1951).

3.6.3 Fish sexual development test

The test procedures are described in detail in Paper IV. In general, juvenile zebrafish were exposed to PFOA from 8 dpf to 100 dpf, at the termination of experiment. The fish were euthanized as mentioned above and their body weight and length were measured. The liver was dissected, weighed, preserved in eppendorf tubes and stored at -80 °C after freezing in liquid nitrogen until analysis. The body part of each fish was preserved in formalin for histological evaluation of gonads. The condition factor (K) for each fish and the liver somatic index (LSI) were calculated according to the formulae "(K) = [body weight (mg)/ (body length (mm)) 3] x 100" and "LSI= [liver weight (mg)/body weight (mg)] x100", respectively.

3.6.4 Histology and gonad maturation

In both the FSTRA and FSDT tests the fish trunks were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin blocks. Then, 5 µm thick longitudinal sections along the entire dorso-ventral axis were taken through the gonadal region of the fish by a microtome with 20 µm increment, and collected on glass slides. The haematoxyline-eosin (HE) stained slide-mounted sections were evaluated by light microscope.

In the FSTRA test, sections were used for phenotypic sex determination of each fish. In the FSDT test, sections were analyzed on coded slides to determine and evaluate the gonadal tissue and phenotypic sex. Testis or ovary was recognized by the presence of spermatogenetic cells or oocytes, respectively. The presence of oocytes in testicular tissue was categorized as intersex (ovotestis). The specimens where either of the sex tissues could not be observed in any field of microscope were declared as non-determined. The testes were further classified as immature, maturing or mature depending on the histological appearance and amount of spermatozoa in sperm duct (Kinnberg *et al.,* 2007). The ovaries were categorized as immature by the presence of only perinucleolar oocytes, declared maturing if the oocytes were at cortical alveolar stage, and mature by the presence of one or more vitellogenic oocytes according to the stages described by Selman *et al.* (1993).

3.6.5 Gene expression

Hepatic expression of PPARα and Vtg-1 were studied to investigate whether PFOA can activate gene transcription in zebrafish. In brief, total RNA from liver samples of individual fish were isolated, and the quantitative expression of mRNA of above mentioned genes was measured by real-time RT-PCR as described in Paper IV.

3.7 Statistical analysis

The differences between the groups at the end of exposure were tested by statistical analyses. For continuous data, mean values were used and proportional data were *arcsin* square root transformed (Paper IV).The normality of the data was tested using the Anderson-Darling test, and the homogeneity of variance of the data was tested using Levene's test prior to the analyses. Data failing these criteria were transformed accordingly to meet the ANOVA requirements. General Linear Models (GLM) followed by Dunnet`s *post hoc* test or Tukey`s pairwise comparisons were used to determine any significant differences among the groups. The data failing to follow the assumptions of parametric analysis were subjected to non-parametric Kruskall-Wallis tests of ranks. The significance differences were analyzed by *post hoc* Dunn`s test of multiple comparison with control.

In paper I on the embryo toxicity test, the analyses were based on individual embryos as experimental units. Continuous data were analysed using one way ANOVA with two sided Dunnet`s *post hoc* test. Lowest observed effect concentration and no observed effect concentration parameters were determined on the basis of Dunnet`s test. The 50% effective concentration

(EC_{50}) values with 95% confidence intervals were calculated using probit analysis.

For the locomotor behavior of zebrafish larvae (Paper II), there were multiple response parameters, including the ambulatory, observatory and inferential behavioral endpoints. Furthermore, there were several groups of explanatory variables. The relationships between these two datasets (response and explanatory) were analyzed using multivariate data analyses. The effects of the different explanatory characteristics on the zebrafish behavior were analyzed by redundancy analysis (RDA; ter Braak, 1995). The RDA analysis used the CANOCO version 4.54 program (Biometrics, Wageningen, the Netherlands). The statistical significance of the explanatory variables was tested using multivariate analysis of variance via permutation (Anderson, 2001). The permutation tests used the DISTLM version 5 programs (Department of Statistics, University of Auckland, New Zealand).

All of the data presented here (Paper I, IV) are means ± SD, unless stated otherwise. All of the univariate statistical analyses were employed using the Minitab® 16 program. The differences were accepted at 95% significance level ($p \leq 0.05$).

4 Results

The detailed results are presented in Papers I-IV included in this thesis. However, the main findings are presented in this section.

4.1 Zebrafish embryo toxicity test (Paper I)

PFAAs were not highly acutely toxic to early life stage zebrafish, based on established toxicity endpoints. PFAAs exposure affected some of the endpoints only at some observation times. One common sublethal effect was pericardial edema, which was often observed after exposure to TFAA, PFBA, PFBS and PFOS. Another common malformation was spinal curvature, which was frequently observed in embryos exposed to PFNA, PFDA and PFOS. Heart rate at 48 hpf and hatching at 144 hpf were also affected in PFBS and TFAA exposed embryos, respectively. The concentration response relationships based on combined lethal and sublethal effects at 144 hpf were determined for all PFAAs. The order of toxicity for the PFAAs in the present study was: PFOS > PFDA > PFNA >PFOA >PFBS>TFAA >PFBA (Paper I).

4.2 Larval locomotor behavior test (Paper II)

PFAAs altered the locomotor activity in the behavioral analysis of zebrafish larvae. The locomotor activity generally increased in the first dark phase (D1) after transition from the acclimation (A) light phase. In the following light phase (L1), the increment in activity was reduced. A similar pattern was observed in the subsequent alternate phases (D2 and L2). In the highest tested concentrations of PFOS, PFNA and PFBS, the general trend of elevated larval activity in darkness (as compared to that of light) was not observed. Overall, the activity in the highest tested concentrations of TFAA, PFBS, PFOS and PFNA was reduced as compared to those of the controls (Paper II).

The behavioral characteristics formed three groups of correlated behavior: (i) swimming distance, average swimming speed, and activity count were highly correlated, as were, (ii) swimming time and relative swimming time, with (iii) active swimming speed being separate (Paper II). The multivariate RDA showed a relatively weak relationship between the behavioral characteristics and the explanatory characteristics, with the two axes shown accounting for 97 % of the total sum of squares of the first two axes of the equivalent unconstrained ordination (Table 3 in Paper II). In spite of the weak relationship, all three of the types of explanatory variables were statistically significant at P=0.0001 (Table 5 in Paper II). The behavioral response to concentration was relatively small (Fig. 2c in Paper II), as was that of light/dark phase. The strongest effect on behavior was associated with the differences between the chemicals (Fig. 2b in Paper II), notably their differences in carbon chain length and attached functional group (Fig. 2d in Paper II).

4.3 Non-compartmental analysis (Paper III)

The calculated concentration of the test substance PFOA in water was maintained at > 90% of each nominal exposure concentrations throughout the exposure period of the experiments. The measured LSC values for PFOA in male and female zebrafish are presented in Paper III, Figure 3. For both genders, PFOA was absorbed into the organisms from the aqueous medium into the organism. The equilibrium concentrations occurred between days 20-30 of exposure. At day 40 of exposure, any gender related difference in PFOA concentrations was not significant. The toxicokinetic parameters were comparable between the genders (Paper III, Table 1).

The non-compartmental estimates of body clearance, effective- and terminal half-lives were estimated from uptake/washout data and the accumulation factor in male and female zebrafish at steady-state. The uptake and disposition kinetics of PFOA demonstrated an average total body clearance 50 mL/day, with terminal and effective half-lives of 13-14 and 7-8 days, respectively.

4.3.1 Tissue disposition and bioconcentration

Whole-body autoradiograms show the distribution of ^{14}C-PFOA. The fish accumulated substantial amount of ^{14}C-PFOA even after 24 h exposure (Fig. 6 Paper III). Whole-body autoradiograms of the fish sampled at days 1, 5 and 10 of exposure showed a similar and progressive pattern of radioactivity distribution. The fish sampled on days 10 and 30 of exposure were thought to be in the peak period of distribution phase, as the effective half-life was

determined as approximately 8 days (Paper III, Table 1). Moreover, the equilibrium was also attained around the same period.

The bile and the intestinal contents concentrated the highest amounts of radioactivity. The blood-perfused structures were highly labeled, mainly the heart, gills and the blood vessel along the spinal cord. The labeling in the liver was relatively low but was uniform. A diffuse and weak labeling could also be seen in other tissues. Development of labeling in the oocytes over the time of exposure was observed. The spleen showed a heterogeneous and relatively weak labeling. The skeletal muscles and brain showed a faint or lack of labeling. However, the lines in the muscles were rather strongly labeled, as were some structures in the brain probably corresponding to blood vessels.

The whole-body autoradiograms from 4 days after the end of exposure showed the same distribution picture and proportion of radioactivity as shown on days 5, 10 and 30 of exposure. However, labeling of the tissues decreased as elimination proceeded. In the fish sacrificed on days 8, 16 and 30 post-exposure, the radioactivity declined relatively in almost all tissues. In the olfactory rosette and the oocytes, there was a slower decrease of the radioactive intensity. The slowest decrease of radioactivity was seen in the gall bladder, at 30 days after exposure the bile still contained the considerable labeling. On day 30 post-exposure, only the bile was still showing a very high intensity of the radioactive labeling, whereas the other tissues showed only weak labeling.

The bioconcentration of PFOA was analyzed in the whole body and in dissected liver, intestines and brain of exposed zebrafish for 40 days (Fig. 4 and 5). The BCFs calculated at steady-state were in a range of 20-30 with a variation of ±12.

The steady-state (actual) tank concentration was plotted against the tissue concentration of ^{14}C-PFOA after 40 days of constant exposure. A linear relationship was obtained between external (tank) exposure concentration and whole body tissue homogenate (internal) concentration to ^{14}C-PFOA within a 100-fold (0.3 – 30 µg/L) exposure range. Apparent gender differences in bioconcentration were observed neither in whole body homogenate nor in the individual tissues (Paper III, Figure 4 and 5).

4.4 Reproduction and sexual development (paper IV)

During the FSTRA experiment no mortality was recorded. Sex of each fish was determined histologically at the end of the experiment, to confirm the number of females per replicate. The reproductive behavior like spawning and number of eggs laid later were determined on the basis of the actual number of females per replicate.

4.4.1 Reproductive parameters

Generally, the number of successful spawning was slightly higher in the controls than in all of the exposed groups during the first week of exposure. However, successful spawning events decreased over time in all of the groups, except the highest exposed concentration. On the contrary, the average number of eggs laid per female increased over time. In fish exposed to 20 mg/L of PFOA, both spawning success and the average number of eggs spawned increased. The average number of eggs laid per female at each successful spawning was 65-84 in the controls and 18-120 in the highest exposed concentration. Approximately 60-80% of spawned eggs were fertilized in all of the treatment groups. An increase of fertilization with the time of exposure was noted. However, none of these observations were confirmed as statistically significant. Neither the malformation nor the mortality of maternally exposed offspring was significantly different at 6 dpf.

4.4.2 Fish sexual development

In the FSDT test, the growth parameters, such as body weights, body lengths, condition factors or LSIs, were not different between exposed and control fish, regardless of the sex classification. Neither significant shift in sex ratio nor any intersex fish were observed. The estrogenic activity in terms of vitellogenin (Vtg) mRNA expression in the liver of male fish was not induced by PFOA. Fatty acid β-oxidation regulator PPARα gene expression in zebrafish liver was only slightly inhibited in the highest PFOA exposed group.

5 Discussion

Perfluoroalkyl acids (PFAAs) are well known environmental chemicals found globally, and are identified and suspected to act in endocrine disruption (White *et al.,* 2011). The mechanism(s) of action for these chemicals are the subject of on-going research, but they are presumed to have influence on several hormonal axes of vertebrates (White *et al.,* 2011). PFAAs and their precursors have been produced for decades, and are still being used in broad range of applications. Among the halogenated chemicals, they still remain the least studied chemicals from an ecotoxicological standpoint.

5.1 Developmental toxicity (Paper I & II)

5.1.1 Zebrafish embryo toxicity (Paper I)

In determining the effects of environmental exposures, the accumulation of the chemicals and the time of exposure are equally significant. Periods in early life are more sensitive to environmental exposures in developing organisms (Fenton, 2006). The developmental exposures or nutritional conditions have adverse effects on neonates. Embryonic zebrafish is a good model for detecting environmental contaminants.

It has already been demonstrated that the developmental effects of PFAAs in zebrafish increased in delayed exposure (Hagenaars *et al.,* 2011; Shi *et al.,* 2008). Therefore, prolonged tests up to 144 hpf were conducted in this thesis and more endpoints, like locomotor behavior of larvae, were included. The toxicity of the different PFAAs recorded in the study was generally low concerning lethal and sublethal effects on morphology endpoints (Paper I). PFAAs were not highly acutely toxic to early life stage zebrafish in the experimental setup. They induced malformations, including pericardial edema, which was highly prevalent after exposure to TFAA, PFBA, PFBS and PFOS,

and spinal curvature was frequently observed in embryos exposed to PFNA, PFDA and PFOS.

The malformation of spine and the edema are common to PFAAs, as already detected in exposed zebrafish larvae (Hagenaars *et al.,* 2011; Huang *et al.,* 2010; Shi *et al.,* 2008). A possible mechanism for the deformation of the spine may be apoptosis and alteration in muscle fibers, as attributed previously in PFOS exposed zebrafish larvae (Huang *et al.,* 2010; Shi *et al.,* 2008).

Heart rate and hatching were also affected in PFBS and TFAA exposed embryos, respectively. In PFBS exposed larvae, at 72 hpf, lower heart rate is already observed (Hagenaars *et al.,* 2011). Pericardial edema is also a sign of compromised cardiac output, which was observed in TFAA, PFBA, PFBS and PFOS exposed embryos. Therefore, the malformations caused by the PFAAs could be the result of several mechanisms reinforcing each other.

Similar sublethal effects in zebrafish embryos have already been observed (Zheng *et al.,* 2012; Hagenaars *et al.,* 2011; Huang *et al.,* 2010; Shi *et al.,* 2008), although the parameters are slightly dissimilar. A significant delay in hatching of 1000 and 3000 mg/L TFAA exposed embryos was observed. Hatching is a critical phenomenon that depends on digestions of chorion by hatching gland enzymes, and the movement of the embryo to open it. A deleterious effect on any of the processes can delay or inhibit the hatching (von Westernhagen, 1988). Previously, a hatching delay in PFOA exposed zebrafish embryos was reported (Hagenaars *et al.,* 2011). However, in our experimental setup, the hatching was not affected in PFOA exposed embryos.

Evaluation of the PFAAs in our study followed the endpoints established for developmental toxicity in fish. The statistical evaluations were based on the sum of the total effects, since the statistical power was too low for evaluating correlations between individual endpoints and chemical concentrations. The results are generally in agreement with those reported in the literature. In Zheng *et al.* (2012) the 72 hpf 50% lethal concentration (LC_{50}) and / or EC_{50} values generated for zebrafish embryos were approximately 200, 80 and 40 mg/L for PFOA, PFNA and PFOS, respectively. In another study by Hagenaars *et al.* (2011), the 120 hpf EC_{50} values for PFBA, PFOA, PFBS and PFOS were approximately >3000, 100, 1500 and 1 mg/L, respectively. In comparison to the aforementioned studies, at 144 hpf the EC_{50} values of the corresponding PFAAs in our study (Table 1, Paper I) and (Figure 1) were within the same range.

The concentrations of PFAAs in surface waters have usually been detected at ng/L levels as summarized by Clara *et al.* (2009). The levels of PFOA and PFOS are usually 1-100 ng/L, with maximum levels of 7500 and 1090 ng/L, respectively, near hotspots. When the reported toxic effect levels are compared

with the measured levels of PFAAs in natural water systems, these chemicals do not indicate a risk of acute toxicity to aquatic organisms. The acute toxic levels for fish are approximately a factor of 1000 higher than the measured environmental levels. However, some of these compounds are highly bioaccumulative in aquatic organisms. Therefore, a focus on chronic toxicity testing is suggested.

5.1.2 Altered locomotor behavior (Paper II)

In order to enhance the knowledge of PFAAs` toxicity on fish development, the chemicals were comparatively evaluated further for potential behavioral effects. To better understand this, we measured several properties of locomotor activity in zebrafish larvae (Table 2 in Paper II). The strategy adopted to standardize the chemicals in relation to their EC_{50} values is presented in Paper II. Differences in toxicities were thus not evaluated between the chemicals but rather correlations between chemical structure and type of activity response.

Locomotor behavioral analysis often serves as a sensitive tool for detection and evaluation of sublethal effects of chemicals (Kane, 2005). Moreover, behavior endpoints can be used to provide important information about the ecological consequences of environmental pollutants. Zebrafish locomotor activity has previously been used for specific high throughput behavioral profiling, leading to the discovery and characterization of psychotropic drugs (Kokel *et al.,* 2010; Rihel *et al.,* 2010). In Paper II, the behavioral analysis showed that exposure of zebrafish larvae to PFAAs caused disturbances in locomotor activity, as observed in the embryo toxicity test. In light to dark transitions, the normal elevated larval activity pattern was not observed in the highest tested concentrations of some PFAAs whereas; during the same scenario a further reduced locomotor activity was observed. Similar patterns of behavior in zebrafish larvae exposed to known neuroactive chemicals have already been reported (Irons *et al.,* 2010). PFAAs have affected the possible mechanism that may be responsible for the elevation of activity in response to darkness.

5.1.3 Toxicity ranking (Paper I & II)

Seven PFAAs were selected for their toxicity impact due to having similar structure. A structure-activity relationship of their effects has been documented previously. The toxicity and accumulation of the PFAAs are correlated to the number of fluorinated carbons in the carbon chain length and the functional group attached (Buhrke *et al.,* 2013; Dai *et al.,* 2013; Liu *et al.,* 2013; Reistad *et al.,* 2013; Zhao *et al.,* 2013; Inoue *et al.,* 2012; Zheng *et al.,* 2012; Hagenaars *et al.,* 2011; Liu *et al.,* 2011; Jeon *et al.,* 2010; Yeung *et al.,* 2009a;

Wolf *et al.,* 2008a; Kleszczynski *et al.,* 2007; Matsubara *et al.,* 2006; Martin *et al.,* 2003a; Martin *et al.,* 2003b). The long-chained PFAAs are highly persistent and bioaccumulative, as shown by their world-wide distribution in various organisms and environments.

PFOS and PFOA are the most frequently detected pollutants in different metrics. Their highest concentrations have also been measured (Rudel *et al.,* 2011; Kannan *et al.,* 2005a). The toxicity level of PFOS as compared to that of PFOA to several fresh water organisms, including zebrafish embryos has previously been demonstrated approximately as ≥10 times higher (Hagenaars *et al.,* 2011; Ji *et al.,* 2008). In this thesis, the EC_{50} values demonstrated an even higher-factor toxicity of PFOS compared to that of PFOA. The concentration levels of these PFAAs started to decline in the environment because of some regulatory measures agreed upon by both manufacturers and environmental protection regulators (Rudel *et al.,* 2011). At the same time other new replacement homologues, such as PFBA and PFBS, are becoming predominant PFAA pollutants in the environment (Giesy *et al.,* 2010; Renner, 2001), which calls for their urgent toxicological evaluation in aquatic organisms.

Among all PFAAs, PFOS has been the one most evaluated for its toxicity. As summarized by Giesy *et al.* (2010), the concentration of PFOS having acute and /or chronic toxicity to aquatic organisms generally ranges between 1 and 100 mg/L. Our findings overlap this range. Furthermore, our results indicate that the developmental toxicity of PFDA was similar to that of PFOS, suggesting that this compound should be further evaluated using chronic tests and other test species, particularly keeping its persistent and bioaccumulative properties in mind. Some aquatic toxicological information on the PFOS replacement analogue PFBS also exists. PFBS exposure demonstrated 5-40 fold lower tissue levels in mice as compared to that of PFOS (Sundström, 2012a). Generally, the PFBS clearly seems to be less toxic than PFOS. In our study, a 300-fold difference in developmental toxicity was calculated, which is also in agreement with the values summarized in Giesy *et al.* (2010). Comparing the toxic effect levels calculated in the literature as well as in our study, with measured levels in natural waters, it does not indicate any risk for acute toxicity to aquatic organisms.

The order of toxicity for the PFAAs based on fish embryo toxicity test was calculated as: PFOS > PFDA > PFNA > PFOA > PFBS > TFAA > PFBA. Identical rankings of PFAA toxicity have been observed in corresponding published studies. In Zheng *et al.* (2012) the PFAAs caused toxicity of zebrafish embryos in a concentration-dependent manner with ranking of the PFAAs in the order PFOS > PFNA > PFOA. In Hagenaars *et al.* (2011) the

rank of the toxicities of the PFAAs were PFOS > PFOA > PFBS > PFBA. The results from both of our studies suggest that PFAAs affect embryo development as well as behavior, although at concentrations higher than commonly measured in natural surface waters.

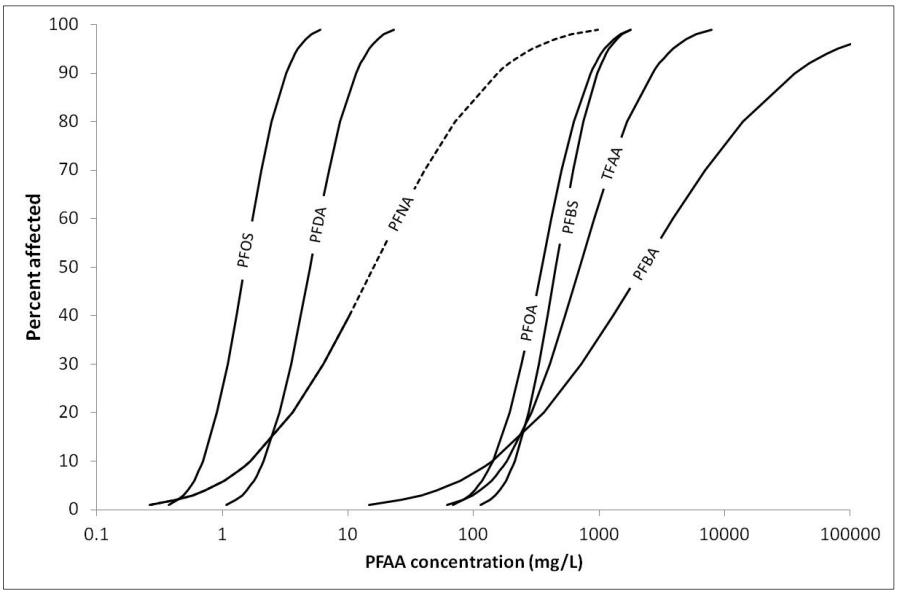

Figure 1. Concentration-response relationships of combined data of lethal and sublethal effects at 144 hpf in zebrafish embryos exposed to seven different PFAAs. Curve for PFNA is extrapolated due to lack of effects higher than 50%.

In our studies, the length of the fluorinated carbon chain and the functional group seem to be related to the developmental toxicity of PFAAs in zebrafish embryos (Figure 1). Generally, PFAAs with longer carbon chain lengths had higher toxic potential than did PFAAs with shorter chain lengths. Further, PFAAs with a sulfonic group were more toxic than were PFAAs with a carboxylic group of the same carbon chain length.

The increased toxicity might be explained by the increased hydrophobicity resulting in higher bioaccumulation potential for chemicals with longer carbon chains and with sulfonic groups. Similar findings have been observed in two other studies evaluating toxic effects of PFAAs on zebrafish embryos (Hagenaars *et al.,* 2011; Zheng *et al.,* 2012). In both studies the toxicities were related to the length and functional groups of the four PFAAs evaluated. Similar correlations between toxicity and the carbon chain length, as well as

the functional groups, have been shown when summarizing toxicity tests for other fish species and for *Daphnia magna* (reviewed in Giesy *et al.,* 2010).

As for the toxicity, also the bioconcentration potential of PFAAs in fish has been correlated with the length of the fluorinated carbon chain and the functional group (Inoue *et al.,* 2012; Jeon *et al.,* 2010; Martin *et al.,* 2003a). If PFOS and other long chain PFAAs can be replaced with shorter carbon chain length PFAAs, then this could be beneficial for aquatic health based on the correlations between bio concentration and also toxicity with fluorinated carbon chain length.

5.2 Non-compartmental analysis and disposition

The tissue distribution and kinetic behavior of PFOA is quite varied in laboratory animals. Variation in kinetic parameters is reported among species and also within genders of the same species. In the present thesis, a study was conducted to examine the kinetics, distribution and bioconcentration of radiolabeled PFOA in adult zebrafish (Paper III).

The fish absorbed the PFOA from water and the steady state was reached in 20-30 days (Paper III, Figure 3). After 40-days of exposure at the nominal concentration of PFOA in water, the accumulation ratio was 20-30 times in the whole fish homogenate (Paper III, Table 1). The BCFs calculated at steady state in Experiment 2, were in the same range.

There was no difference in accumulation of PFOA between the genders. Contrary to this, Hagenaars *et al.* (2013) have reported a gender related difference in accumulation as well the bioconcentration rate of PFOA in zebrafish. They found that males have accumulated more chemical at higher rates than their counter females. They also demonstrated that males accumulate PFOA at a lower rate than females in longer exposure periods (28 days) as compared to shorter periods of exposure (4 days). However, in their higher tested exposure concentration a gender difference in PFOA accumulation was documented. This gender difference has been explained by the difference in elimination rate of PFOA in rats and fathead minnows (*Pimephales promelas*). Females eliminate the chemical rapidly, as compared to that in males, where its elimination is inhibited by the testosterone level (Lee & Schultz, 2010; Vanden Heuvel *et al.,* 1991). In PFOA exposed fathead minnows, decreased levels of testosterone have already been reported (Oakes *et al.,* 2004). Hagenaars *et al.* (2013) have also suggested that the reduced gender difference might be caused by an altered steroid metabolism. They found gender related alterations in gene transcripts involved in the biosynthesis of cholesterol and progesterone which can influence the levels of testosterone and thereby elimination of PFOA. In

the last, the inhibitory effect of testosterone on elimination of PFOA in males is masked by a decrease level of the steroid hormone in male fish. Monitoring data showing a similar trend are reviewed by Lau *et al.* (2007).

The non-compartmental kinetic analysis of the data of Fig.3 (Paper III) showed that at 10 µg/L exposure level the PFOA was first taken up rapidly (effective half-life 7-8 days) and eliminated more slowly (terminal half-life 13-14 days) in zebrafish (Table 1, Paper III). The effective half-life of 7 days indicates that the PFOA exposure was a chronic period of exposure (>5 half-lives).

The concentration versus-time profile of PFOA displayed a bi-exponential decline during the wash-out period. The terminal half-life of PFOA in zebrafish (13-14 days) was greater than those reported in rainbow trout and Nile tilapia (Han *et al.,* 2011; Lee & Schultz, 2010). This variation might be due to species differences. A relatively long half-life combined with the detection of high PFOA concentrations in the bile and intestines may indicate enterohepatic re-circulation of PFOA in zebrafish. For PFOA, this phenomenon has already been demonstrated in fish (Martin *et al.,* 2003b) and rodents (Kudo and Kawashima, 2003; Johnson, 1984). Large species differences have been reported for the biological half-life of PFOA in laboratory animals. The differences in elimination between aquatic and terrestrial animals may be explained by their difference in elimination processes. Fish have a relatively rapid elimination rate from gills to water compared to that from lungs to air in terrestrial animals.

Moreover, large gender related differences within the same species have also been reported, both in fish and mammals (Han *et al.,* 2011; Lee & Schultz, 2010; Hundley *et al.,* 2006). Females eliminate PFOA more rapidly, and have a shorter half-life as compared to that of males. PFOA is excreted into urine by active tubular secretion, and certain organic anion transporters are responsible for the secretion (Kudo & Kawashima, 2003). These gender differences in elimination may also be explained by these solute carrier molecules in renal handling of PFOA, which are under the influence of steroid hormones release (Lee and Schultz, 2010; Vanden Heuvel *et al.,* 2006). However, the zebrafish in the present study did not show an overall gender difference in uptake and disposition of PFOA.

To describe the fate of the chemical, WBA was employed for quantification in tissues and organs. Determination of PFOA accumulation in target tissues can assist understanding of the physiological response, or any change in realistic environmental exposures. Detection and labeling of PFOA in the brain and olfactory apparatus of the brain indicated the access of the chemical to the central nervous system. The uptake of metals via the olfactory pathway has

already been observed in rats and fish (Persson *et al.,* 2003; Tjälve *et al.,* 1996; Tjälve *et al.,* 1995). The axonal transport of PFOA or other PFAAs has not to our knowledge been reported.

The uptake and transport of such environmental chemicals to the CNS via the olfactory system may be related to neurological dysfunctions. Access to brain region is also possible because PFAAs can cross the blood brain barrier to certain extent (Harada *et al.,* 2007; Maestri *et al.,* 2006). PFAAs have already been detected in brain regions of wild fish and freshwater farmed fish (Shi *et al.,* 2012; Kannan *et al.,* 2005). The nervous system is a sensitive target, and PFAA levels are linked to certain neurological disorders (Hoffman *et al.,* 2010). An association between blood levels of PFAAs and impulsivity and ADHD prevalence in children has been reported in cross-sectional studies (Gump *et al.,* 2011; Hoffman *et al.,* 2010). PFAAs are considered developmental neurotoxicants and their effects on spontaneous behavior, habituation and learning later at adulthood have been observed in prenatally or neonatal exposed mice (Onishchenko *et al.,* 2011; Johanson *et al.,* 2008).

The increasing levels of PFOA in oocytes over the time of exposure indicated that the chemical may be transported through yolk proteins such as vitellogenin. PFAAs have been detected in eggs, and this suggests the maternal transfer of the chemical to the F1 generation in oviparous species (Shi *et al.,* 2012; Kannan *et al.,* 2005). Furthermore, the observation of labeling in oocytes even on day 8 of washout indicated the slow release of the chemical from the tissue.

The bioconcentration of PFOA at steady state demonstrated substantial variation across the tissues. A 100-fold constant external exposure revealed the bioconcentration profile, which is similar to information on PFOA in prior studies conducted in zebrafish, *Daphnia magna* green mussel, earthworm, common carp and rainbow trout in their respective experimental setups of experiments (Dai *et al.,* 2013; Hagenaars *et al.,* 2013; Ng and Hungerbuhler, 2013; Liu *et al.,* 2011; Martin *et al.,* 2003). The intrinsic accumulation of PFOA in individual tissues was 20-100 fold i.e. C_{tissue} –to- C_{tank}. The relationship between external exposure and internal body burden may ideally be used for exposure risk assessment. For compounds that highly bind to body proteins, their internal exposure levels are a reliable index of exposure that can be related to health and risk assessment. The equilibrium model employed in the present study not only explained the better estimation of tissue accumulation but a more stringent risk assessment.

5.3 Reproduction and sexual development (Paper IV)

Developmental and reproductive effects of PFOA exposure were evaluated in zebrafish. The impact of PFOA exposure on the population level reproductive endpoint was not significantly toxic to zebrafish. However, a decrease in successful spawning over time was observed for all groups except the highest exposed concentration group, where the opposite trend was observed. Adult zebrafish spawn several times in a month. Most teleost species with asynchronous ovaries have protracted spawning seasons, with multiple spawning (Selman *et al.,* 1993). Contrary to the decrease in successful spawning events, the average number of eggs laid per female increased over the time of exposure. The progeny was more pronounced in fish exposed to 20 mg/L of PFOA. The average number of eggs laid per female at each successful spawning was 65-84 in the controls and 18-120 in the highest exposed concentration. Similarly, in PFOA exposed fathead minnows (*Pimephales promelas*) no significant differences in spawning events or average number of eggs per female have previously been reported (Oakes *et al.,* 2004). The fertilization ratio was approximately 60-80% for all treatment groups. An increased trend of fertilization with the time of exposure was noted. However, none of these observations were confirmed significant statistically.

Overall, the present study suggests that the tested concentrations of PFOA well above the routinely observed levels in environment do not substantially interfere with reproduction in zebrafish. In a similar experimental exposure period, no significant effects of PFOA exposure on reproductive capability, like fecundity and fertility of exposed zebrafish were found (Hagenaars *et al.,* 2013). However, the authors found the evidence of effects on reproduction only at the transcriptional level during the 28-day exposure.

The results from offspring survival indicate maternal transfer of PFOA; and the reduction in survival might have been more pronounced if even higher PFOA concentrations had been tested. Maternal transfer of PFAAs have been observed both in wild fish as well as in zebrafish exposed experimentally to radiolabelled PFOA by us (Paper III) and others (Kannan *et al.,* 2005a; Shi *et al.,* 2008). Moreover, the adult exposure could have a significant effect on the F1 generation`s survival.

In the FSDT test, PFOA does not appear to affect the health and somatic fitness of zebrafish. As, no significant differences were recorded in the measured physiological parameters, such as body weights, body lengths, condition factors or LSIs between exposed and control fish, regardless of the sex classification. Condition factor is a general index of relative fish health. It reflects the changes in body development (Goede & Barton, 1990). Previous studies have shown that zebrafish sex ratio is a sensitive endpoint for

evaluation of endocrine disrupters. Phenotypic sex determination is a dynamic process, in zebrafish, that might be regulated by both genes and environmental factors (Baroiller *et al.,* 1999; Delvin & Nagahama, 2002). Complete sex reversals have been observed when testing both estrogens and androgens (Örn *et al.,* 2003, Örn *et al.,* 2006). However, in the present study neither intersex nor any significant shift in sex ratios was observed. It demonstrated that PFOA does not act as an endocrine disrupter at the tested concentrations. Furthermore, PFOA exposure did not result in any significant change in the gonad maturations. Thus, PFOA does not seem to interfere with individual reproductive behavior in zebrafish.

The accumulation and effects of PFOA on liver are reported previously (Liu *et al.,* 2008; Wei *et al.,* 2008; Kannan *et al.,* 2005a; Martin *et al.,* 2003a; Martin *et al.,* 2003b). In rats the mechanism of PFOA hepatotoxicity is reported as activation of the gene expression of peroxisome proliferative activated receptor which is a main regulator of fatty acid β-oxidation (Guruge *et al.,* 2006). We did not observe any significant difference in expression of mRNA level of PPARα in livers of PFOA exposed zebrafish (Paper IV). However, a decreasing trend in mRNA level of PPARα expression in exposed concentrations was observed. Previous studies have reported inconsistencies in expressions of PPAR with both up-and down regulation after exposure to PFOA or other PFAAs (Ren *et al.,* 2009). In perfluorododecanoic acid (PFDoDA) exposed zebrafish, the mRNA level of PPARα in liver was decreased in a dose response manner (Liu *et al.,* 2008). After PFOA exposure, a significant activation of PPARα is reported in several studies (Takacs & Abbott, 2007; Vanden Heuvel *et al.,* 2006). In response to low PFOS exposure, the transcription of PPARα in isolated hepatocytes of Atlantic salmon is inhibited (Krovel *et al.,* 2008). This indicates the species specific expression of PPARα in exposure to different PFAAs.

Induction of vitellogenin (Vtg) in male fish is a sensitive biomarker to determine the estrogenic properties of the environmental chemicals (Jobling *et al.,* 2004). In our set up of experiment, no significant differences were detected for Vtg1 mRNA expression in liver of exposed fish from that of the control. Inconsistent with our finding, estrogenic effects have been revealed by inducing the hepatic Vtg gene expression in PFOA exposed rare minnows (*Gobiocypris rarus*) (Wei *et al.,* 2007).

The tested concentrations of PFOA did not interfere significantly the reproduction and sexual development of zebrafish. The effects of PFAA exposure to aquatic organisms are contradictory.

6 Conclusions and future perspectives

- The chemicals tested at concentrations well above the routinely observed in the environment were found toxic to the zebrafish at early stages.
- Zebrafish larvae displayed more sensitivity to PFAAs in behavior analysis than the morphology endpoints. All of the exposure concentrations altered the larval locomotor behavior.
- A relationship between higher toxicity with longer carbon chain length and attached functional group was noted for both early development and behavior.
- In an equilibrium model the radiolabeled PFOA accumulated in the body 20-30 times higher than the exposure concentrations in water and distributed to liver, intestine, gall bladder, as well as brain, kidney and oocytes.
- PFOA was not a traditional endocrine disruptor at the tested concentrations and did not affect the reproduction and sexual development in zebrafish.
- Regardless of the mechanism behind behavior alterations, changed locomotor behavior will likely have ecological implications. Future studies are required to find the factors that are involved in the structure activity relationship and mode of action of different PFAAs.
- Future work is required to elucidate the possible links between PFAAs exposure and mental disorders like ADHD and Alzheimer`s disease.
- The long term toxicity of PFOA is possibly mediated through bioaccumulation. The future studies should be designed to investigate mechanism(s) and impact of maternal transfer on offspring.
- A prime focus was given on endocrine disruption and reproduction, the effects on other endpoints cannot be ruled out. For future studies, metabolic disorders and neurotoxic monitoring of PFAAs in life cycle studies in zebrafish would be an interest.

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Summary in Urdu

صنعتی انقلاب کے ساتھ ہی مصنوعی نامیاتی مرکبات کی پیداوار میں اضافہ ہو گیا۔ گزشتہ صدی میں ان مصنوعی مرکبات کی پیداوار میں بے تحاشہ اضافہ ہوا ہے۔ یہ مرکبات کسی نہ کسی طور آبی ذخائر میں ہی پہنچ کر اختتام پزیر ہوتے ہیں۔ ان کی موجودگی آبی حیات کو حد درجہ متاثر کر رہی ہے۔ آبی جاندار چونکہ ہمہ وقت اس آلودہ پانی میں رہتے ہیں اس لیے وہ ان کے خطرناک اثرات سے زیادہ متاثر ہوتے ہیں۔ مچھلیاں ایسے جاندار ہیں جن میں بہت زیادہ تنوع پایا جاتا ہے۔ علاوہ ازیں مچھلیوں کو اپنی آکسیجن کی ضرورت پوری کرنے کے لیے اوسط وزن سے ہزار گنا زیادہ پانی اپنے جسم سے گزارنا ہوتا ہے۔ اس لیے مچھلیوں میں ان مرکبات کی بڑی مقدار موجود ہو سکتی ہے۔

پرفلوروالکائل (Perfluroalkyle) ایسے ہی نامیاتی مرکبات ہیں جو کہ مصنوعی طور پر ۱۹۴۰ء کے عشرے میں تجربہ گاہوں میں تیار کیے گئے۔ جب یہ تیار کیے گئے تھے تو انہیں محفوظ تصور کیا گیا تھا۔ یہ اپنی غیر معمولی خصوصیات کی وجہ سے صنعتی اور روزمرہ کی اشیاء میں بہت زیادہ استعمال ہوتے ہیں۔ آگ بجھانے کے سامان سے لیکر کھانا پکانے کے برتنوں تک ان کا استعمال وسیع ہے۔ عمومی طور پر یہ مرکبات توڑ پھوڑ کا شکار نہیں ہوتے۔ اس جیسی دوسری خصوصیات کی بناء پر زیادہ تر ماحول یا حیوانی جسم میں برقرار رہتے ہیں۔ عالمی طور پر ان کی مقدار انسانوں، جنگلی جانوروں اور ماحول میں معلوم کی گئی ہے۔ جانوروں میں ان کے اثرات جگر، تولیدی نظام اور دوران افزائش دیکھے گئے ہیں۔ اب تک تیس کے قریب ان سے ملتے جلتے مرکبات انسانی خون میں پائے گئے ہیں۔

ان مرکبات کے انسانی جسم میں پھیلنے کے بارے تفصیلی معلومات جو کہ ان مرکبات کے زہریلے اثرات کو سمجھنے کے لئے کارآمد ہو سکتی ہیں بہت حد تک محدود ہیں۔ ان اثرات کو سمجھنے کے لئے مختلف جانوروں پر آزمائشی تجربات کیے گئے ہیں۔ ایسی ہی ایک کاوش گورخر ماہی (Zebrafish) میں ان کے اثرات کو جانچنا ہے۔ زیبرفش ایک چھوٹی سی مچھلی ہے جو کہ برصغیر کے ندی نالوں میں پائی جاتی ہے۔ یہ اپنی گونا گوں خصوصیات کی وجہ سے ان آزمائشی تجربات کے لیے نہایت کارآمد ہے۔

ہم نے اپنی تحقیق میں ان نامیاتی مرکبات کے زہریلے اثرات کا اس مچھلی کے انڈوں اور افزائش پانے والے جنین میں جائزہ لیا ہے۔ ہم نے دیکھا کہ یہ مرکبات دوران افزائش خاطر خواہ اثر انداز ہوتے ہیں۔ ان اثرات سے نہ صرف نوزائیدہ مر جاتے ہیں بلکہ یہ ناقابل تلافی بگاڑ بھی پیدا کرتے ہیں۔ ہم نے ان مرکبات کا چھوٹی مچھلیوں میں تیرنے کی صلاحیت پر اثرات کا بھی مشاہدہ کیا۔ ہم نے ان مرکبات کی ساخت اور ان کے زہریلے اثرات کے درمیان براہ راست تعلق کو دریافت کیا ہے۔

ان نامیاتی مرکبات کا ایک نمائندہ مرکب (PFOA) ہے۔ جس کو بہت زیادہ تحقیق کا موضوع بنایا گیا ہے۔ ہم نے اس تابکاری مرکب کو مچھلیوں میں تحقیق کے لئے استعمال کیا۔ تابکاری مرکبات دوا یا کسی بھی کیمیائی مرکب کے جسم میں تحلیل اور مختلف اعضاء میں نفوس پزیری کے عمل کو سمجھنے میں مددگار ہوتے ہیں۔ چونکہ اس کی تابکاری موجودگی کو جسم میں کسی بھی جگہ دیکھا جا سکتا ہے۔ ہم نے دیکھا کہ مرکب جسم میں جلدی جذب ہوتا ہے۔ اور اسکا اخراج پہلے تیز اور پھر آہستہ آہستہ ہو جاتا ہے۔ اس کا علاوہ یہ مرکب جگر، دماغ اور دوسرے اعضاء میں جمع ہوتا رہتا ہے۔ ہماری تحقیق کا اہم مشاہدہ اس مرکب کا مادہ مچھلیوں سے اگلی نسل میں منتقل ہونا تھا۔ جو کہ نوزائیدہ جانداروں میں افزائش نسل کے دوران خطرے کی گھنٹی ہو سکتی ہے۔

ہم نے اپنی ایک اور تحقیق میں اسی مرکب کا تولیدی نظام پر اثرات کا جائزہ بھی لیا۔ اس مرکب کے مچھلیوں کے تولیدی نظام پر بظاہر اثرات نہیں دیکھے گئے۔ مگر مچھلیوں کے انڈے دینے کی صلاحیت اور بارآوری متاثر ہوتی نظر آئی ہے۔ اس ضمن میں مزید تحقیق کی ضرورت ہے۔ ہم نے مچھلیوں میں اس جنسی صلاحیت کی نشوونما پر بھی اثرات کا جائزہ لیا۔ اس پہلو پر بھی ہمیں ملے جلے اثرات نظر آئے۔ تاہم ایک اچھی پیش بندی کے ساتھ مزید تحقیق کی ضرورت ہے۔ ہم نے ان مرکبات کو مجموعی طور پر نوزائیدہ مچھلیوں میں دوران افزائش مہلک اور خطرناک پایا۔

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