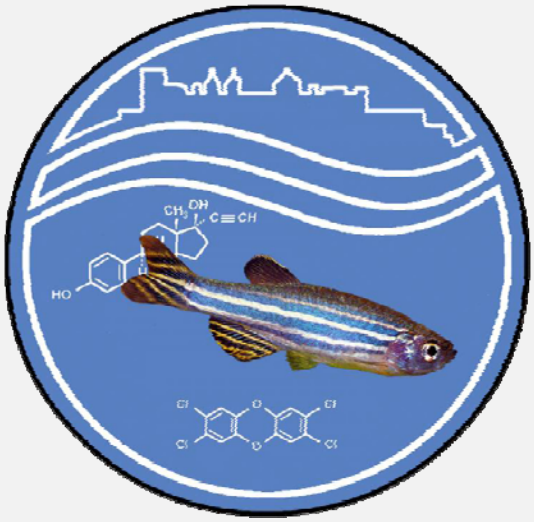

Doctoral Dissertation

Long-term effects and synergistic potential of perfluorinated chemicals (PFCs) in zebrafish (*Danio rerio*)

Susanne Keiter

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DISSERTATION

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Long-term effects and chemosensitizing potential of perfluorinated chemicals (PFCs) in zebrafish (*Danio rerio*)

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“Maybe mistakes are what make our fate... without them what would shape our lives? Maybe if we had never veered off course we wouldn't fall in love, have babies, or be who we are. After all, things change, so do cities, people come into your life and they go. But it's comforting to know that the ones you love are always in your heart... and if you're very lucky, a plane ride away”

-Carrie-

To Sven and Steffen

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Abstract

Increased awareness of the human and environmental health risks associated with perfluorinated chemicals (PFCs) has raised intensive discussions among authorities and policymakers. Yet, despite declined use of PFCs in industrial and consumer products, these emerging contaminants are still being detected in aquatic environments worldwide. In light of the persistent properties of PFCs, fairly little attention has been given to long-term effects. Another challenging topic in the context of PFCs concerns their potential to interact synergistically; a relevant matter given the complex exposure scenarios in aquatic systems.

This thesis aimed at increasing the knowledge and understanding of PFCs and their toxicity towards an aquatic vertebrate model, the zebrafish (*Danio rerio*). Focus was on multixenobiotic resistance (MXR) and toxicity following chronic exposure. Selected PFCs were evaluated in transporter efflux assays serving to determine the synergistic potential *via* P-glycoprotein (P-gp) transporter inhibition. Long-term effects following single and binary exposures of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) were evaluated over two full generations with emphasis on survival, histological alterations, vitellogenin (Vtg) and reproductive success.

Among the tested PFCs, PFOS induced the strongest accumulation of the standard P-gp transporter substrate rhodamine B (RhB) in zebrafish embryos. An up to fourteen-fold RhB-retention was found in PFOS-exposed embryos if compared with control animals. In comparison, the effect of PFOS on the uptake of the P-gp substrate calcein-AM by MDCKII cells overexpressing human P-gp was substantially smaller than that of the reference compound verapamil indicating that PFOS only weakly interacts with human P-gp. In the long-term study, the most prevalent effects following waterborne PFOS-exposure were decreased survival in off-spring generations and hepatocellular alterations. The hypothesis that the presence of PFOS increases the endocrine potential of BPA could not be confirmed in zebrafish.

This thesis provides further evidence of the chemosensitizing potential of some PFCs in zebrafish. Although the exact mechanisms of action behind the increased uptake of P-gp substrates remain unclear, the results obtained further highlight the importance of mixture toxicity when investigating the hazardous potential of PFCs. Adverse long-term effects on liver structure and survival in zebrafish were only seen at concentrations well above

ecologically relevant concentrations. Yet, the decline in survival rates following PFOS-exposure seen over generations again documents the necessity of long-term approaches within the assessment of persistent environmental pollutants.

Keywords: Long-term effects, Mixture toxicity, Multixenobiotic resistance (MXR), Perfluorinated chemicals (PFCs), Zebrafish

Zusammenfassung

Ein immer stärker werdendes Bewusstsein für Gefahren für die menschliche Gesundheit und die Umwelt durch perfluorierte Chemikalien (PFCs) hat zu intensiven Diskussionen zwischen Behörden und Politik geführt. Doch trotzt einer reduzierten Verwendung von PFCs in Industrie- und Konsumgütern werden diese Schadstoffe noch immer weltweit in der aquatischen Umwelt nachgewiesen. In Anbetracht ihrer persistenten Eigenschaften wurde den langfristigen Wirkungen von PFCs bisher nur eine geringe Aufmerksamkeit gewidmet. Eine weitere Herausforderung im Zusammenhang mit PFCs stellt deren synergistisches Effektpotential dar, was angesichts eines komplexen Expositionsszenarios in der aquatischen Umwelt ein Thema von hoher Relevanz darstellt.

Ziel dieser Dissertation war es, das Wissen und Verständnis verschiedener PFCs und deren Toxizität gegenüber einem aquatischen Modellorganismus, dem Zebrabärbling (*Danio rerio*), voranzutreiben. Ein besonderer Schwerpunkt lag hierbei auf der Untersuchung der multixenobiotischen Resistenz (MXR) und der Toxizität nach chronischer Exposition. Ausgewählte PFCs wurden in Transporter-Efflux-Assays untersucht, um das synergistische Wirkpotenzial mittels über eine Hemmung der P-glycoprotein (P-gp)-Transporter zu bestimmen. Die langfristigen Auswirkungen nach isoliertes und gemeinsames Belastung mit Perfluoroctansulfonat (PFOS) und Bisphenol A (BPA) wurden über zwei volle Generationen getestet. Hauptsächlich wurden hierbei die Überlebensrate, histologische Veränderungen, Vitellogenin (Vtg) und der Reproduktionserfolg untersucht.

Die Ergebnisse zeigen, dass unter den getesteten PFCs, PFOS die stärkste Akkumulation des Standard P-gp-Transportersubstrates Rhodamin B (RhB) in Zebrabärblingen induziert. Im Vergleich war die Wirkung von PFOS auf die Calcein-AM-Farbstoffaufnahme von MDCKII-Zellen mit einer humanen P-gp Überexpression wesentlich geringer, als die der Referenzsubstanz Verapamil. Dies deutet auf eine geringe Interaktion zwischen PFOS und dem humanen P-gp hin. In der Langzeitstudie waren die am häufigsten beobachteten Effekte nach PFOS-Exposition eine Verminderung der Überlebensrate in nachkommenden Generationen sowie hepatozelluläre Veränderungen. Die Hypothese, dass PFOS das endokrine Potential von BPA erhöht, konnte in Zebrabärblingen nicht bestätigt werden.

Abschließend kann jedoch festgestellt werden, dass diese Arbeit weitere Beweise für das chemosensibilisierende Potenzial von PFCs liefert. Obwohl der genaue Wirkmechanismus

hinter der erhöhten Aufnahme des P-gp-Substrates unklar bleibt, unterstreichen die Befunde die Bedeutung von Toxizitätsstudien mit Mischungen bei der Bewertung des Gefährdungspotenzials von PFCs. Obwohl nachteilige Effekte auf die Struktur der Leber und die Überlebensrate nur bei Konzentrationen deutlich über ökologisch relevanten Konzentrationen beobachtet wurden, zeigt der beobachtete Rückgang der Überlebensrate über den Verlauf der Generationen nach PFOS-Belastung die Notwendigkeit von Langzeitstudien bei der Beurteilung von persistenten Chemikalien.

Sammanfattning

Ökad förståelse för de risker som perfluorerade kemikalier (PFCs) utgör för hälsa och miljö har lett till en intensiv debatt bland auktoriteter och beslutsfattare. Trots en reducerad användning av PFCs i industri- och konsumentprodukter kan mätbara halter fortfarande hittas globalt i akvatiska system. Med hänsyn till PFCs persistens i miljön är långtidsstudier av högsta relevans, dock är kunskapen om detta ännu otillräcklig. En annan problematik vad gäller PFCs är deras potentiella förmåga att öka toxiciteten av andra kemikalier; något som är högst relevant med tanke på den komplexa föroreningssituation som råder i akvatiska system.

Det övergripande syftet med avhandlingsarbetet var att på olika sätt öka förståelsen och kunskapen om PFCs och deras toxicitet med hjälp av zebrafisk (*Danio rerio*) som akvatisk modellorganism. Fokus lades på multixenobiotisk resistens (MXR) och toxicitet till följd av kronisk exponering. Utvalda PFCs inverkan på speciella transportpumpar undersöktes för att fastställa eventuell toxicitetsökande effekt via transportinhibiering. Långtidseffekter av perfluoroktansulfonat (PFOS), med eller utan bisfenol A (BPA), studerades över två generationer med tyngdpunkt på överlevnad, histologiska förändringar, vitellogenin samt reproduktion.

Resultaten visar att PFOS inducerade den starkaste ackumuleringen av P-glycoprotein (P-gp) substratet rhodamine B (RhB) i zebrafiskembryon med en upptill 14 gånger högre RhB-signal jämfört med kontrollgruppen. Dock visade sig PFOS påverka ackumulationen av calcein-AM i MDCKII celler med överexpression av P-gp i betydligt lägre grad än standardinhibitorn verapamil. Detta tyder på en svag interaktion mellan PFOS och humant P-gp. De mest framträdande effekterna till följd av PFOS-exponering i långtidsstudien var mortalitet i nästkommande generationer samt levertoxicitet. Hypotesen att PFOS ökar den endokrina effekten av BPA kunde inte stärkas i försök med zebrafisk.

Sammanfattningsvis stödjer det här arbetet tidigare indikationer på den synergistiska potentialen av PFCs. Trots att den exakta verkningsmekanismen bakom det ökade upptaget av P-gp substrat fortfarande är oklar visar det här arbetet återigen på vikten av att studera kombinationseffekter vad gäller PFCs. Allvarliga effekter på leverstruktur och överlevnad observerades bara vid betydligt högre koncentrationer än de som normalt uppmäts i miljön. Ökad mortalitet i efterföljande generationer understryker dock betydelsen av långtidsförsök vid riskbedömning av persistenta ämnen.

List of papers

The work in this thesis has been summarized in the following papers:

Susanne Jernbro, Paula Suares Rocha, Steffen Keiter, Dirk Skutlarek, Harald Färber, Paul D. Jones, John Giesy, Henner Hollert & Magnus Engwall. (2007). Perfluorooctane Sulfonate Increases the Genotoxicity of Cyclophosphamide in the Micronucleus Assay with V79 Cells: Further Proof of Alterations in Cell Membrane Properties Caused by PFOS. *ESPR - Environ. Sci. Pollut. Res.* 14 (2): 85-87

Susanne Keiter, Lisa Baumann, Harald Färber, Henrik Holbech, Dirk Skutlarek, Magnus Engwall & Thomas Braunbeck. 2012. Long-term effects of a binary mixture of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish. *Aquat. Toxicol.* 118–119: 116– 129

Susanne Keiter, Harald Färber, Britta Kais, Dirk Skutlarek, Magnus Engwall, Thomas Braunbeck &Till Luckenbach. Is chemosensitization of zebrafish embryos (Danio rerio) by PFCs caused by efflux transporter inhibition? *Manuscript*

Contents

1. Introduction

Despite the complex exposure scenario in the natural environment of aquatic organisms, most research has tended to focus on single chemicals using a compound-by-compound strategy. In 2001, approximately 300 peer-reviewed papers addressed the topic of aquatic mixture toxicity. A decade later, the number of available papers had more than doubled. This development clearly points towards a shift in attention regarding mixture toxicity in ecotoxicological research. With respect to drug-drug interactions, the assessment of chemical mixture effects is crucial to achieve an appropriate view of the hazardous potential of environmental pollutants. Previous indications of the toxicity-modifying properties of perfluorinated chemicals (PFCs; Hu et al., 2003; Stevenson et al., 2006; Rodea-Palomares et al., 2012) together with their ubiquitous presence in aquatic systems have spurred further research concerning mixture effects. Given the persistence of PFCs, potential long-term effects represent another issue where the current state of knowledge is far from being satisfactory. Many fish populations are chronically exposed to complex mixtures of pollutants and the zebrafish model, therefore, represents an excellent test organism for the above-mentioned purposes.

1.1 Environmental pollution and mixture toxicity

"What has already silenced the voices of spring in countless towns in America? This book is an attempt to explain" is a famous excerpt from the book *Silent spring* by Rachel Carson (1962). The controversial book of Carson aimed to highlight the ecological consequences of pesticide use and bring them to public attention. As a result, the general attitude regarding environmental pollution underwent a shift of paradigm; the dilution paradigm ("the solution to pollution is dilution") was replaced by the boomerang paradigm ("what you throw away can come back and hurt you"; Newman and Unger, 2003). The increased awareness of the risks associated with environmental pollution led to the creation of a new discipline of science: ecotoxicology. Multiple definitions have been applied to describe the context of ecotoxicology (Newman and Unger, 2003); however, according to the earliest definition by Truhaut (1977), ecotoxicology represents "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal, vegetable and microbial, in an integral context". Over the years, the science of

ecotoxicology has developed further and constitutes today a key element in environmental risk assessment (Kahru and Dubourguier, 2010).

The number of chemicals and chemical products currently circulating in the modern society is unknown, however, chemicals production is projected to increase until 2020 (OECD, 2001). Given the worldwide manufacture and use of chemicals, environmental release is inevitable to occur. The introduction of anthropogenic chemicals into the ecosystem can take place at any stage in their life-cycle such as during production, distribution, use and during final waste disposal (Harrison, 2001). Aquatic ecosystems are *via* different routes of exposure challenged with multiple chemicals originating from human activities such as accidental release, wastewater effluent and agricultural run-off (Backhaus et al., 2003). Consequently, the aquatic environment has been considered as a final disposal of industrial waste (Kime, 1998).

Despite the complex exposure scenario in the natural habitat of aquatic organisms, most research has tended to focus on single chemicals using a compound-by-compound strategy. As such, safety levels of chemicals mostly do not account for mixture toxicity effects (Celander, 2011). Within pharmacology, drug-interactions are well known for their potential to generate effects greater than (synergism) or less than (antagonism) the total effect as predicted by each chemical individually (Newman and Unger, 2003). Synergistic chemical-chemical interactions can be described as either pharmacodynamic, i.e., chemicals asserting identical or opposite action, or pharmacokinetic, i.e., interactions inducing altered absorption, distribution, biotransformation or excretion (Celander, 2011). Commercially, the phenomenon of synergism has long been applied as a method to enhance the efficiency of e.g., insecticides (Bernard and Philogene, 1993). As single-component assessments clearly bear the risk of underestimating the true hazardous potential, the importance of mixture toxicity has gained increased awareness within ecotoxicological research. The relevance of mixture approaches is further emphasized by recent studies confirming the potential of environmental contaminants to produce synergistic toxic effects when combined with other compounds both *in vivo* (e.g., Norgaard and Cedergreen, 2010; Caldwell et al., 2011; Xu et al., 2011; Boltes et al., 2012) and *in vitro* (Hu et al., 2003; Harris et al., 2009).

1.2 Multixenobiotic resistance

Organisms inhabiting contaminated areas have often been shown to express a higher activity of various detoxification mechanisms involving e.g., cytochrome P450 enzymes (Nyman et al., 2000; Lavado et al., 2006; Wills et al., 2010), chaperones (Muller et al., 1998; Padmini et al., 2009) and metal binding proteins (reviewed by Amiard et al., 2006). Within ecotoxicology research, increased attention has been given a new group of cellular defense proteins, namely the ATP-binding cassette (ABC) transporters (Bard, 2000; Davidson and Maloney, 2007). The ABC-transporters have been identified in a variety of taxons including fish (reviewed by Bard, 2000) and represent a subclass of ATP-driven membrane proteins involved in the active transportation of exogenous as well as endogenous molecules across cell membranes (Fig. 1.; Davidson and Maloney, 2007; Licht and Schneider, 2011). The preventive expulsion of foreign substances, the so-called multixenobiotic resistance (MXR), has acknowledged the ABC-transporters as a first line of cellular defense against a broad range of natural and man-made pollutants (Epel, 1998). The 170 kDa permeability glycoprotein (P-glycoprotein, P-gp) and the multidrug-resistance protein (MRP), both members of the ABC-transporter subclass, represent two of the best described transporter proteins associated with MXR (Regev et al., 1999; Luckenbach and Epel, 2005). The environmental importance of these transporters was first highlighted by Kurelec and colleagues (Kurelec, 1992), who later on demonstrated that the transporter activity in snails from a polluted site was more than twice as effective compared with snails populating an uninpacted site (Smital and Kurelec, 1998).

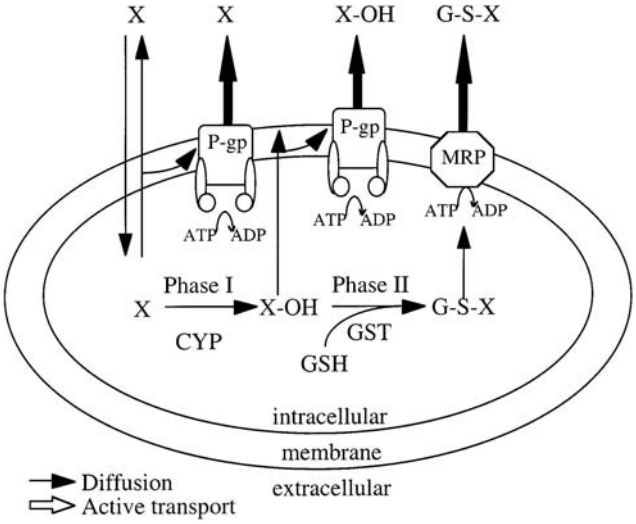


Fig. 1: Illustration of xenobiotic resistance provided by membrane integrated efflux pumps together with phase I and II detoxification enzymes, cytochromes P450 (CYP) and glutathione-S-transferase (GST), respectively. Intracellular accumulation of a moderately hydrophobic compound (X) is prevented by active P-gp transporters. At high concentrations, accumulated X will undergo biotransformation via phase I and be expelled by P-gp transporters or continue to phase II involving GST-catalyzed conjugation to glutathione (GSH). Efflux of the glutathione conjugate (G-S-X) will be carried out by multidrug.resistance protein- (MRP) mediated transport. Figure from Bard (2000).

The MXR defense mechanism is similar to the early discovered multidrug resistance in tumor cell lines displaying resistance towards chemotherapeutic drugs (Smital and Kurelec, 1997). The search for agents arresting the pump activity, so called chemosensitizers, is an ongoing research topic in the field of cancer therapy (Peer and Margalit, 2006). In recent years, it has become increasingly clear that environmental pollutants of different origin and structure can disturb the sensitive MXR defense mechanism (Kurelec, 1995; Luckenbach and Epel, 2005; Stevenson et al., 2006), thus allowing previously exported chemicals to enter the cell and accumulate (reviewed by Kurelec, 1995, 1997; Epel et al., 2008). Environmental pollutants with such MXR-reversing behavior have been suggested to be top-ranked among hazardous chemicals given their potential to inhibit basic biological defense systems (Smital and Kurelec, 1998). The way in which a xenobiotic can act as a chemosensitizer has been described as either direct or indirect. A direct inhibition of the MXR can occur by substances acting as pump substrates, thus saturating the pump capacity causing an increased intracellular accumulation of the substrates normally exported (Hofsli and Nissenmeyer, 1990). Compounds blocking the ATPase activity or influencing the cell membrane fluidity can indirectly induce a similar pump arrest (Litman et al., 1997; Romsicki and Sharom, 1999).

1.3 Perfluorinated chemicals

Perfluorinated chemicals (PFCs) constitute a group of emerging contaminants which have received increased attention within environmental research. PFCs have been synthetically produced over several decades as their surface-active properties offer great advantages when applied to industrial and commercial products such as e.g., fire fighting foams and coatings for textiles and paper products approved for food contact. The chemical structure of PFCs is characterized by an alkyl chain of varying length with fluorine substitutions forming strong carbon-fluorine bonds (C-F). Due to these high-energy bonds, PFCs are showing resistance towards hydrolysis, photolysis, microbial degradation as well as metabolism by vertebrates and are therefore considered to be persistent in the environment (Giesy and Kannan, 2002).

Since organofluorine compounds were first detected in human sera in 1968 (Taves, 1968), improved analytical capabilities have identified PFCs in a wide range of environmental matrices at a global scale including biota, solids and aqueous systems (Bossi et al., 2005; Higgins et al., 2005; Tseng et al., 2006). Perfluorooctane sulfonate (PFOS) is frequently reported as one of the most commonly detected PFCs in biotic and abiotic samples (Martin et

al., 2004; Ahrens et al., 2009; Naile et al., 2010; Houde et al., 2011; Li et al., 2011; Thompson et al., 2011). In aquatic ecosystems, PFOS is normally detected in the lower ng/L range; however, concentrations of 0.65 - 43.5 μg/L have been reported in water environments affected by industrial waste (Rostkowski et al., 2006; Skutlarek et al., 2006; Zainuddin et al., 2012). Confirmed presence of PFOS in drinking water worldwide further highlights the environmental importance of this compound (Skutlarek et al., 2006; Ericson et al., 2009; Quinete et al., 2009; McLaughlin et al., 2011).

Giesy and Kannan (2001) were the first to demonstrate the global distribution of PFOS. Investigated locations within their study included the Arctic where detectable concentrations of PFOS were found in the liver of polar bears and blood plasma of ringed seals (350 ng/g and 110 ng/mL respectively). Given that the volatility of PFOS is considered low or possibly negligible (OECD, 2002b), it is unlikely that the migration to such remote areas is performed *via* long-range atmospheric transport. One explanation is that PFOS is formed following reactive processes of volatile precursors (Renner, 2001). This was also implied in two independent studies where PFOS was detected as a possible end-product following biodegradation of the polyfluorinated alcohol N-EtFOSE (2-(N-ethylperfluorooctane sulfonamido) ethyl alcohol; Cleston, 2000) and the polyfluorinated sulphonamide N-EtPFOSA (N-ethyl perfluorooctanesulfonamide; Tomy et al., 2004).

With respect to the persistent, bioaccumulative and toxic (PBT) properties of PFOS (OECD, 2002a), a restriction for commercialization and use was implemented within the EU in 2008 (EU, 2006). PFOS was also added to the list of priority substances to be controlled within the European Water Framework Directive (EU, 2008). However, due to a continued production of PFOS and its precursors in non-EU member countries (UNIDO, 2009) combined with the long-range transport potential of PFCs (Young et al., 2007; Dreyer et al., 2009), a sustained global emission of PFOS is likely to occur.

Numerous studies have investigated the hazardous potential of PFOS in organisms and confirmed the liver as one of the target organs (Hagenaars et al., 2008; Ivan et al., 2008; Cui et al., 2009; Du et al., 2009). Further effects associated with PFOS exposure include, e.g., abnormal development (Han and Fang, 2010), reduced offspring survival (Lau et al., 2003; Newsted et al., 2007; Han and Fang, 2010), endocrine disruption (Oakes et al., 2005; Liu et al., 2007; Du et al., 2009) and oxidative stress responses (Arukwe and Mortensen, 2011; Chen et al., 2012). To date, the majority of PFOS-related studies with fish have focused on acute

effects, leaving chronic and reproductive effects largely unexplored. Given the aquatic occurrence and non-metabolizing properties of PFOS, chronic low-dose exposures in fish have been warranted (Oakes et al., 2005). Ankley et al. (2005) performed a partial life-cycle test with the fathead minnow (*Pimephales promelas*) and observed deviating sex steroid levels and histopathological alterations in ovaries following exposure to 300 µg/L PFOS. The discovery of such sublethal effects has great ecological importance. Yet, the continued lack of studies over multiple generations with aquatic vertebrates limits a comprehensive assessment of possible risks of PFOS. Notable concentrations of PFOS in eggs of field-sampled fish have suggested maternal effects (Kannan et al., 2005), thus further emphasizing the relevance of long-term studies with PFOS to explore potential population-relevant effects.

Another aspect of PFOS toxicity concerns the complexity of joint effects. Due to the amphiphilic nature of PFOS, its potential to modulate the toxicity of other chemicals has repeatedly been investigated and confirmed (Hu et al., 2003; Liu et al., 2009; Kim et al., 2011). Thus, in light of the environmental persistence of PFOS, long-term assessment of combinations with other pollutants appears a promising strategy to shed more light on the complex toxicology of PFOS.

1.4 Zebrafish as a test organism in ecotoxicological research

The zebrafish (*Danio rerio*; Fig. 2) is a tropical freshwater fish belonging to the family Cyprinidae, native to Southeast Asia, e.g. Thailand, Burma, India, Pakistan and Bangladesh, where it lives in stagnant or flowing water; e.g. paddy fields or rivers (Laale, 1977). The zebrafish grows to an average length of 3-5 cm and is characterized by five longitudinal blue stripes along both sides of the compressed body. The sexes are easily differentiated during spawning period due to the swollen bellies of the females in addition to their lack of reddish tint along the longitudinal stripes. The zebrafish is an oviparous species and under favorable conditions, the female zebrafish can spawn 100 to 500 eggs every 2-3 days all year around (Lohr and

Fig. 2: Adult zebrafish (*Danio rerio*). Female: upper individual, Male: lower individual. Picture by Erik Leist

Hammerschmidt, 2011). The zebrafish has frequently been utilized in scientific experiments since the nineteen thirties given its inexpensive and ready maintenance in addition to its short generation cycle of approximately 2-3 months (Bresch, 1991; Scholz et al., 2008). The transparency of the eggs makes them well suitable for embryo-larval (EL) toxicity tests which are generally more sensitive than toxicity tests with juvenile and adult fishes (McKim, 1977; Hutchinson et al., 1998). EL toxicity studies with zebrafish are also ideal considering the short time period of 72-96 h from fertilization until hatching. Today, zebrafish eggs are commonly chosen for toxicity testing as they represent a good alternative to fish acute toxicity testing (Lammer et al., 2009). In addition to toxicological screening of chemical compounds, the zebrafish model system has been found suitable for developmental, genetic and transgenetic approaches as well as for endocrine research (Segner, 2009; Lohr and Hammerschmidt, 2011). Moreover, findings revealing preserved key endocrine functions in zebrafish previously recognized in mammals have implied the zebrafish as a potential model for human endocrine systems (Lohr and Hammerschmidt, 2011).

1.5 Bioassays for the evaluation of chronic and chemosensitizing effects

Micronucleus test

The micronucleus test is one of the most popular assays in genotoxicity testing (Bolt et al., 2011). Originally, the micronucleus test was developed for toxicological screening of genotoxic agents towards mammalian cells (Heddle et al., 1983). Over the years, the test has been considered suitable as an early warning signal for genotoxic hazards towards both aquatic organisms and humans (Al-Sabti, 2000). The micronucleus test offers a fast and invasive method for the detection of genotoxic agents of which the convenience is emphasized by the possibility to score data at any time (Al-Sabti, 2000). Due to the complexity behind micronuclei formation, the possibility to detect the action of both clastogenic and aneugenic chemicals represent a further advantage. Micronuclei consist of whole or fragmented chromosomes that fail to integrate into the daughter nuclei following anaphase (Al-sabti and Metcalfe, 1995). Micronucleation represents an endpoint of high toxicological significance (Kirsch-Volders et al., 2011) and has furthermore been implemented by OECD as guideline 478 (OECD, 1997).

Fish embryo toxicity test

The fish embryo toxicity (FET) test represents an alternative approach to traditional acute toxicity testing with live fish (Lammer et al., 2009); since the year 2005, it is compulsory when conducting whole effluent testing in Germany (DIN, 2001). In addition to several technical benefits, the FET test allows the implementation of sublethal effects making it more compatible with current animal welfare legislation (Lammer et al., 2009). Teratogenic effects investigated following sub-lethal exposure involve among others abnormalities such as malformations of head, tail, notochord and yolk as well as retarded growth (Nagel, 2002).

Dye uptake assays for determining efflux activity

Reduced or inhibited efflux of an ABC transporter substrate by a test compound, a so-called chemosensitizer, will result in an increased intracellular accumulation of the test compound (Peer and Margalit, 2006). By using fluorescently labelled substrates, any negative impact on the efflux activity of the transporters may be quantitatively determined by means of fluorescence microscopy (Hamdoun et al. 2004). Rhodamine B (RhB) and calcein acetoxymethyl ester (calcein-AM) are both fluorescent substrates of specific ABC transport proteins (Smital and Kurelec, 1997; Essodaigui et al., 1998) and have frequently been used to study MXR in aquatic organisms (e.g., Hamdoun et al., 2004; Stevenson et al., 2006; Faria et al., 2011; Fischer et al., 2011; Della Torre et al., 2012). To confirm the presence of transport activity in a test specimen, a known MXR-reversing compound must be run in parallel. To date, numerous agents such as vincaalkoloids, cyclosporins and calcium channel antagonists are known for their potential to reverse MXR (Kurelec, 1995). Transporter activity assays with fluorescently labelled substrates offer a fast and fairly simple technique for detection of chemosensitizers and are favourable as they can be used in whole organisms as well as in isolated cells and tissues (Kurelec et al., 2000).

Multi-generation study and relevant endpoints in fish

Multi-generation approaches allow monitoring of reproductive success which is considered one of the most ecologically relevant endpoints in fish life-cycle studies (Arcand-Hoy and Benson, 1998). Furthermore, endocrine-disruptive effects may be induced during early development; however, subsequent expression of related effects may lag until adulthood or even offspring generations (Kavlock et al., 1996; Allen et al., 1999). Thus, a chronic exposure

design over generations represents a promising approach to gain population-relevant information as to the toxic potency of a chemical or chemical mixture.

Over the years, an increasing number of environmental pollutants have been identified as endocrine disruptive chemicals (EDCs) towards a wide range of organisms (e.g., Jensen and Leffers, 2008; Mann et al., 2009; Flint et al., 2012; Soffker and Tyler, 2012). For the detection of EDCs in fish, altered levels of vitellogenin (Vtg) constitutes an acknowledged biomarker (Tyler et al., 1999) and is moreover one of the main endpoints in the newly adopted OECD test guidelines no. 229, 230 and 234 (OECD, 2009a, b, 2011). The yolk protein Vtg is under the influence of estradiol synthesized by the liver for subsequent incorporation into the developing oocyte (Fig. 3.; Kime, 1998). Vtg is produced by female fish and is under normal circumstances absent in males. However, as the necessary receptors for estradiol are present in the livers of male fish, Vtg may under xenoestrogenic influence be detectable in males as well (Kime, 1998).

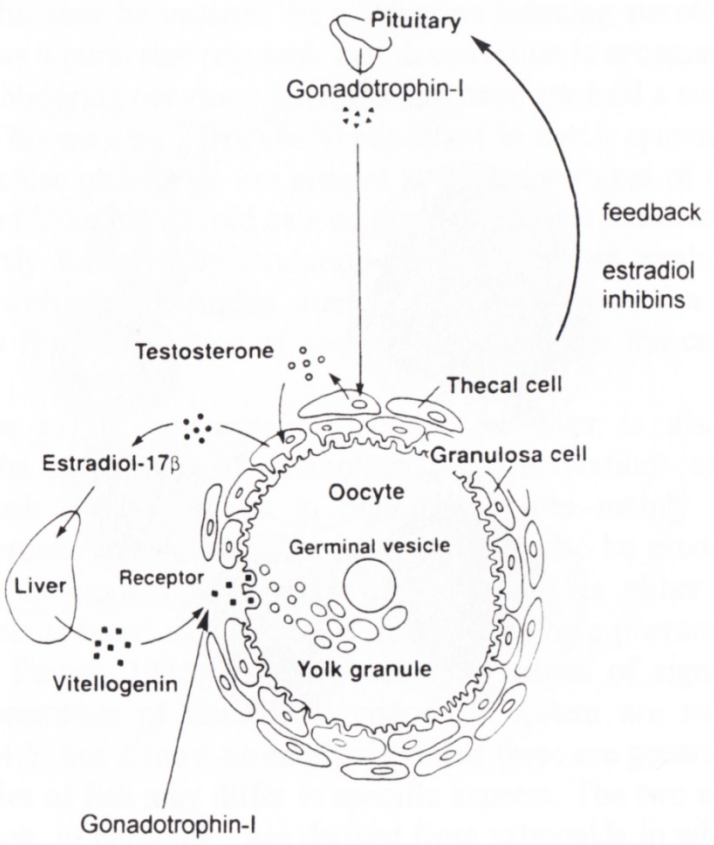


Fig. 3. Regulation of vitellogenesis in fish. Illustration from Kime (1998).

Within aquatic toxicity testing, histopathology has been acknowledged as a tool of high diagnostic power providing superior information regarding target organs of toxicity and

mechanisms of action (Wester et al., 2002). As histopathological alterations are known to occur as a result of toxic effects at lower levels of biological organization (Chavin, 1973; Vogt, 1987), histological monitoring in fish is associated with a higher sensitivity compared with toxicological endpoints such as growth, survival and reproduction (Segner and Braunbeck, 1988; Wester et al., 2002).

1.6 Aims of the present study

The overall aim of this PhD thesis was to evaluate the chemosensitizing potential and long-term effects of selected PFCs with focus on PFOS. For these purposes, the zebrafish (*Danio rerio*) was used as model organism.

The specific aims were:

- to investigate the P-gp inhibiting potential of selected PFCs;
- to evaluate any chronic effects of PFOS over generations with focus on survival, growth, endocrine disruption, reproduction and histological alterations;
- to elucidate possible joint effects of PFOS and BPA following long-term exposure

2. Materials and methods

2.1 Chemicals

The chemicals used in the experiments described in this thesis are shown in Table 1. In the micronucleus assay, PFOS and cyclophosphamide monohydrate (CPP) were prepared in DMSO with a maximum solvent concentration of 1 %.

In the transporter activity assays, the PFCs, vinblastine sulfate salt and vanadate were prepared in double-distilled water and in the case of PFOS, also in DMSO. Prior to use, vanadate was prepared by pH 10 adjustment and boiling cycle until clear at pH 10. Stock solutions of calcein acetoxymethyl ester (calcein-AM), cyclosporine A (CsA), MK571 sodium salt and verapamil were prepared in DMSO. DMSO concentration in the final test solutions never exceeded 0.5 %. Stock solutions of rhodamine B (RhB) were always freshly prepared in double-distilled water and kept dark. For the transporter activity assay, a hypotonic lysis buffer (10 mM KCL, 1.5 mM $MgCl_2$, 10 mM Tris HCL) was prepared in double-distilled water and adjusted to pH 7.4.

In the long-term test with zebrafish, PFOS and bisphenol A (BPA) were both delivered to the test vessels without use of a carrier solvent. A first stock solution of PFOS (300 mg/L) was prepared by dissolving 1.5 g PFOS in 5 L of deionized water with over-night magnetic stirring. The solution after the first dilution step, hereafter named second stock solution (0.016, 2.6 and 7.8 mg/L), was prepared by diluting the first stock solution (300 mg/L) with deionized water. Nominal concentrations of PFOS in the test vessels were 0.6, 100 and 300 µg/L. Stock solutions of BPA (500 mg/L) were freshly prepared twice a week by adding 500 mg in 1 L of dechlorinated water. Complete solubilisation was achieved by alkalinization and rigorous over-night stirring as previously described by Pickford et al. (2003). The pH of the first BPA stock solution ranged from 10.97 to 11.55 throughout the test. The second stock solution of BPA (0.26, 5.2 and 10.4 mg/L) were prepared by dilution of the first stock solution (500 mg/L) using deionized water. Nominal concentrations of BPA in the test vessels were 10.0, 200 and 400 µg/L. For the co-exposure treatments, all tested BPA concentrations (10, 200 and 400 µg/L) were combined with the lowest and the highest PFOS concentrations (0.6 and 300 µg/L), respectively.

Table 1. Trade name, abbreviation, CAS number and supplier of investigated chemicals.

Chemical	Abbreviation	Chemical formula	CAS no.	Supplier
Bisphenol A	BPA	$C_{15}H_{16}O_2$	80-05-7	Sigma-Aldrich, Taufkirchen, Germany
Calcein acetoxymethyl ester	Calcein-AM	$C_{46}H_{46}N_2O_{23}$	148504-34-1	Biozol, Eiching, Germany
Cyclophosphamide monohydrate	CPP	$C_7H_{15}Cl_2N_2O_2P{\cdot}H_2O$	6055-19-2	Sigma-Aldrich, Schnelldorf, Germany
Cyclosporin A	CsA	$C_{62}H_{111}N_{11}O_{12}$	59865-13-3	Enzo Life Sciences, Lörrach, Germany
Heptadecafluorooctane-sulfonic acid potassium salt	PFOS	$C_8HF_{17}O_3S$	2795-39-3	Sigma-Aldrich, Schnelldorf, Germany
MK571 sodium salt	MK571	$C_{26}H_{26}ClN_2NaO_3S_2 \cdot xH_2O$	115104-28-4	Enzo Life Sciences, Lörrach, Germany
Pentadecafluorooctanoic acid	PFOA	$C_8HF_{15}O_2$	335-67-1	Sigma-Aldrich, Schnelldorf, Germany
Perfluorodecanoic acid	PFDA	$C_{10}HF_{19}O_2$	335-76-2	Sigma-Aldrich, Schnelldorf, Germany
Perfluorononanoic acid	PFNA	$C_9HF_{17}O_2$	375-95-1	Sigma-Aldrich, Schnelldorf, Germany
Potassium nonafluoro-1-butanesulfonate	PFBS	$C_4HF_9O_3S$	29420-49-3	Sigma-Aldrich, Schnelldorf, Germany
Rhodamine B	RhB	$C_{28}H_{31}ClN_2O_3$	81-88-9	Sigma-Aldrich, Schnelldorf, Germany
Sodium orthovanadate	Vanadate	Na_3O_4V	13721-39-6	Enzo Life Sciences, Lörrach, Germany
Verapamil	Verapamil	$C_{27}H_{38}N_2O_4$	52-53-9	Sigma-Aldrich, Schnelldorf, Germany
Vinblastine sulfate salt	Vinblastine	$C_{46}H_{58}N_4O_9 \cdot H_2SO_4$	143-67-9	Sigma-Aldrich, Schnelldorf, Germany

2.2 Test organisms and maintenance

Cell lines

The V79 cell line applied in the micronucleus assay is a mammalian cell line derived from cultured embryonic lung fibroblasts of the Chinese hamster (*Cricetulus griseus*). The cells were cultured in Minimum Essential Medium (MEM) supplemented with 10 % foetal calf serum and antibiotics. The cultivation was obtained in 25 cm^2 culture flasks under sterile conditions in a humidified incubator (37 ºC, 5.0 % CO_2 in air).

Madin-Darby canine kidney (MDCKII) cells overexpressing human P-gp (MDCKII P-gp [=ABCB1]) along with non-transfected MDCKII cells were used in the calcein assay. The

MDCKII cell lines were cultured at the Department of Bioanalytical Ecotoxicology, UFZ-Helmholtz Centre for Environmental Research, and were a kind gift from Dr. Piet Borst from the Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital. MDCKII-derived cell lines were cultured under sterile conditions in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum.

Zebrafish

In the transporter activity assay as well as in the long-term study, eggs from a brood stock consisting of mature non-exposed zebrafish (*Danio rerio*; Westaquarium strain) were used. The brood stock was reared in the laboratory of the Aquatic Ecology and Toxicology section, Heidelberg University and maintained in a light-isolated room with an artificial 14/10 h light/dark period. Adult fish were fed twice daily with freshly hatched *Artemia* nauplii (Aquafauna Bio-Marine Inc., Hawthorne, CA) complemented with TetraMin flake food (Tetra, Germany). Larvae were initially fed twice daily with liquid starter food (Hobby liquizell, Gelsdorf, Germany) followed by Sera micron powder food (Sera, Heinsberg, Germany) and freshly hatched *Artemia* nauplii. Tap water and deionized water were mixed until a conductivity of 600 – 750 µS, hardness (276 ± 17.8) and pH (8.0 – 8.2) were stably balanced. The water mix was supplied from an aerated reservoir and used for culture of all embryos and fish. The final test water was routinely characterized for pH (8.25 - 8.75), total hardness (167 - 356 mg/L), ammonium (< 5 mg/L) and nitrite (< 1 mg/L). Temperature (26.0 ± 1.0 °C) and dissolved oxygen (6.45 - 10.97 mg/L) was checked weekly. In order to ensure high water quality, food remains and debris were removed daily and vessel surfaces were gently scraped once a week with the exception of sensitive periods during early larval development.

2.3 Micronucleus assay

The micronucleus assay with the V79 cell line served as a first screening of the toxicity increasing potential of PFOS (23.2 µM) in the presence of the genotoxicant CPP (4.79 and 9.58 µM). The micronucleus assay was performed according to the ISO Draft International Standard (ISO/DIS 21427-2). V79 cells were washed with Hank´s Balanced Salt Solution (HBSS) and seeded at a density of 5.0 x 10^4 cells/ml onto ethanol-cleaned slides in culture dishes and incubated at 37 ºC for 6 h allowing attachment of the cells. MEM with and without

DMSO (1 %) served as solvent and negative controls, respectively. Five ml of each test solution and controls were added to the culture chambers followed by incubation at 37 ºC for 4 h. After 4 h, the medium of the S9-treated cultures was removed and the cultures were washed twice with HBSS followed by addition of 5 ml MEM before continued incubation. After a total incubation of 24 h, the culture medium of each plate was removed completely and each culture was treated with 5 ml pre-heated (37 ºC) solution of sodium citrate (Merck; 1.5 % in double distilled water) for 3 min. After removal of the sodium citrate solution, cells were fixed for 10 min with 5 ml 4 ºC glacial acetic acid/ethanol solution (1/3) with 1 % formaldehyde (37 %). After the first fixation, the solution was removed and replaced by an equal amount of fresh fixative for additional 10 min. The slides were removed from the culture chambers and allowed to dry before the staining procedure. After 3 min in May-Grünwald-solution (Sigma-Aldrich, Schnelldorf, Germany) the slides were washed in WEISE-buffer (Merck) in order to remove excess staining. In the final staining step, slides were submerged in 2.6 % Giemsa (Gurr, Poole, UK) in WEISE-buffer. After 20 min incubation, slides were washed twice in WEISE-buffer. After dryness, each slide was mounted with DePeX (Serva, Heidelberg, Germany) and covered with a coverslip (24 x 70 mm; Langenbrink). The documentation of micronuclei was performed with a CKX41 inverted microscope (Olympus, Hamburg, Germany) equipped with a digital Olympus C5060 camera and the digitizing software Analysis 5.0 (Soft Imaging Systems, Olympus). For the evaluation of micronuclei, the following scoring criteria were used: (a) cells with oval appearance and intact cytoplasm, (b) oval nuclei with intact nuclear membrane, (c) micronuclei less than or equal to one-third the size of the main nuclei, (d) micronuclei clearly separated from the main nuclei (Huber et al., 1983; Titenko-Holland et al., 1998). Each treatment was performed in duplicates and per treatment replicate, a total of 1000 cells were scored. The test was repeated twice on separate days. PFOS recovery was determined using LC-MS/MS (Skutlarek et al., 2006).

2.4 Transporter activity assays

Fish embryo toxicity test

Prior to the transporter activity experiments, the embryotoxic potential of the PFCs was determined in a 48 h range-finding FET test according to DIN 38415-T6 and ISO 15088 (DIN, 2001; ISO, 2007). Artificial water as specified in ISO 7346-1 (ISO, 1996; 294.0 mg/L

$CaCl_2 \times 2\ H_2O$; 123.3 mg/L $MgSO_4 \times 7\ H_2O$; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl) was prepared and aerated to oxygen saturation prior to use. At the day of the experiment, viable eggs at cell stage 8-32 were carefully selected with a plastic Pasteur pipette and transferred to 24-well plates (Renner, Dannstadt, Germany) containing 2 ml test solution per well (10 eggs per treatment). Artificial water served as negative control. In order to prevent interaction between adjacent wells, the plates were covered with self-adhesive film (Nunc, Wiesbaden, Germany) before incubation at 26.0 ± 1.0 °C. The embryos were inspected 24, 48, 72 and 96 h after the onset of exposure using an inverted microscope (CK-2; Olympus, Hamburg, FRG) equipped with a digital camera (Canon EOS D60; Canon, Grießen, FRG). Normal development along with lethal and sub-lethal effects were documented for each individual egg (Table 2). The FET test was considered valid if the negative control did not reveal more than 10 % mortality.

Table 2. Documented endpoints of lethal (+) and sublethal (o) toxicity in zebrafish embryos.

	Exposure time			
	24 h	**48 h**	**72 h**	**96 h**
Coagulation	+	+	+	+
Lack of heartbeat		+	+	+
Lack of somite formation	+	+	+	+
Lack of blood circulation		+	+	+
Deformation	o	o	o	o
Edema	o	o	o	o
Disturbed pigmentation		o	o	o

The standard FET was also conducted to evaluate the lethal toxicity of the vinca alkaloid and P-gp substrate vinblastine in the absence or presence of PFOS. Freshly spawned embryos were incubated in artificial water with either vinblastine (0.5-3 µM), PFOS (21 µM) or with different binary mixtures of the two compounds. Artificial water alone served as a negative control. After 48 h of incubation, lethal effects, as depicted by coagulation or lack of heart beat, were documented among 10 embryos per treatment group. Each experiment was repeated four times on separate days with embryos of different batches.

Rhodamine B accumulation assay

Accumulation of the fluorescent P-gp substrate rhodamine B (RhB) in the absence or presence of PFBS, PFOS, PFOA, PFNA or standard transporter inhibitors was used to measure the transporter activity in zebrafish embryos. As an inhibited transporter activity will

lead to an increased intracellular RhB accumulation, measurements of the fluorescence signal will provide information regarding the chemosensitizing potential of the investigated compounds.

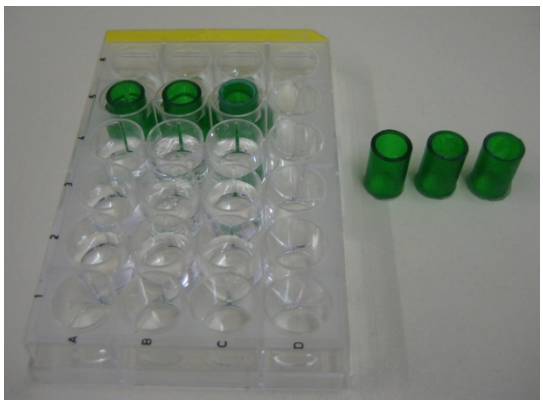


Fig. 4. Nylon mesh baskets.

To measure dye accumulation, embryos at 24 hpf were incubated in artificial water with RhB (0.5 or 1µM) in the absence or presence of PFOS (10-100 µM), PFBS, PFOA, PFNA (35 and 50 µM) or the inhibitor compounds cyclosporine A (CsA; 30 µM), MK571 sodium salt (MK571; 50 µM) or sodium orthovanadate (vanadate; 500 µM). Artificial water with RhB (0.5 or 1µM) in the absence or presence of DMSO (0.05 %) served as water and solvent controls, respectively. Each treatment was performed in pseudo-triplicates with 5 eggs (n = 15). Self-made nylon mesh baskets (3 per treatment; mesh size 100 µm; inner core diameter 10 mm; Fig. 4) were placed in 24-well plates, where they served as mobile incubation chambers throughout the duration of the test (based on the original idea by Fischer, 2007).

At the day of the test, all plates were refilled with freshly prepared test solutions. Viable eggs were selected under a stereo microscope and immediately transferred to the mesh baskets containing 1 mL artificial water. The mesh baskets, each containing 5 eggs, were relocated to the neighboring wells containing 2 mL of freshly prepared test solutions with RhB and incubated in darkness for 1 h at 26.0 ± 1.0 °C. After incubation, excess dye from the surface of the eggs was rinsed off *via* a three-step washing series with artificial water. Immediately after washing, the eggs were gently transferred to 1.5 mL microcentrifuge tubes, where excess water was carefully pipetted out. After addition of 200 µL hypotonic lysis buffer, the contents of each tube were sonicated (2 x 10 sec) and briefly centrifuged (~ 10 sec). Of the supernatant, 150 µL were transferred to a black 96-well microplate (Nunclon, Nunc, Wiesbaden; Germany), where the RhB fluorescence was measured at 595 nm (emission) / 530 nm (excitation) at optimal gain. Each experiment was repeated at a minimum of three times on separate days with embryos of different batches. Dye accumulation in the treatments was quantified as fold increase over the water control.

To assess detergent-like effects of PFOS and its consequences for RhB uptake/efflux, 30 zebrafish embryos at 24 hpf were incubated in artificial water with RhB (0.5 µM) and PFOS

(20 - 100 µM). After 1 h of incubation, all embryos were washed as described above. After washing, RhB fluorescence was immediately measured in10 embryos, whereas the remaining embryos were directly transferred to clean artificial water with or without PFOS (20-100 µM) and further incubated for 1 h. After 1 h post-incubation, RhB fluorescence was measured in the remaining embryos. Each treatment was performed in pseudo-duplicates with 5 eggs (n = 10). Artificial water with 0.5 µM RhB in the absence or presence of DMSO (0.05 %) served as water and solvent controls, respectively. The experiment was repeated twice on separate days with embryos of different batches. Dye accumulation in the treatments was quantified as fold increase over the water control.

In order to investigate the auto-fluorescence of PFOS and its potential influence on the fluorescence intensity of RhB, fluorescence of artificial water with PFOS (100 and 1000 µM) was measured at gain 95 both in the absence and presence of RhB (1 µM; no embryos included).

Calcein-AM assay

The calcein-AM assay with PFOS, PFNA and PFDA was performed by Peggy Wellner at the Department of Bioanalytical Ecotoxicology, UFZ-Helmholtz Centre for Environmental Research in Leipzig.

Two hundred µl of MDCKII cells suspended in DMEM with 5% FBS were seeded in each well of 48 multi-well plates and cells were left to attach over-night. Before exposure, cells were washed with 200 µl of PBS (with Mg^{2+} and Ca^{2+}). Two-hundred µl of ice-cold, serum-free DMEM with 0.5 µM calcein-AM and test compounds were added to each well and incubated at 37 °C in a humidified atmosphere with 5 % (v/v) CO_2 for 30 min, then washed with 200 µl of PBS and lysed in 200 µl of 0.2 % Triton X-100 in PBS. Fluorescence was measured at 485 nm/530 nm (excitation/emission) with a Tecan Genios microplate reader (Tecan, Männedorf, Switzerland). Non-transfected MDCKII cells and the reference P-gp inhibitor verapamil were used as negative and positive controls, respectively. Each control and PFC concentration was set up as duplicates on the plate, and the assay was performed on three different days. The DMSO concentration in all treatments during calcein-AM exposures was 0.5 %, which was not found to affect transporter activity in the cells.

2.5 Long-term study with the zebrafish

Experimental design

During the course of the study, fish were continuously exposed to PFOS and BPA, either singly or in combination. Each treatment group was replicated twice holding a starting number of 80 fish per replicate (160 individuals per treatment). At 2 - 4 h post-fertilization (hpf), eggs were transferred to glass dishes (12 cm diameter, 8 cm high) and exposed to the different treatments at 26 ± 1 °C under semi-static conditions (complete renewal of solutions after 24 h) until 48 hpf, when they were transferred to respective test vessel. Whole-glass tanks (18x40x40 cm) adjusted for a 10 L working volume were utilized as test vessels (Fig. 5). The test vessels were placed on top of serial-connected heating mattresses ensuring constant heating conditions. A flow-through system with a three-fold water exchange per day was applied throughout the study in order to provide adequate supply of fresh test solution. External aeration by pressurized air was installed for each test vessel. Test solutions were daily refilled into light-isolated 10 L glass bottles located above the test vessels. Each test solution was constantly held in solution by magnetic stirring. Peristaltic pumps (M312; Gilson, Villiers-le-Bel, France) were used for a continuous delivery of test solution (50 ml/h) from each glass bottle to paired test vessels serving as replicates A and B for each treatment group. For each test vessel, a water flow rate of 1.25 L/ h was adjusted by means of rotameters (Rota Yokogawa, Wehr, Germany). Flow rates of water supply and test solutions were controlled daily. Outflow water passed through active carbon filters (Prantner, Reutlingen, Germany) before release to the sewer.

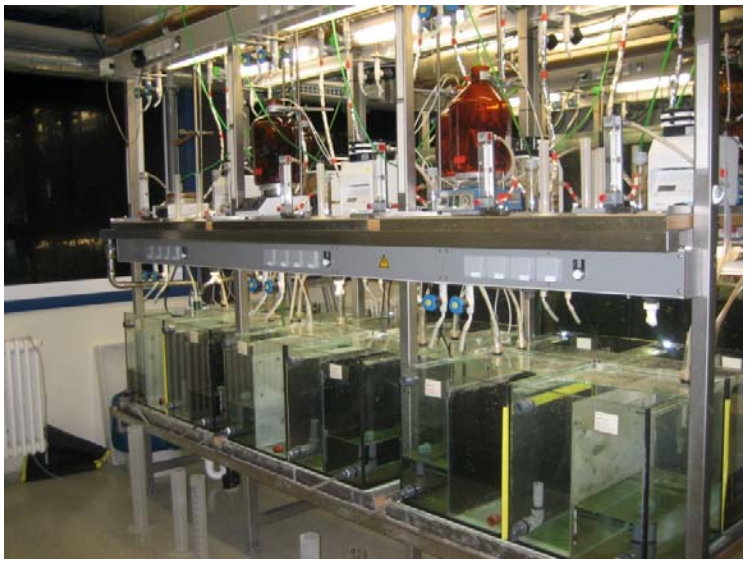


Fig. 5. Experimental facility used for the long-term experiment with zebrafish.

Each replicate of the F1 and F2 generations was sub-sampled at 30 and 90 d post-fertilization (dpf). At 30 dpf, the number of fish in each replicate test vessel was reduced by 35 individuals for measurements of length and weight. Sampled fish were anaesthetized and euthanized in a saturated solution of 4-ethylaminobenzoate (benzocain). At 90 dpf, each replicate was further reduced by 25 individuals for measurement of length, weight and vitellogenin (Vtg) as well as for histological evaluation of liver, thyroid and gonads. After sacrifice, length and weight were documented, and fish were transversely trimmed behind the anal fin separating the tail for Vtg measurements and the rest of the body for histology (Fig. 6). Tails were instantly frozen in liquid nitrogen, and the rest of the body was placed in histology cassettes and submerged in cooled Davidson´s fixative (Romeis, 1989) for a minimum of 24 h before histological processing. For each replicate test vessel, a total of 10 males and 10 females were retained for reproduction experiments and breeding of the F2 and F3 generations. After termination of the breeding experiments (approximately at 180 dpf), remaining adults were sampled following the exact procedure as described above for sub-sampling at 90 dpf. Post-hatching survival was documented for the F3 generation at 14 dpf, when the experiment was terminated without subsequent sampling.

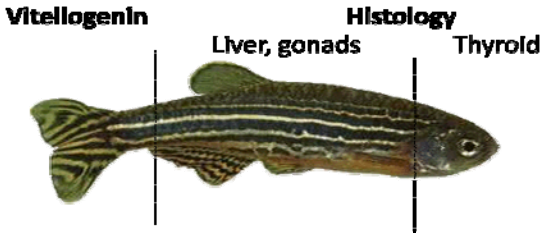


Fig. 6. Illustration of the body parts sampled for vitellogenin measurements and histological analyzes in the zebrafish (*Danio rerio*).

Vitellogenin

Deep frozen tails of sampled zebrafish males and 10 x the tissue wet weight of ice-cold homogenate buffer (50 mM Tris-HCL, pH 7.4, 1 % protease inhibitor cocktail) were added to 2 ml Eppendorf tubes containing one stainless steel bead each (5 mm diameter; Qiagen, Hilden, Germany). The tissue homogenization was performed with a tissue lyser II (30 s, 15 Hz, Qiagen). The homogenate was centrifuged for 30 min at 24, 650 g at 4 °C and the supernatant for Vtg measurements was collected and stored in aliquots of 50 µl at -20 °C until further analysis. Concentrations of Vtg were measured by a method based on a direct non-

competitive sandwich ELISA previously described by Holbech et al. (2001) with the following modifications as described by Morthorst et al. (2010): Dextran-HRP conjugated antibodies were replaced by a two-step process in order to enhance sensitivity of the assay. First, 150 µL of biotin-conjugated antibody were added to each well, and the plate was incubated on a horizontal shaker (100 vibrations / min) for 1 h at room temperature. After rinsing five times with washing buffer (PBS, 0.1 % Tween-20, 0.1 % BSA) 150 µL of streptavidin HRP-conjugated antibody was added to each well, and the plate was incubated on a horizontal shaker for 1 h at room temperature.

Histology

For histological examination of liver, thyroid and gonads, tissues (central body portions without tail) fixed in Davidson´s fixative were processed in a Leica TP 1020 Tissue Processor (Leica Microsystems, Wetzlar, Germany) and embedded in Histoplast S (Serva, Heidelberg, Germany). Four micron sections were mounted on glass slides, stained with periodic acid-Schiff (PAS) staining (Romeis, 1989) and examined using a light microscope (Leitz Aristoplan). For further details on embedding and staining procedures, see Schmidt and Braunbeck (2011). Gonadal staging and severity grading was semi-quantitatively assessed according to the criteria outlined by Braunbeck et al. (2010). Briefly, testes and ovaries were staged based on the abundance of specific gametogenic cell types by use of a staging system ranging from 0 (undeveloped) to 4 (spent) or 5 (post-ovalutory) for males and females, respectively. Severity grading was based on the degree to which a histomorphological change was present in a tissue section and was employed according to a system ranging from grade 1 (minimal) to grade 4 (severe). In order to allow a comparison between treatments and generations regarding severity and maturation, the maturity index developed and described by Baumann (2008) was adopted. Briefly, each stage of maturity was accorded to a fixed value which increases with increasing maturity of the fish (maturity stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The values of each replicate aquarium were summed up, divided by the number of female and male fish, respectively and the mean value of each treatment group was calculated. The same principle was applied for the severity index. Sections investigated for the presence of *Mycobacterium* spp. were stained according to the Ziehl-Neelsen technique.

Breeding

Breeding experiments for evaluation of fecundity and fertilization rate were performed with F1 and F2 adults starting at approximately 4 months of age. Breeding trials for each treatment group were repeated six to seven times (F1) and nine to ten times (F2) with a minimum of one week of recovery in between to avoid stress related bias. At the day before spawning, five individuals of either sex from each test vessel were randomly selected and transferred to breeding tanks prior to the onset of darkness. The spawning facility was constructed of six breeding tanks (15x16x25 cm) which were held together under un-exposed semi-static conditions with constant air supply (7.37 - 8.10 mg/L) and heating (25.0 ± 1.0 °C). Each breeding tank was equipped with green nylon netting serving as a breeding stimulant. The bottom of the breeding tanks was covered by a stainless steel grid (mesh size 1.25 mm) to allow the eggs to pass through into separate spawning trays and thus to avoid cannibalism by parental fish. About 20 - 30 minutes after the onset of light, spawning trays were removed and the eggs were collected and any further debris was removed. Eggs were counted and visually inspected under a stereo microscope and transferred to petri dishes (18 cm diameter) containing freshly prepared artificial water according to ISO (1996; maximum 100 eggs/200 ml water). The eggs were incubated at 26.0 ± 1.0 ºC over night, after which coagulated and fertilized eggs were counted.

2.6 Chemical analysis

In the micronucleus test and long-term experiment, samples for chemical analysis were collected in order to assess deviations between nominal and true exposure concentrations. PFOS concentrations were determined using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS; Skutlarek et al., 2006).

Prior to the micronucleus assay, test solutions containing 23.2 µM PFOS (nominal concentration) were incubated in empty well plates at 26.0 ± 1.0 °C aiming to investigate potential substance loss *via* plastic adsorption. Samples for chemical analysis were collected at 0 and 24 h.

Throughout the long-term test, water samples for chemical analysis of PFOS concentrations were collected directly from the test vessels on a monthly basis and stored at minus 80 °C prior to chemical analysis. BPA concentrations were not analytically verified. In

a previous study, no degradation of BPA was detected in natural waters within 1 day (Dorn et al., 1987). As the flow-through system in the present study was maintained to provide three tank volume changes per day, the rate of fresh test solution was considered enough to compensate for any bacterial degradation occurring in the test vessels.

2.7 Statistical methods

In order to test differences in the frequency of micronucleated cells, the non-parametric Kruskal-Wallis ANOVA on-ranks test was applied followed by multiple comparisons *versus* the control group (Dunn´s method). When no normality or equal variance was found, this test was also used to detect differences in RhB fluorescence accumulation as well as in total body length and weight, survival and Vtg concentration. In case assumptions of normality and equal variances held true, differences between data sets were evaluated with one-way ANOVA followed by the post-hoc Holm-Sidak method. To compare groups (in a set less than three), the Student´s t-test or the Mann-Whitney rank-sum test (when no normality or equal variance) were applied. To allow comparison of fluorescence data between different measurements, raw values of each plate were normalized to the fluorescence intensity of the water controls of the respective plate and expressed as fold increase over control.

A level of p less than 0.05 was considered statistically significant with the exception of the long-term test where differences were considered significant at three different levels (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$) relative to controls or to the single exposure treatments. Analyses were performed with SigmaStat® Statistical Software version 3.5 (Systat-Jandel Scientific, Erkrath, Germany).

3. Results

3.1 Chemosensitized micronucleus induction by PFOS *in vitro*

PFOS alone did not induce any increase in the frequency of micronucleated V79 cells relative to the control (Fig. 7). Co-exposure of cells to PFOS and cyclophosphamide monohydrate (CPP; 4.79 and 9.58 µM) caused a significant increase in micronucleated cells. Binary mixtures with 23.2 µM PFOS showed a clear increase in micronucleated cells compared with the same concentrations of CPP and PFOS alone. Furthermore, addition of 23.2 µM PFOS to 4.79 µM CPP caused a higher frequency of micronucleated cells than exhibited by 9.58 µM CPP alone.

Chemical analyses indicated a good recovery of PFOS (100- 110 %), as measured prior to and after incubation, thus confirming a negligible loss of substance during the incubation period (data not shown).

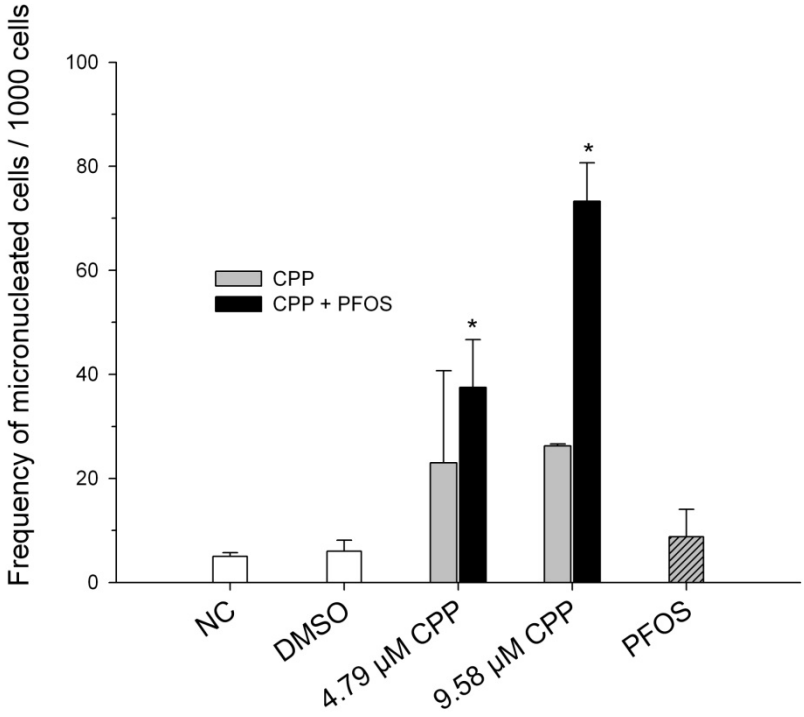


Fig. 7. Frequency of micronucleated V79 cells following exposure to PFOS (23.2 µM), CPP (4.79 and 9.58 µM) or to a binary mixture of the two compounds. All treatments are with metabolic activation. Minimum Essential Medium in the absence and presence of DMSO (1 %) served as a negative and solvent control, respectively. Data are means ± SD of two independent experiments with 2 replicates each. For each replicate, 1000 cells were assessed. Asterisks indicate significant genotoxicity compared to negative controls ($p < 0.05$) following Kruskal-Wallis ANOVA on-ranks and Dunn´s multiple comparison test.

3.2 PFC-modulated uptake of P-gp substrates in zebrafish

Lethal toxicity of vinblastine and PFOS

Mortality of zebrafish embryos at 48 hpf exposed to the P-gp substrate vinblastine in the absence or presence of PFOS (21 µM) is shown in Fig. 8. Despite little mortality in the 21 µM PFOS exposure group (2.5 ± 5 %), the presence of PFOS significantly increased the toxicity of vinblastine (1-2.5 µM) causing a two- to five-fold increase in mortality, if compared with the single exposures of vinblastine. No mortality was observed in the negative control group throughout the 48 h exposure period.

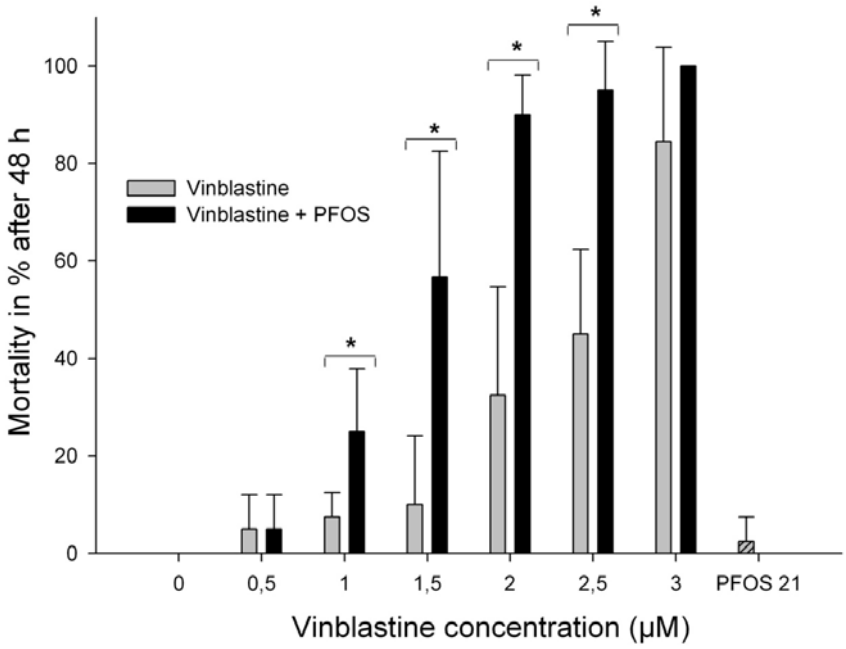


Fig. 8. Relative mortality among zebrafish (*Danio rerio*) embryos (48 hpf) after 48 h exposure to vinblastine alone and in combination with PFOS (21 µM). Asterisks indicate significant mortality in binary mixtures compared to single exposures of vinblastine ($p < 0.05$) following Student´s t-test. Data are given as average ± S.D. of four experiments (10 eggs per treatment).

Rhodamine B accumulation and efflux following PFC-exposure

RhB accumulation (fluorescence) in the presence of PFOS, PFBS, PFOA or PFNA (35 and 50 µM) was investigated in zebrafish embryos at 24 hpf (Fig. 9). The highest RhB accumulation following 1 h of incubation was observed in embryos exposed to 35 and 50 µM PFOS showing an eight- to eleven-fold increase in RhB fluoresence compared with water controls.

Although without statistical significance, a two- and threefold RhB increase was observed in embryos exposed to 35 µM PFOA and 50 µM PFNA, respectively.

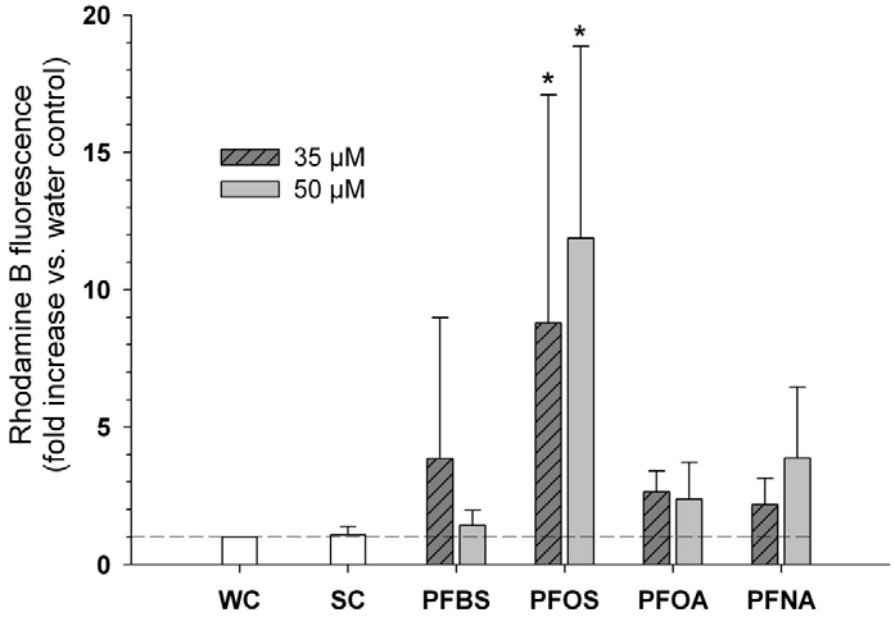


Fig. 9. Rhodamine B (RhB) accumulation in 24 hpf zebrafish (*Danio rerio*) embryos given as fold change in fluorescence compared to water control (0.5 µM RhB). Zebrafish embryos were incubated for 1 h in RhB (0.5 µM) in combination with selected PFCs (35 and 50 µM). Solvent control: 0.5 µM RhB in 0.05 % DMSO. Asterisks indicate significant fluorescence increase compared to water control ($p < 0.05$) following Kruskal-Wallis ANOVA on-ranks and Dunn´s multiple comparison test. Data are given as average ± S.D. of four experiments (15 eggs per treatment).

All reference inhibitors tested (CsA, vanadate and MK571), enhanced RhB accumulation in zebrafish embryos (increase by factors of 1.56 ± 0.69, 1.23 ± 0.13 and 2.26 ± 0.83, respectively; Fig. 10), however, significance was only found for vanadate. An extended concentration range of PFOS (10 – 100 µM) revealed a concentration-dependent fluorescence intensity up to 70 µM PFOS where after dye accumulation leveled off. Uptake of RhB was significantly increased in the presence of 70 to 100 µM PFOS demonstrating an up to fourteen-fold increase in RhB fluorescence compared with solvent controls (Fig. 10).

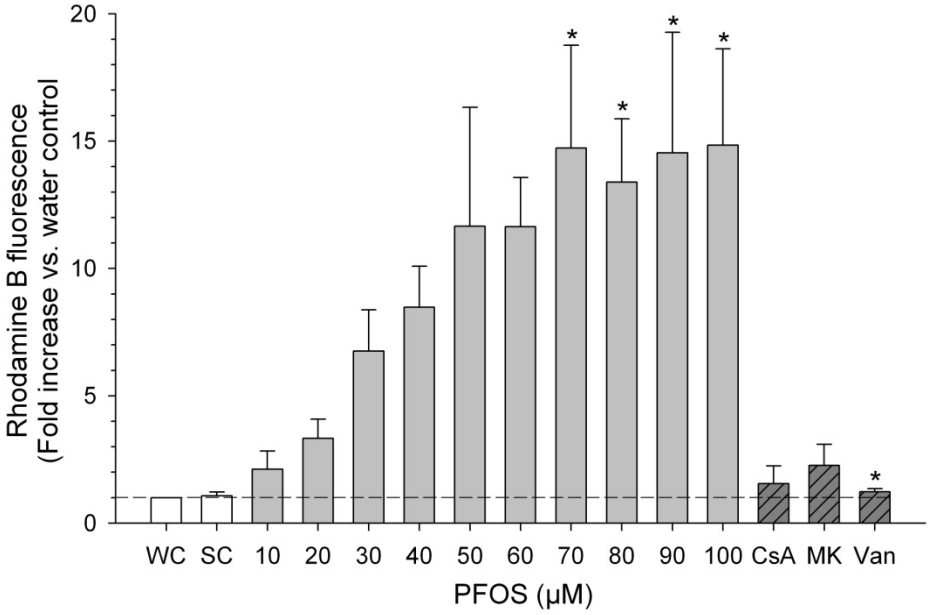


Fig. 10. Rhodamine B **(**RhB) accumulation in 24 hpf zebrafish (*Danio rerio*) embryos given as fold change in fluorescence compared to water control (1 µM RhB). Zebrafish embryos were incubated for 1 h in RhB (1 µM) in combination with PFOS (10 - 100 µM) or reference inhibitors CsA (30 µM), MK571 (50 µM) and vanadate (500 µM). Asterisks indicate significant fluorescence increase compared to solvent control (0.05 % DMSO; $p < 0.05$) following Kruskal-Wallis ANOVA on-ranks and Dunn´s multiple comparison test. Vanadate was statistically compared to water control ($p < 0.05$; Mann-Whitney Rank-Sum test). Data are given as average ± S.D. of four (CsA and MK571: three) experiments (15 eggs per treatment).

In order to investigate whether the detergent-like properties of PFOS could influence the RhB uptake/efflux in zebrafish embryos, RhB accumulation in embryos incubated in binary mixtures with RhB and PFOS was measured both prior to and after post-incubation in pure water with or without PFOS (Fig. 11). RhB accumulation following 1 h incubation, increased in a PFOS-dependent manner with a nine-fold higher RhB fluorescence in the highest 100 µM PFOS treatment compared with the water control group. After 1 h post-incubation in pure artificial water, all zebrafish embryos previously exposed to PFOS displayed reduced RhB fluorescence with an elimination rate ranging from 35 to 73 %. In contrast, individuals post-incubated in artificial water with PFOS did not show any sign of RhB elimination, but displayed a RhB content in the same order of magnitude as measured prior to the post-incubation.

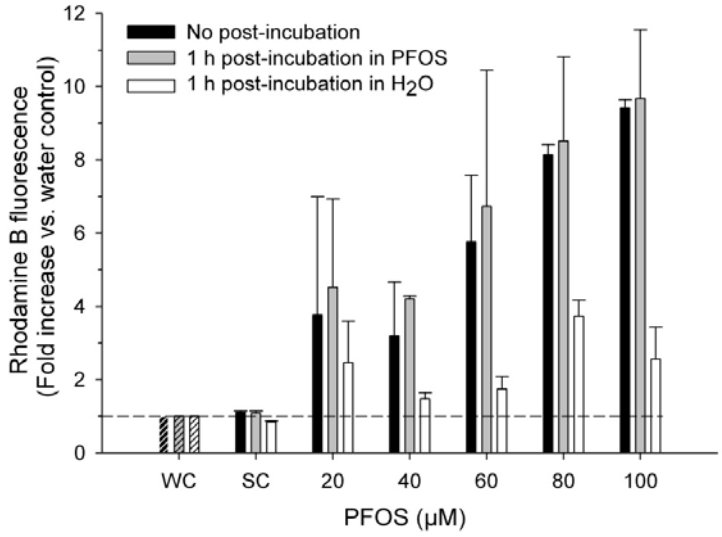


Fig. 11. Rhodamine B (RhB) accumulation in 24 hpf zebrafish (*Danio rerio*) embryos given as fold change in fluorescence compared to water control (0.5 µM RhB). The graph illustrates the modulation of RhB clearance by PFOS in zebrafish embryos after 1 h of incubation in RhB (0.5 µM) in combination with PFOS (20-100 µM; solid bars) followed by 1 h post-incubation in PFOS (20 - 100 µM) without RhB (grey bars) or in clean water (open bars). Solvent control: 0.5 µM RhB in 0.05 % DMSO. Data are given as average ± S.D. of two experiments (10 eggs per treatment).

In order to exclude the possibility of a PFOS-modified RhB fluorescence, the fluorescence of different test solutions containing the two compounds alone or in combination was measured (no embryos included). As illustrated in Fig. 12, results indicate no difference in the intensity of RhB fluorescence in the absence or presence of different PFOS concentrations (100 and 1000 µM).

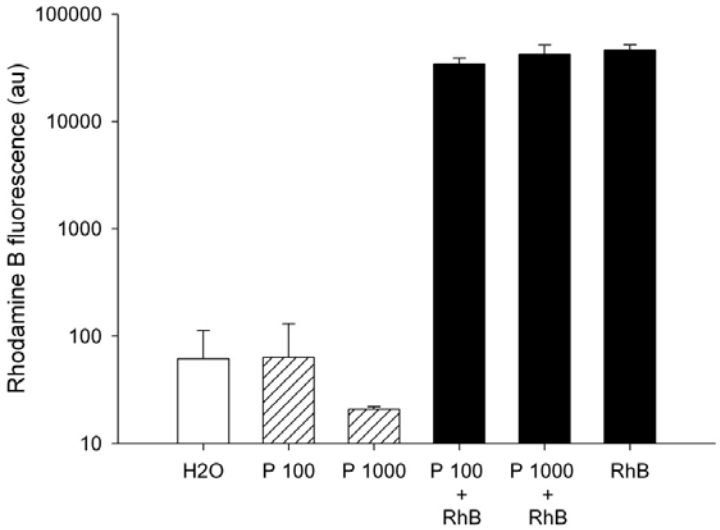
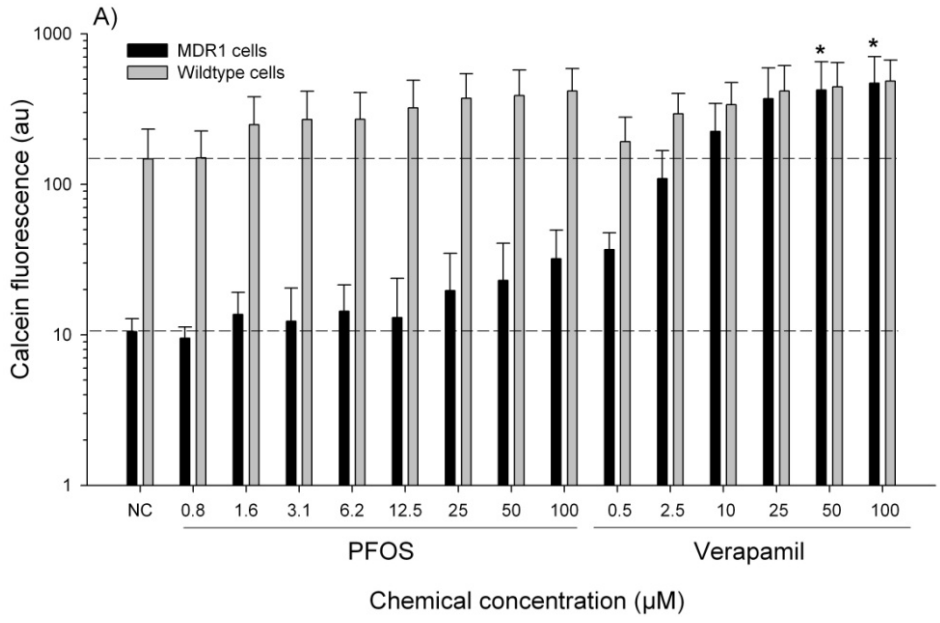
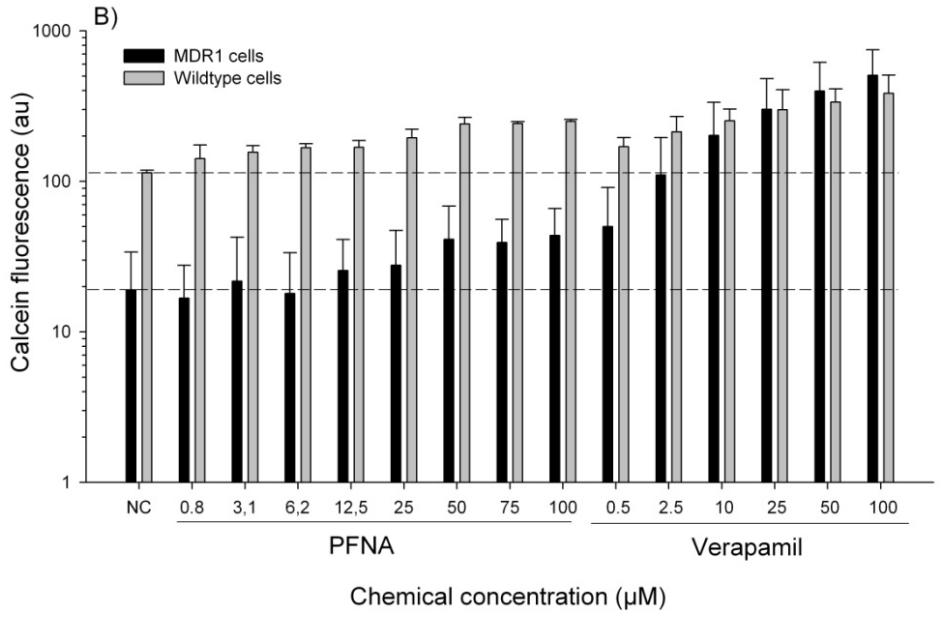


Fig. 12. Autofluorescence of PFOS (100 and 1000 µM) singly and in combination with 1µM rhodamine B (RhB). Results indicate no influence of the fluorescence intensity of RhB in the presence of PFOS. Data are given as average ± S.D. of two experiments.

PFC-induced inhibition of P-gp activity in MDCKII cells

Calcein accumulation in MDCKII cells overexpressing human P-gp (MDR1 cells) was not significantly affected by the tested concentrations of PFOS, PFNA or PFDA (0.8 - 100 µM, Figs. 13a-c). A significant calcein uptake of up to forty-fold was demonstrated in MDR1 cells exposed to the transporter inhibitor verapamil (50 and 100 µM; Fig. 13a). This shows that the investigated PFCs do not considerably affect efflux transporter activity of human P-gp.





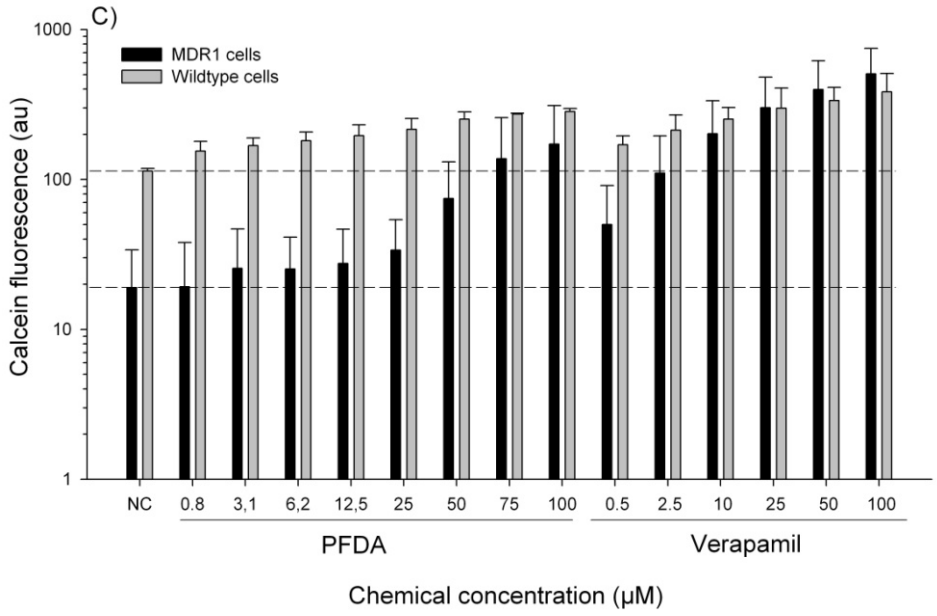


Fig. 13. Calcein accumulation in MDCKII cells overexpressing human P-gp (MDR1 cells) along with non-transfected MDCKII cells (wild-type cells) after exposure to PFOS (A), PFNA (B) or PFDA (C). Verapamil was used as reference P-gp inhibitor. Asterisks indicate significant fluorescence increase compared to control (0.05 % DMSO; $p < 0.05$) following Kruskal-Wallis ANOVA on-ranks and Dunn´s multiple comparison test. Data are given as average ± S.D. of two (PFOS: three) experiments.

Microscopy imaging of rhodamine B accumulation in zebrafish embryos

To qualitatively visualize the PFOS-induced RhB accumulation in zebrafish embryos, light-microscopy images were taken at 48 hpf following 1 h incubation with RhB (200 µM) alone and in combination with 0.05 % DMSO or PFOS (50 or 100 µM). In accordance with the obtained results from the transporter activity assay, the most intensive dye accumulation, (as indicated by the pink color of stained tissues) was recorded in the PFOS-exposed embryos (Fig. 14d, h, l and p). Removal of the chorion demonstrated that PFOS-exposure induced a stronger RhB accumulation both in the chorion (Fig. 14g-h) as well as in the embryo itself, primarily in the yolk (Fig. 14k-l). The 1 h re-incubation of dechorionated embryos revealed a continued dye accumulation in all treatments including the control group (Fig. 14m-p). In embryos exposed to 100 µM PFOS, a continued RhB accumulation was also clearly visible in other body parts such as the head, notochord and tail, indicating a non-tissue-specific dye accumulation.

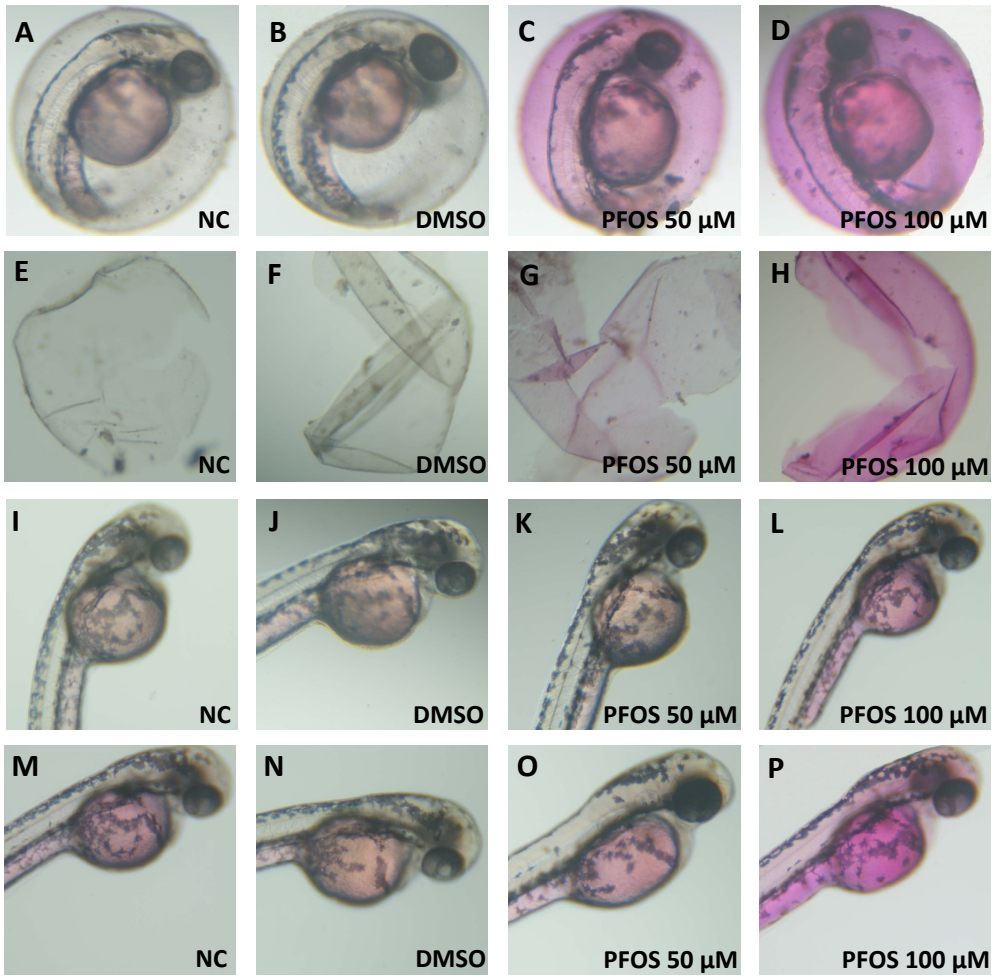


Fig. 14. Light-micrographs illustrating rhodamine B (RhB) accumulation in zebrafish (*Danio rerio*) embryos at 48 hpf incubated in RhB (200 µM) alone (**A, E, I, M**) and in combination with 0.05 % DMSO (**B, F, J, N**), 50 µM PFOS (**C, G, K, O**) or 100 µM PFOS (**D, H, L, P**). Stronger staining of pink indicates higher RhB fluorescence in embryo tissue. Embryos were incubated for 1 h (**A-D**) where after the chorion was mechanically removed to allow a clear view of the RhB accumulation in the chorion (**E-H**) and in the embryo (**I-L**). In order to investigate the RhB accumulation in the absence of the chorion, dechorionated embryos were re-incubated for 1 h (**M-P**).

Epifluorescence images of RhB accumulation in zebrafish embryos at 48 hpf are shown in Fig. 15. As previously illustrated by light-microscopy imaging, embryos exposed to RhB in combination with PFOS (50 and 100 µM) displayed a stronger fluorescence signal (RhB accumulation) in the chorion compared with the control group (Fig. 15a-d). When non-exposed embryos without chorion were incubated with RhB and PFOS (Fig. 15i-l), PFOS-exposed embryos again displayed the highest fluorescence signals.

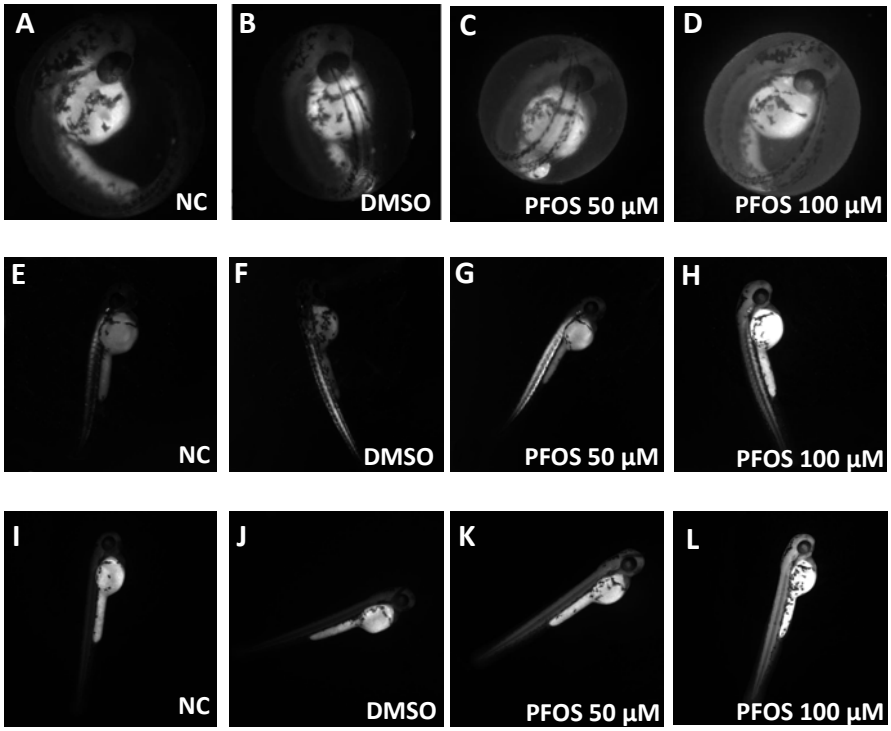


Fig. 15. Epi-fluorescence images of rhodamine B (RhB) accumulation in zebrafish (*Danio rerio*) embryos at 48 hpf. Stronger brightness indicates higher RhB fluorescence in embryo tissue. Zebrafish embryos were incubated with RhB (200 µM) alone (**A, E, I**) and in combination with 0.05 % DMSO (**B, F, J**), 50 µM PFOS (**C, G, K**) or 100 µM PFOS (**D, H, L**). Embryos were incubated for 1 h (**A-D**) where after the chorion was mechanically removed to provide a clear view of the RhB accumulation in the embryo (**E-H**). In order to investigate RhB accumulation in the absence of the chorion, non-exposed dechorionated embryos were incubated for 1 h in the same exposure solutions described above (**I-L**). Exposure time in (**A-H**) and (**I-L**) was 42 and 14 ms, respectively.

3.3 Long-term effects of PFOS and BPA in zebrafish

Chemical analysis

Mean measured concentrations of PFOS throughout the experimental period are shown in Fig. 16. Monthly mean concentrations (standard deviation; total number of sampled test vessels) of PFOS in controls, 0.6, 100 and 300 µg/L treatment groups were 0.073 (0.080; n = 10), 0.734 (0.131; n = 26), 106.9 (16.26; n = 9) and 267.6 (44.99; n = 27) µg/l, respectively. The measured PFOS concentrations in the control vessels already at day 1 and later on are believed to reflect the background concentration in the water supply to the test facility. The peak in the control group at exposure day 142 was due to a handling error causing a temporarily higher PFOS concentration (0.29 µg/L) compared with the background level. At

the subsequent sampling time (exposure day 183), the measurements of PFOS (0.076 µg/L) indicate a return to the range of the background level.

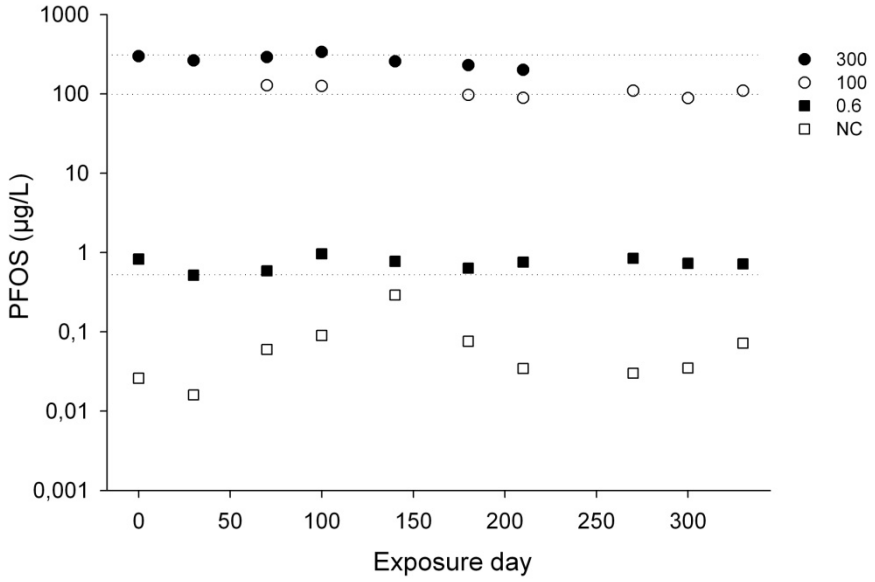
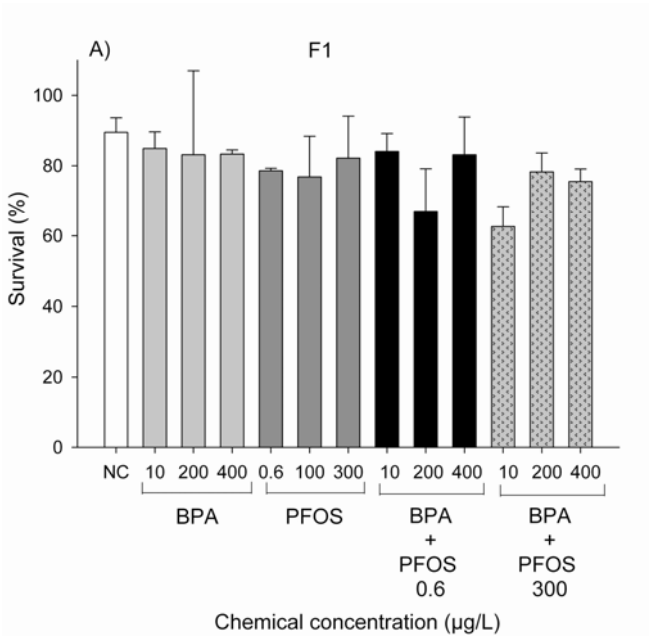


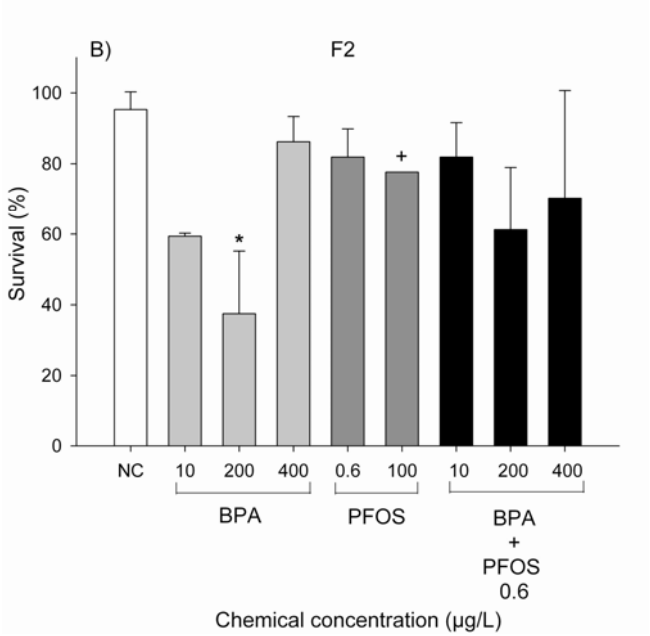
Fig.16. Mean water concentrations of PFOS measured over the course of the 330 d exposure trial of the F1, F2 and F3 generation zebrafish (*Danio rerio*). Nominal concentrations of PFOS were 0 (□; n = 1), 0.6 (■; n = 3), 100 (○; n = 1) and 300 µg/L (●; n = 4), where n represents the amount of sampled test vessels within each exposure group at each sampling time. Concentrations of PFOS were measured using LC-MS/MS.

Survival in the F1, F2 and F3 generation

Survival for the F1, F2 and F3 generations is summarized in Fig. 17. No significant mortality or malformations were observed in the F1 generation over the course of the 180-day exposure. In the subsequent F2 generation, post-hatch malformations such as body flexure followed by 100 % mortality was documented within a period of 14 dpf in the highest PFOS (300 µg/L) treatment, with or without BPA. Given this, all treatment groups with the highest PFOS exposure (300 µg/L) had to be terminated and were not further investigated in subsequent F2 and F3 generation. During the same time period, an unexplained and significant drop in survival (survival rate 37.5 ± 17.7 %) was observed in the 200 µg/L BPA exposure group (Fig. 17). Furthermore, a decrease in fish density shortly after swim-up was observed in one of the two replicates of PFOS 100 µg/L (survival rate 5 %). In the F3 generation, no post-hatch malformations were documented throughout the examined 14 dpf period. Although not significant, the lowest survival rate (41.8 ± 29.2 %) in the F3 generation

was observed in the PFOS 100 µg/L treatment group, whereas survival rates for the remaining treatments ranged between 77.5 and 91.6 %.





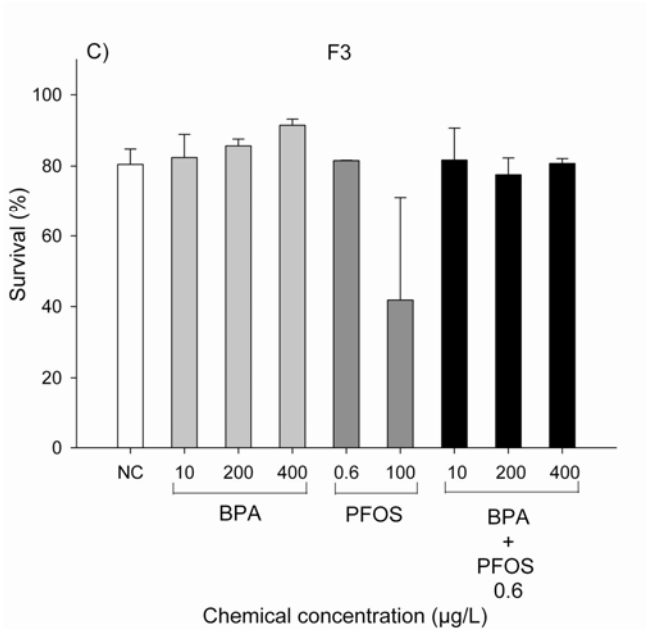


Fig.17. Survival of the F1 (**A**), F2 (**B**) and F3 (**C**) generation zebrafish at 180 (**A** and **B**) and 14 (**C**) dpf after exposure to PFOS, BPA or a binary mixture of PFOS and BPA. Data are given as average ± S.D. of two replicate aquaria. Significant differences from control are shown (*, $p < 0.05$). Statistic significant differences were detected with Kruskal-Wallis ANOVA on ranks (*post-hoc* Dunn´s method). $^{+}$ Due to a low survival rate (5 %) in one of the two replicates of the PFOS 100 µg/L treatment group in the F2 generation (**B**), only the second replicate is illustrated in the figure.

Growth in the F1 and F2 generation

Growth data (total length and body weight) for male and female F1 and F2 zebrafish at 30-, 90- and 180 dpf are summarized in Figs. 18 and 19, respectively. Pooled growth data for males and females at 30 dpf in F1 and F2 generation.

PFOS

F1 generation: At 90 dpf, growth (length and weight) in male zebrafish was significantly reduced in all PFOS exposures (0.6, 100 and 300 µg/L), if compared to the controls (Figs. 18b, d). In adult males at 180 dpf, length was significantly suppressed at the higher PFOS exposure groups (100 and 300 µg/L; Fig. 18b). In adult females at 180 dpf, length and weight was significantly reduced in all PFOS exposure groups (0.6, 100 and 300 µg/L; Figs. 18a, c).

F2 generation: At 90 dpf, growth in male (Figs. 19b, d) and female zebrafish (Figs. 19a, c) was significantly reduced in both PFOS exposure groups (0.6 and 100 µg/L) (weight not significant in males in the 100 µg/L exposure group). At 180 dpf, growth in adult males and females exposed to PFOS 100 µg/L was significantly lower than in controls.

BPA

F1 generation: After exposure to 200 µg/L BPA, growth reduction was evident for both males and females at 90 dpf (length not significant in females; Figs. 18a-d). However, this growth inhibition was most likely due to a counting error at the first time of sampling (30 dpf), resulting in a slightly higher fish density throughout a two month period in one replicate (61 *versus* approx. 44 individuals in the other treatment groups).

F2 generation: At 90 dpf, all BPA treatment groups (10, 200 and 400 µg/L) showed reduced growth in both males and females compared to the controls (Figs. 19a-d). In adult males and females at 180 dpf, growth at the highest BPA concentration (400 µg/L) was significantly lower than in controls.

PFOS and BPA mixture

F1 generation: At 90 and 180 dpf, the highest tested PFOS concentration (300 µg/L) in combination with BPA (10, 200 and 400 µg/L) significantly reduced growth in males (Figs. 18b, d). A significant growth reduction was noted for females at 180 dpf following co-exposure to PFOS (0.6 and 300 µg/L) and BPA (10, 200 and 400 µg/L). Length changes were not significant in the PFOS 300 µg/L + BPA 10 µg/L exposure group (Fig. 18a).

F2 generation: At 90 dpf, growth in both males and females in all binary mixture groups (0.6 µg/L PFOS + 10, 200 and 400 µg/L BPA) was significantly lower than in controls (Figs. 19a-d). At 180 dpf, growth was significantly reduced in males and females exposed to a binary mixture with 0.6 µg/L PFOS and the two highest tested BPA concentrations (200 and 400

µg/L); weight changes in males not significant in the PFOS 0.6 µg/L + BPA 200 µg/L exposure group.

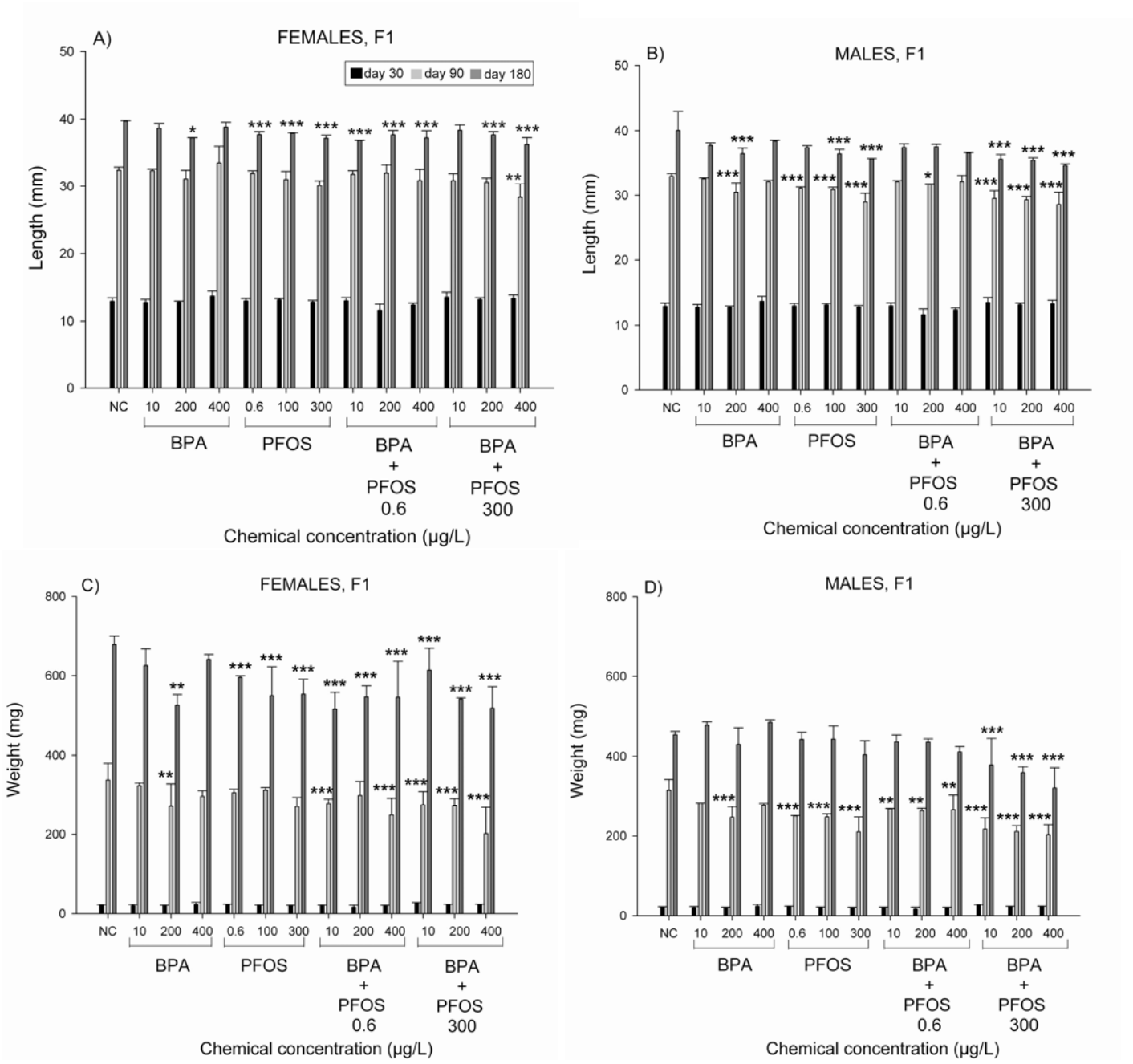


Fig. 18. Length and weight of female (**A**, **C**) and male (**B**, **D**) zebrafish in the F1 generation exposed to PFOS, BPA or a binary mixture of PFOS and BPA after 30, 90 and 180 dpf. Length and weight data at 30 dpf consist of males and females pooled together. Data are given as average ± S.D. of two replicate aquaria. Significant differences from negative controls are shown (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). Statistic significant differences were detected with one-way ANOVA (*post-hoc* Holm-Sidak method) or Kruskal-Wallis ANOVA on ranks.

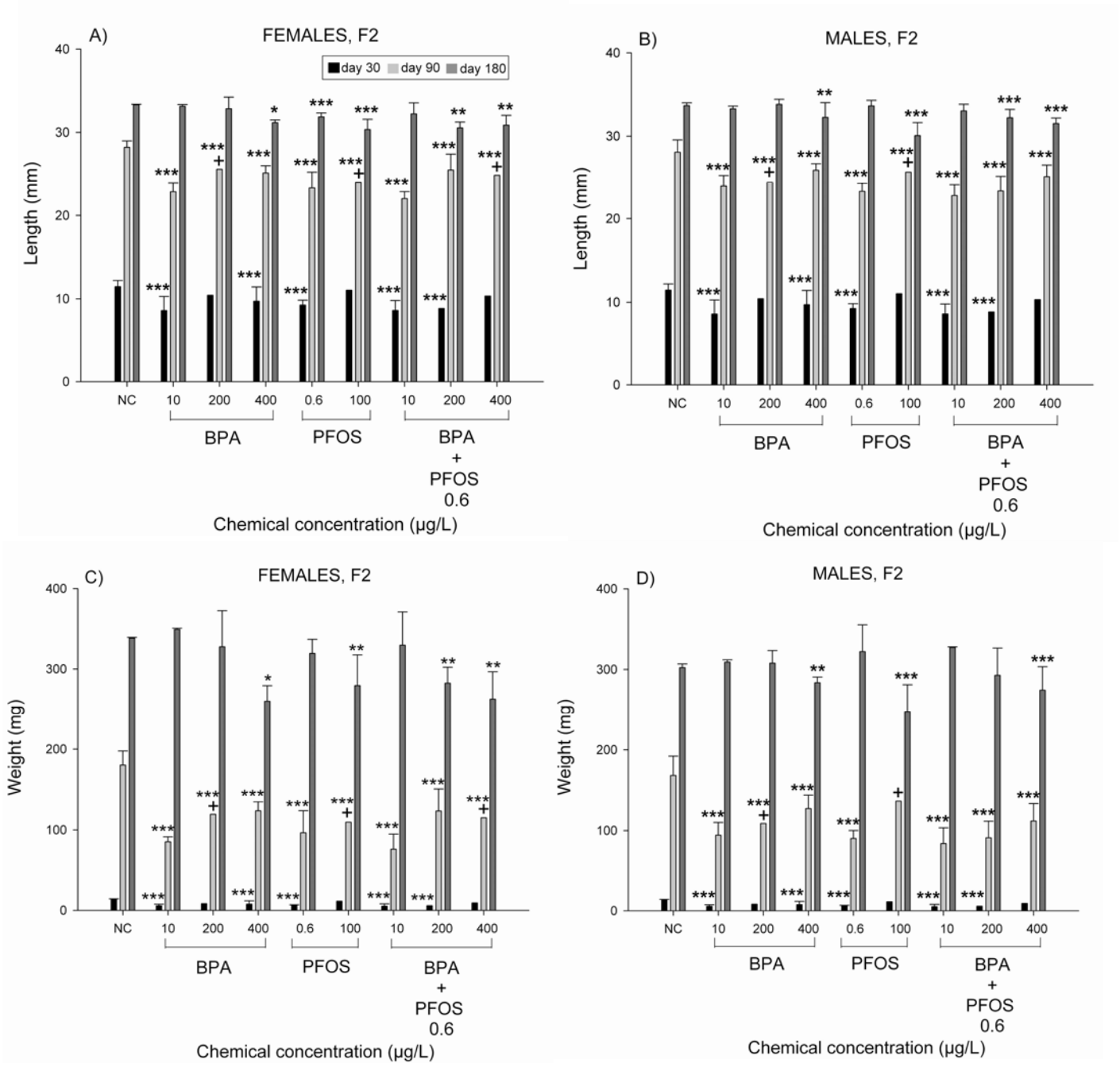


Fig. 19. Length and weight of female (**A**, **C**) and male (**B**, **D**) zebrafish in the F2 generation exposed to PFOS, BPA or a binary mixture of PFOS and BPA after 30, 90 and 180 dpf. Length and weight data at 30 dpf consist of males and females pooled together. Data are given as average ± S.D. of two replicate aquaria. Significant differences from negative controls are shown (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). $^{+}$ Only one replicate aquaria was sampled. Statistic significant differences were detected with one-way ANOVA (*post-hoc* Holm-Sidak method) or Kruskal-Wallis ANOVA on ranks (*post-hoc* Dunn´s method).

Vitellogenin induction in males of the F1 and F2 generations

Concentrations of Vtg (outliers excluded) measured in F1 and F2 male zebrafish at 90 and 180 dpf are summarized in Fig. 20. Any Vtg value greater than 1.5 times the spread outside the closest hinge of the boxplot of each treatment was considered an outlier.

PFOS

F1 generation: At 90 dpf, males exposed to 0.6 µg/L PFOS showed a significantly induced Vtg synthesis if compared to controls (Fig. 20a). In the presence of outliers (11 448 and 13 723 ng/g Vtg in controls and 66 253 ng/g Vtg in the PFOS 0.6 µg/L exposure group) this difference was not great enough to be statistically significant. Vtg levels generally tended to decrease in a concentration-dependent manner; however, Vtg synthesis in F1 males exposed to 100 and 300 µg/L PFOS was never significantly different from controls (Figs. 20a-b).

F2 generation: Comparable with the corresponding F1 males, F2 males exposed to 0.6 µg/L PFOS for 90 d displayed a statistically significant Vtg induction over controls (Fig. 20c). Due to the low Vtg concentrations in adult control males, both PFOS exposure groups (0.6 and 100 µg/L) were statistically higher at 180 dpf (Fig. 20d).

BPA

F1 generation: At 90 dpf, the Vtg synthesis was significantly induced in males exposed to the highest tested BPA concentration (400 µg/L; Fig. 20a). Overall, Vtg levels in the BPA treatment groups tended to increase in a concentration-dependent manner at both sampling times (Figs. 20a-b).

F2 generation: Exposure to 10 and 400 µg/L BPA significantly induced Vtg levels in male zebrafish over controls at 90 dpf (Fig. 20c). In presence of outlier value (985.2 ng/g Vtg), the Vtg induction in the BPA 200 µg/L group was also significantly higher compared to controls. Due to the low Vtg concentrations in the adult control males, all BPA exposure groups were statistically higher at 180 dpf (Fig. 20d). In adult males exposed to 400 µg/L BPA at 180 dpf, there was a clear, but non-significant decrease in Vtg synthesis, if compared with the lower BPA exposure groups (10 and 200 µg/L).

PFOS and BPA mixture

F1 generation: At 90 dpf, males in the lowest PFOS (0.6 µg/L) binary mixture groups displayed a BPA-dependent increase in Vtg synthesis; however, no statistical significance was found compared to controls (Fig. 20a). At 90 dpf, Vtg levels in males co-exposed to 0.6 µg/L PFOS and 10 µg/L BPA were significantly lower, if compared with the single exposures of the single compounds. In contrast to the lowest PFOS (0.6 µg/L) binary mixture groups, Vtg levels at 90 dpf in males exposed to the highest PFOS (300 µg/L) binary mixture decreased in a concentration-dependent manner with a significant reduction in the highest binary mixture group (PFOS 300 µg/L + BPA 400 µg/L), if compared with 400 µg/L BPA alone. As seen at 90 dpf, adult males at 180 dpf co-exposed to 0.6 µg/L PFOS and 10, 200 and 400 µg/L BPA displayed a BPA concentration-dependent increase in Vtg synthesis with the highest binary mixture group (PFOS 0.6 µg/L + BPA 400 µg/L) being significantly higher compared with the single exposures of both compounds as well as compared to controls (Fig. 20b). In presence of outlier in control males (10 976 ng/g Vtg) no significant difference between PFOS 0.6 µg/L + BPA 400 µg/L and controls was found. At 180 dpf, adult males in all highest PFOS (300 µg/L) binary exposure groups displayed significantly lower Vtg levels compared to controls. In presence of outliers in the two highest binary exposure groups with PFOS 300 µg/L (123 000 ng/g and 39 864 ng/g respectively) no significance was found.

F2 generation: At 90 dpf, Vtg levels in males co-exposed to 0.6 µg/L PFOS and 10, 200 and 400 µg/L BPA were significantly higher than in controls (Fig. 20c). A significant drop in Vtg synthesis was seen with a mixture of 0.6 µg/L PFOS and 400 µg/L BPA, if compared with isolated exposure of BPA (400 µg/L). Given the low Vtg concentrations in the adult control males, all binary exposure groups at 180 dpf were statistically elevated (Fig. 20d). Similarly to the corresponding F1 adults at 180 dpf, adult F2 males exposed to the lowest PFOS (0.6 µg/L) binary exposure groups showed a slight BPA-dependent induction of Vtg synthesis with significantly higher Vtg levels in males exposed to the highest binary mixture (PFOS 0.6 µg/L + BPA 400 µg/L), if compared with the single exposure of BPA (400 µg/L).

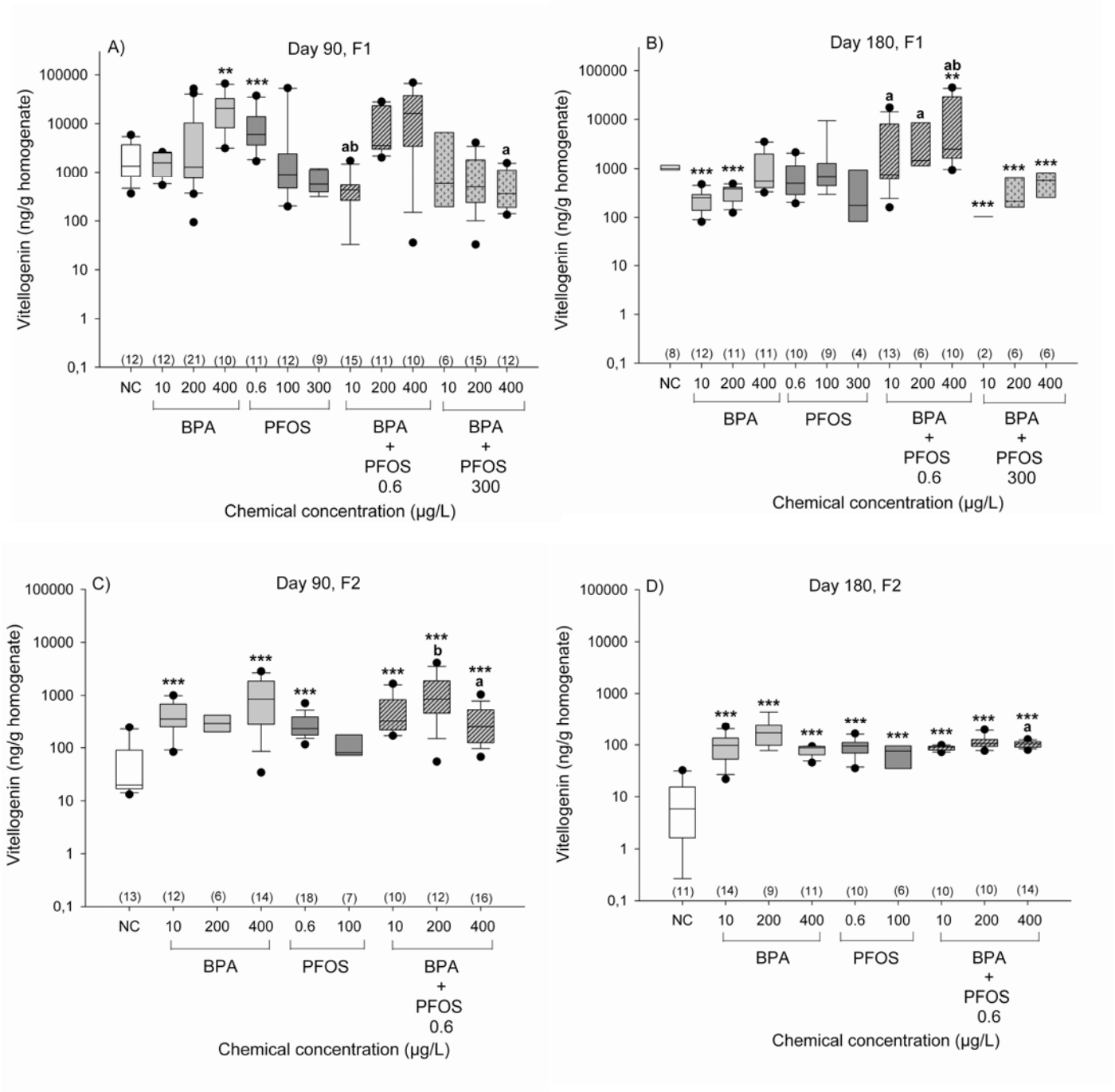


Fig. 20. Measured vitellogenin (Vtg) concentrations in tail homogenate of male zebrafish in F1 and F2 generation at 90 (**A**, **C**) and 180 (**B**, **D**) dpf after exposure to PFOS, BPA or a binary mixture of PFOS and BPA. Vtg concentrations are presented as box plots with median line (line within box), 25th and 75th percentiles (lower and upper boundary of box) and 10th and 90th percentiles (lower and upper whiskers). Significant comparisons *versus* negative control are indicated with *, < 0.05, **, $p < 0.01$ and ***, $p < 0.001$. Significant comparisons *versus* BPA-treated group and PFOS-treated group are indicated with ^{a} and ^{b}, respectively. Statistically significant differences were detected with one-way ANOVA (*post-hoc* Holm-Sidak method) or Kruskal-Wallis ANOVA on ranks (*post-hoc* Dunn´s method). The number of measured male fish in each treatment group (pooled data from all tanks in each treatment) is indicated in parentheses in the bottom of the figure.

Histological alterations

Gonads: Testis maturation stages (Braunbeck et al., 2010) for F1 and F2 male zebrafish did never differ from controls in any exposure group, nor was there a difference between generations except for 200 µg/L BPA where adult F2 males showed slightly advanced maturation, if compared to adult F1 males (details not shown). No obvious difference in number or size of Leydig cells was recorded for any treatment group in either generation. In the F1 generation at 90 dpf, one case of moderate testis-ova (perinucleolar oocytes) was observed in the 10 µg/L BPA exposure group (1 out of 12 males). Likewise, after 90 d exposure in the F2 generation, a minimal case of testis-ova (perinucleolar oocytes) was observed in the control group (1 out of 13 males). No case of testis-ova was recorded for adult males at 180 dpf in any generation. Ovarian maturation stages (Braunbeck et al., 2010) in F1 and F2 females did not differ from controls in any treatment group both at 90 and 180 dpf. With the exception of 90 d old F2 females exposed to the highest BPA concentration (400 µg/L; both alone and in combination with PFOS), all F2 females displayed a transient delay in ovarian maturation, if compared with F1 females (details not shown). At 180 dpf no obvious difference in ovarian maturation stages between the two generations was seen.

Thyroid: No deviations from controls were recorded in any treatment regarding size, structure, distribution and number of thyroid follicles.

Liver: Vacuolization of the liver (Fig. 21) was the major histological alteration found exclusively in PFOS-exposed fish in F1 and F2 generations, both alone and in combination with BPA. At 90 dpf, hepatocellular vacuolization in F1 males was recorded for the upper investigated PFOS range (100 µg/L and 300 µg/L) both in single and binary exposures. At 180 dpf, adult F1 males displayed a similar chemical-related vacuolization, although with fewer individuals affected (Fig. 22a). Vacuolization was also detected in F2 males after 90 d of exposure to 100 µg/L PFOS, whereas no such findings were made in adult F2 males (Fig. 22b). Vacuolization was documented in both sexes; however, compared with males, far fewer females were affected. Female F1 fish at 90 dpf displayed vacuolization following exposure to all binary mixtures with the highest PFOS concentration (300 µg/L PFOS in combination with 10, 200 and 400 µg/L BPA; Fig. 22a). No hepatic alterations were found in adult females at 180 dpf. In F2 females, no case of hepatocellular vacuolization was observed at any time.

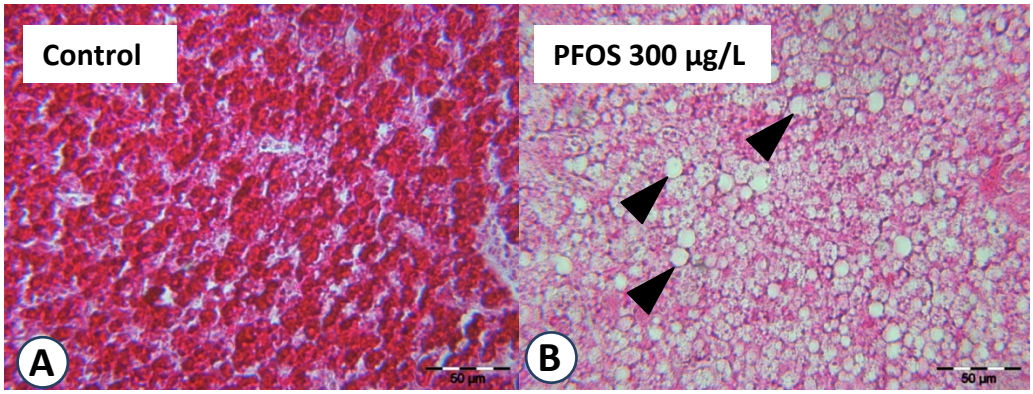


Fig. 21. Light micrographs showing the liver structure in control (**A**) and PFOS-exposed (**B**) males of zebrafish in the F1 generation at 90 d post-fertilization. Hepatocellular vacuolization (arrows) is observed in PFOS-exposed fish (**B**). Identical vacuolization was also detected in binary mixtures with BPA (micrographs not shown). Sections of 4 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin.

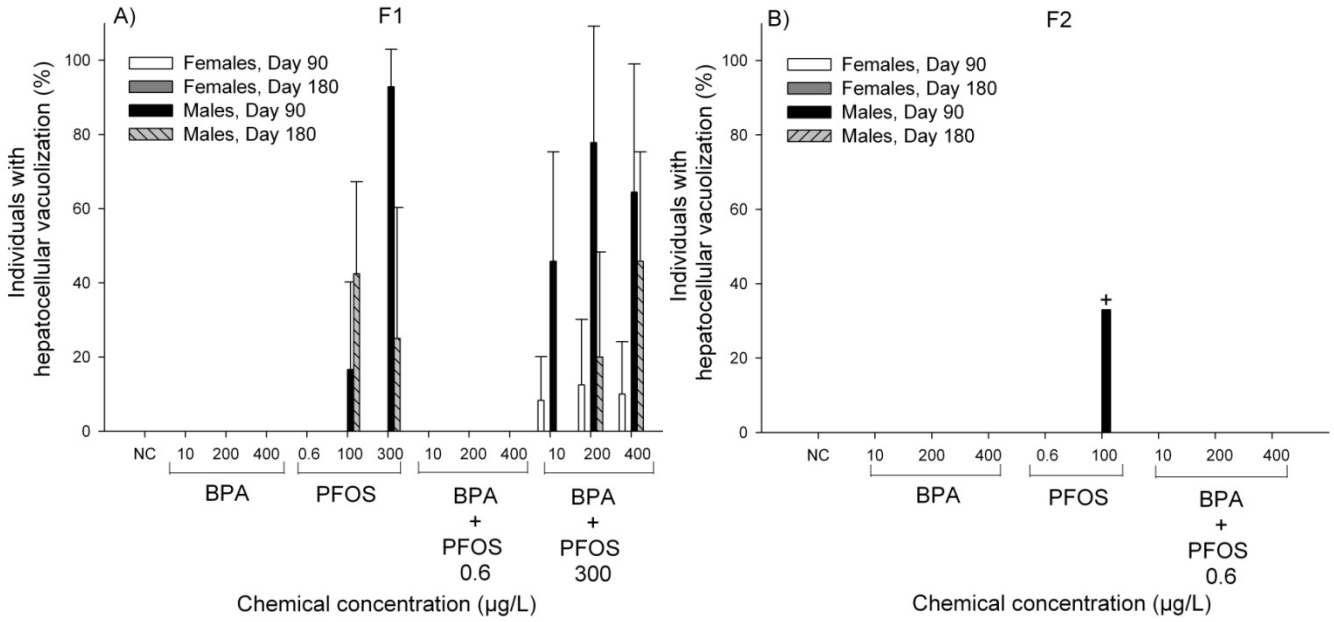


Fig. 22. Hepatocellular vacuolization in male and female zebrafish in F1 (**A**) and F2 (**B**) generation following exposure to PFOS, BPA or to a binary mixture of PFOS and BPA. Data are given as average ± S.D. of two replicate aquaria. $^{+}$ Only one replicate aquarium was sampled.

Granulomatous inflammation: Distinct granulomas (Fig. 23) were frequently observed in fish exposed to 100 and 300 µg/L PFOS both in single and binary exposure groups. Granulomas were mainly located in the liver, but were also documented in other visceral organs such as pancreas and kidney as well as in the reproductive organs of both sexes. Figures 24a and b show the mean histological index of granulomas in the liver of males and females in F1 and F2 generations. The occurrence of granulomas was clearly gender-specific with male fish being more susceptible than female fish in both generations. At 90 dpf, F1 males exposed to PFOS 300 µg/L, alone and in combination with all BPA concentrations, displayed granulomas in liver, pancreas and testis with the order of severity being: liver > pancreas > testis. With an overall higher grade of severity, an identical pattern was documented for adult F1 males with the exception that granulomas in liver and pancreas were noted also with 100 µg/L PFOS. For F2 males, kidney-located granulomas in the 100 µg/L PFOS treatment were observed already at 90 dpf (1 out of 9 males). One case of kidney granuloma was also documented in the lowest PFOS (0.6 µg/L) binary exposure group (0.6 µg/L PFOS in combination with 200 µg/L BPA; 1 out of 13 males). In adult F2 males, the severity grade of granulomas was increased and followed a similar pattern of affected organs as in the F1 generation (liver > pancreas ≈ testis). At 90 dpf, F1 females in the highest PFOS (300 µg/L) binary exposures displayed a minimal case of granuloma in the liver, pancreas and ovary with the order of severity being: liver > pancreas > ovary. In adult F1 females, a higher grade of severity was noted with granulomas primarily located in the ovary and the liver (ovary > liver). A minimal and moderate case of granuloma was furthermore detected in the ovary of two control females (2 out of 11 females). No granulomas were observed in F2 females at 90 dpf in any treatment; however, at 180 dpf, a case of minimal granuloma was detected in the ovary of one F2 female (1 out of 7 females) following exposure to PFOS 100 µg/L.

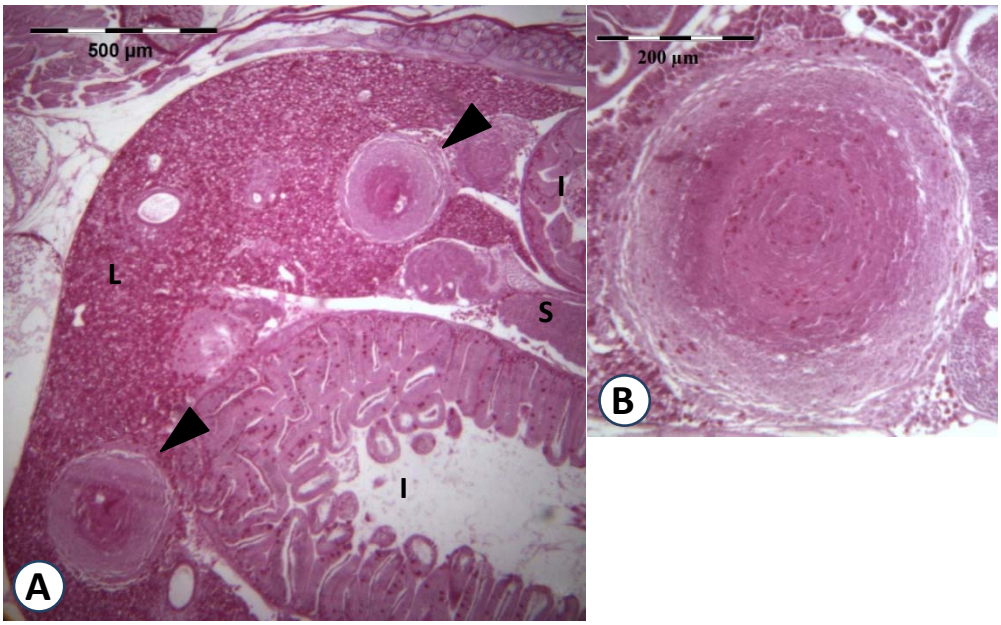


Fig. 23. Light micrographs showing granuloma structures (arrows) located in the liver of PFOS-exposed zebrafish males of the F1 generation at 90 d post-fertilization (**A**). High magnification of a granuloma structure (**B**). Identical granulomas were also detected in binary mixtures with BPA (micrographs not shown). Section of 4 µm thickness stained with periodic-acid Schiff (PAS) and Mayer's hematoxylin. L: liver; I: intestine and S: spleen.

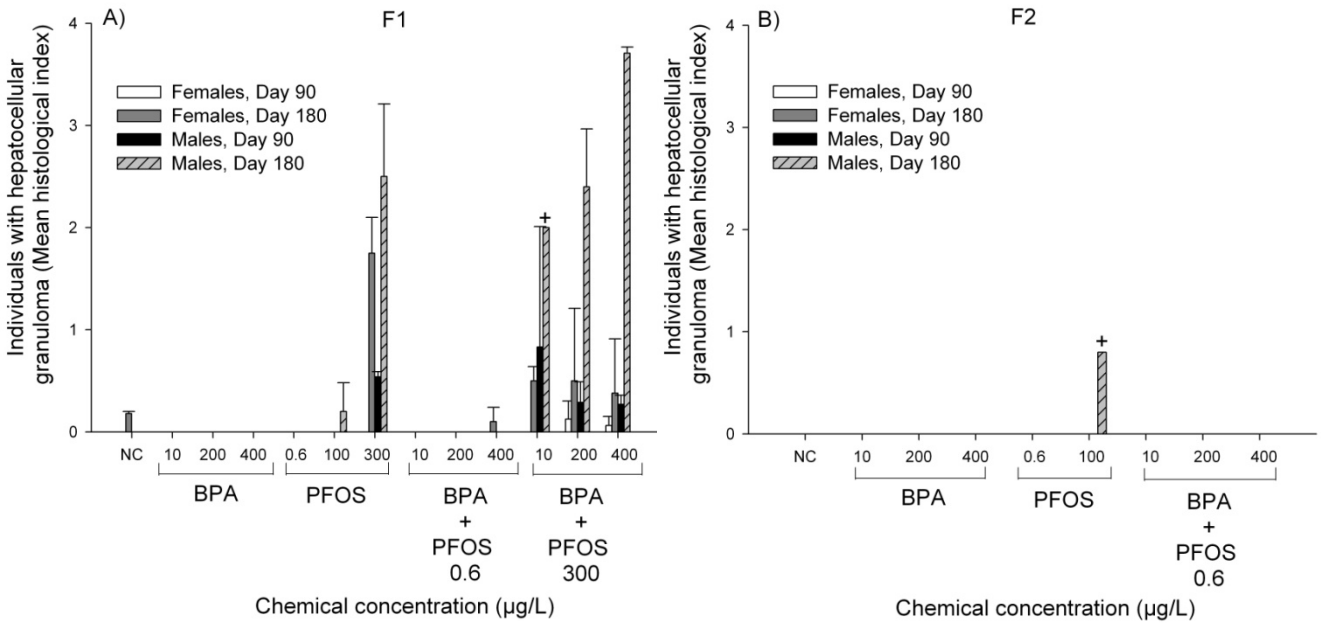


Fig. 24. Granulomas located in the liver of male and female zebrafish in F1 (**A**) and F2 (**B**) generation following exposure to PFOS, BPA or to a binary mixture of PFOS and BPA. Data are given as average ± S.D. of two replicate aquaria. $^{+}$ Only one replicate aquarium was sampled.

Egg production and fertilization rate in the F1 and F2 generation

Fecundity (total amounts of eggs spawned per female) in the F1 and F2 generations is shown in Fig. 25 (mean value ± SD of two replicate aquaria). Mean (± SD) of eggs spawned per control female (190 ± 12) in the F1 generation represented the highest reproductive output among all treatment groups. Co-exposure of 0.6 µg/L PFOS and 400 µg/L BPA significantly reduced fecundity compared to single exposures of the two chemicals as well as compared to controls.

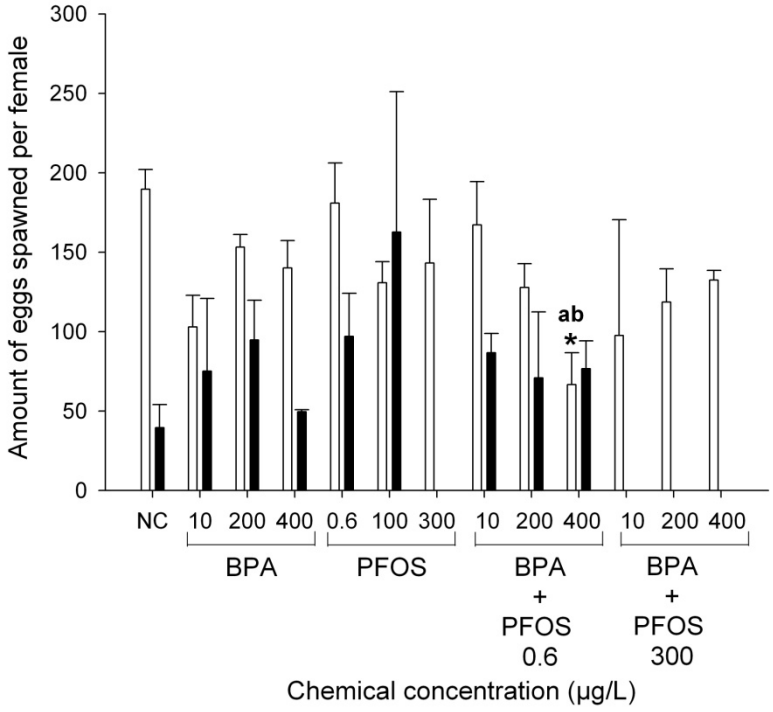


Fig. 25. Total number of eggs spawned by F1 (white bars) and F2 (black bars) generation females exposed to PFOS, BPA or a binary mixture of PFOS and BPA (**A**). Data are given as average ± S.D. of two replicate aquaria. Comparisons significant at < 0.05 are indicated with * (*versus* negative control), [a] (*versus* BPA-treated group) and [b] (*versus* PFOS-treated group). Statistic significant differences were detected with one-way ANOVA (*post-hoc* Holm-Sidak method). Due to technical problems in F2 generation control group, no statistical analyses were carried out.

The scatter plot (Fig. 26a) serves to illustrate the varying amount of eggs spawned per F1 female over the breeding study, thus explaining the high standard deviation which was generally seen in all treatment groups including the control group. As seen in Fig.26b, the total amount of eggs spawned per F2 female (SD ranging between 25 and 60 % of the total mean) varied less than in the F1 generation (SD ranging between 34 and 81 % of the total mean).

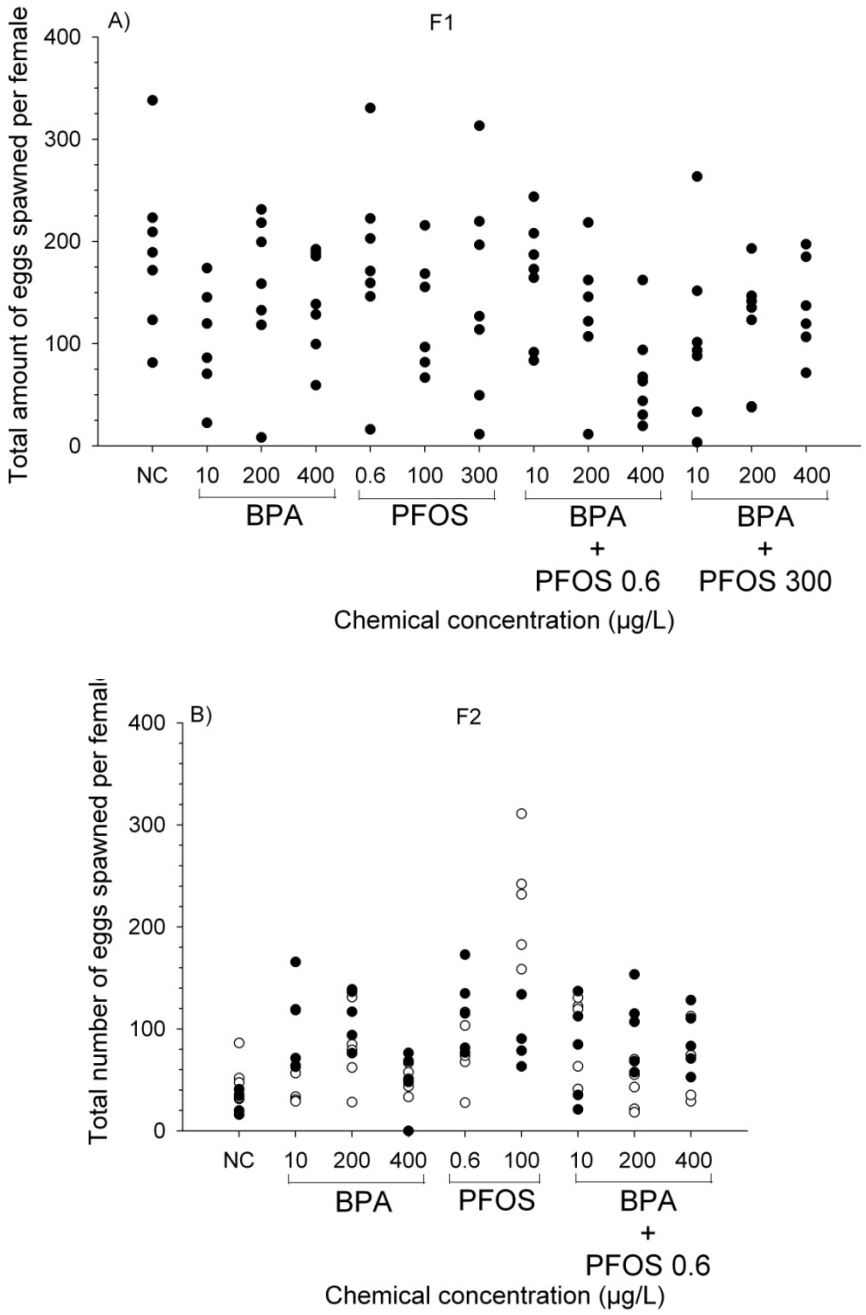


Fig. 26. Scatter plots illustrating the variation in egg production per female within each replicate aquarium in the F1 (**A**) and F2 (**B**) generation (open circles: replicate 1; filled circles: replicate 2).

The fertilization rate of the F1 offspring ranged between 59 and 79 % for all treatment groups with no significant difference compared to controls (72 ± 2; Fig. 27). The fertilization rate of the F2 offspring ranged between 63 and 80 % for all treatment groups including the control. A significantly higher fertilization rate compared to controls was observed in the 0.6 µg/L PFOS exposure group. No statistical significance was found compared with the fertilization rate of the F1 offspring.

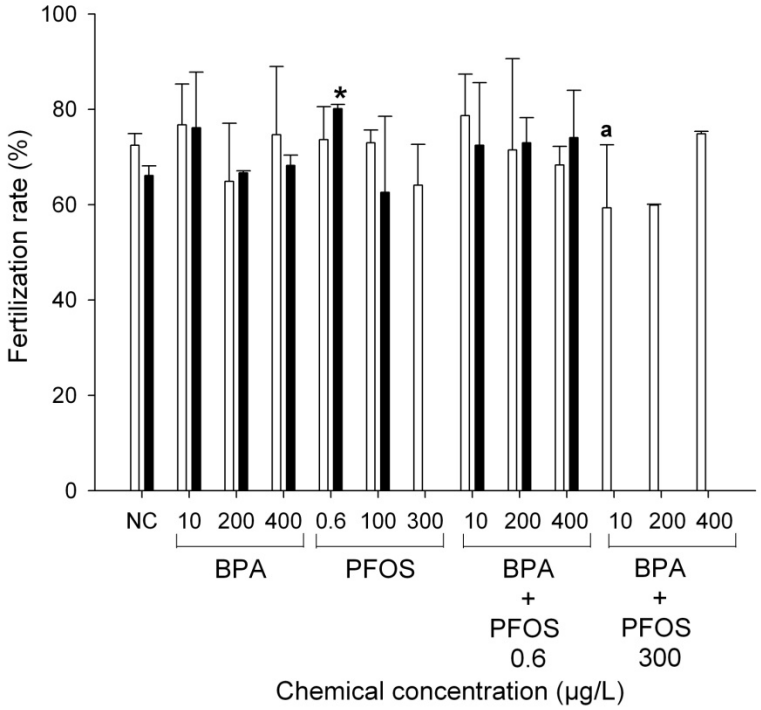


Fig. 27. Fertilization rate of eggs spawned by females of F1 (white bars) and F2 (black bars) generation exposed to PFOS, BPA or a binary mixture of PFOS and BPA. Data are given as average ± S.D. of two replicate aquaria. Comparisons significant at < 0.05 are indicated with * (*versus* negative control) and [a] (*versus* BPA-treated group). Statistic significant differences were detected with one-way ANOVA (*post-hoc* Holm-Sidak method).

4. Discussion

4.1 Emphasized genotoxicity by PFOS in V79 cells

In the present study, PFOS was not found to exert any genotoxic effect towards V79 cells. These results are in agreement with a previous investigation showing no micronuclei induction in a human cell line following PFOS exposure (Florentin et al., 2011). Binary exposure of 23.2 μM PFOS and the genotoxicant CPP (4.79 and 9.58 μM) demonstrated an enhanced frequency of micronucleated cells compared with treatments with CPP and PFOS alone. Combination of PFOS and 9.58 μM CPP tested induced a larger increase of micronuclei than would have been expected if an additive toxicity was present. Based on these results, it appears as if the increased genotoxic action of CPP is caused by a synergistic action of PFOS. A possible explanation for this observation might be found in a previous study by Hu et al. (2003) where PFOS was discovered to increase the permeability of cell membranes for estradiol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. In addition, PFOS was reported to increase membrane fluidity in fish leukocytes, providing strong indications of the membrane alteration abilities of PFOS. From these observations along with the present study, it seems plausible that any alterations in cellular membrane properties by PFOS could have considerable impact on the availability and hence, genotoxicity, of CPP towards V79 cells.

4.2 Chemosensitizing behavior of PFCs in zebrafish embryos

In this study, zebrafish embryos were used to test the hypothesis that the effect of PFCs to enhance chemical uptake and toxic sensitivity of cells and organisms is *via* the MXR efflux mechanism. Results demonstrate that PFOS substantially enhanced uptake and toxic effects of two P-gp specific substrates, RhB and vinblastine. The underlying mechanism can, however, not entirely be explained by direct P-gp inhibition.

All PFCs tested increased accumulation of RhB in zebrafish embryos, however only PFOS induced a significantly enhanced uptake and toxicity of RhB and vinblastine. Co-administration of PFOS and vinblastine induced a more than additive mortality in zebrafish embryos compared with animals in single exposure treatments. Furthermore, co-exposed individuals displayed increased severity of tail defects typically seen in vinblastine mono-exposures. This, in fact, indicates a PFOS-induced increase of the intracellular vinblastine

concentration in zebrafish embryos. Binary mixtures of vinblastine and MXR-reversing compounds have previously been reported to impede embryonic development in aquatic organisms such as the marine worm *Urechis caupo* (Toomey and Epel, 1993) and the zebra mussel (*Dreissena polymorpha*; Faria et al., 2011). The finding that PFCs enhanced RhB uptake in zebrafish embryos is in agreement with a previous study by Stevenson and co-workers (2006), who reported a significant accumulation of RhB in gill tissue of the marine mussel *Mytilus californianus* following exposure to various PFCs. The authors discovered a chain-length-dependent inhibition where PFOA and PFNA, an eight- and nine carbon PFC, respectively, were among the PFCs displaying the highest chemosensitizing potential. Compared with the negative control, the transporter inhibition in mussels exposed to PFOA and PFNA was found to be approximately two-fold higher. These findings are in line with the results obtained in the present study, demonstrating a two- and three-fold increase in RhB accumulation by PFOA- and PFNA-exposed zebrafish embryos, respectively.

An extended evaluation of PFOS in the transporter activity assay revealed a clear concentration-dependent increase in dye retention up to 70 μM PFOS where after the RhB accumulation seemed to reach maximum around a fold increase of fourteen compared to controls. As no significant RhB accumulation was detected in mussels exposed to PFOS (Stevenson et al., 2006), the high accumulation observed in the present study is surprising. A mechanistic question of relevance is whether the increased RhB accumulation is the result of a direct or indirect inhibition of the P-gp transporter activity. If PFCs would behave as P-gp substrates, this would directly lead to an inhibited efflux of RhB as the PFC compounds would compete with the dye as for substrate binding sites. In the work by Stevenson et al., PFNA could not be confirmed to be a P-gp substrate, thus the authors speculated that the inhibited pump activity was more likely an indirect consequence following detergent-like effects on the membrane. In the present study, some reference transporter inhibitors were found to cause only minor to negligible RhB accumulation in zebrafish embryos. Based on these results, the substantially higher dye accumulation generally seen for the PFCs can most likely not be explained by direct P-gp inhibition. The probable involvement of an additional mechanism of action was further implied by the functional assay with MDCKII cells over-expressing human P-gp where PFOS, PFNA and PFDA showed comparatively little effect on cellular calcein accumulation compared with the reference compound verapamil. Taken these results together, it seems plausible to assume that PFCs do not hamper P-gp activity in zebrafish in a direct manner by acting as pump substrates. However, as one cannot presume

that P-gp of mussel, fish and human share the same substrate preferences, care should be taken when drawing parallels between studies using different model species. The importance of this problem was recently shown by Zaja and colleagues (2011) who compared the substrate specificity for fish and human P-gp. Results demonstrated that although most substances tested interacted in a similar manner with P-gp of both species, some interacted specifically with fish or human P-gp only. The fact that PFCs share some of the common properties generally associated with P-gp substrates, e.g. amphiphilicity, the substrate-specificity of zebrafish P-gp needs to be further elucidated. One approach would be to monitor PFC-uptake in zebrafish embryos with and without a standard inhibitor (Stevenson et al., 2006). An influenced PFC accumulation in the presence of an inhibitor would provide evidence that the PFC investigated is indeed recognized as pump substrate.

In a recent study by Kais et al. (in prep.), the uptake of the fluorescent chemical fluorescein in zebrafish embryos was found to be positively correlated with the DMSO concentration, suggesting that DMSO may enhance the permeability of the chorion for fluorescein. With respect to the detergent-like behavior of PFOS (Hu et al., 2003; Matyszewska et al., 2007), a modified transporter activity assay was conducted to elucidate whether the increased RhB accumulation seen by PFCs could be the result of an increased uptake across the chorion: Zebrafish embryos were co-exposed to RhB and PFOS and were, following washing, transferred to artificial water with or without PFOS. In case PFOS would indeed increase the biological uptake of RhB *via* altered chorion permeability, it would appear reasonable to assume a similar action of PFOS during the post-incubation phase. The expected result would be a PFOS-induced leakage of RhB, hence displaying embryos with lower RhB content. Surprisingly, zebrafish embryos post-incubated in artificial water with PFOS displayed no RhB efflux, but demonstrated dye contents in the same order of magnitude as prior to the post-incubation. In contrary, embryos post-incubated in pure artificial water revealed a rapid RhB clearance ranging from 50 to 70 % in embryos previously exposed to 40 - 100 µM PFOS. A similar reversal of inhibitory effects of PFNA was shown in mussel gill tissue following a few hours in clean seawater. This fast reversibility of the PFNA effect was linked with rapid loss of PFNA from the tissue (Stevenson et al., 2006).

The absence of RhB efflux in the presence of PFOS indicates a transporter-related PFOS effect. However, as previously discussed, the dramatic effects on RhB accumulation compared with some standard inhibitors disfavor a direct P-gp inhibition as main reason. A

putative explanation could be linked to the involvement of other ABC transporters. A substrate overlap between P-gp and the MDR associated subfamily ABCG2 has been shown for RhB (Litman et al., 2001). A PFOS-induced blockage of both transporters would thus logically give rise to a stronger RhB accumulation compared to P-gp specific inhibitors. However, as the ABCG2 ATPase has been shown to be vanadate-sensitive (Litman et al., 2001), the low MDR reversing potential by vanadate seen in the present study provides no support for such a multiple transporter blockage by PFOS.

Interestingly, Regev et al. (1999) discovered that the P-gp ATPase activity in cell cultures was inhibited by an increased cell membrane fluidization caused by investigated compounds. Likewise, given that PFOS is a known fluidizer (Hu et al., 2003; Matyszewska et al., 2008), the increased accumulation of MXR substrates in PFC-exposed embryos could be the result of a membrane-mediated disturbance of the P-gp efflux activity. An indirect inhibition *via* an altered membrane environment has previously been shown to cause a disrupted conformation and, hence, function of membrane integrated transporters (Romsicki and Sharom, 1999).

In order to optically determine the PFOS-induced RhB uptake into the egg as well as the accumulation in the embryo, light- and epifluorescence microscopy imaging of zebrafish embryos was performed. Both microscopy techniques enabled the dye retention to be primarily located to the chorion and the yolk; however, with increasing PFOS concentrations, the accumulation of RhB in embryos tended to be less tissue-specific incorporating other body parts such as head, tail and notochord. In a recent study with zebrafish embryos, the accumulation of RhB was determined *via* fluorescence in the absence and presence of the transporter inhibitor CsA (Fischer, 2007). In line with the present observations on PFOS, the intracellular dye accumulation was clearly increased in the presence of CsA; however, in contrast to PFOS-exposed embryos, the dye was not found to be associated with the chorion.

The reason behind the chorion-associated dye retention could be related to the pore canals. The pore canals penetrating the two inner layers of the zebrafish chorion have been shown to hamper the uptake of certain compounds depending, among others, on their molecular size. Creton (2004) demonstrated a physical barrier function for fluorescent dextrans larger than 3 kDa. Likewise, Henn and Braunbeck (2011) found a restricted uptake for a 400 kDa polymer. However, as the molecular weight of both RhB and PFOS is approximately 0.5 kDa, the chorion should, under normal circumstances, not represent a serious obstacle for the uptake of these two compounds. Previous results (data not shown)

have actually demonstrated teratogenic effects of PFOS in un-hatched zebrafish embryos, hence, implying the potential of PFOS to at least partly diffuse across the chorion. A possible explanation for the chorion-integrated RhB could be that a significant fraction of PFOS indeed remains loosely associated with the chorion, inducing a structural change of the pore canals, which in turn causes a portion of the RhB to be trapped.

4.3 Long-term effects and mixture toxicity of PFOS and BPA in zebrafish

Long-term effects and synergizing behaviour of PFOS in binary mixtures with BPA were investigated in two full generations of the zebrafish (*Danio rerio*). Both chemicals were investigated in isolated and combined exposure scenarios with focus on Vtg concentrations, histological alterations and reproductive effects. Whereas PFOS did not increase the endocrine potential of BPA; PFOS-exposure resulted in hepatocellular vacuolization and reduced survival for the F1 offspring.

Growth and survival

A number of studies have investigated the acute and chronic toxicity of PFOS and BPA towards a wide range of aquatic organisms, confirming a lethal potency at doses well above those normally reported in environmental samples (OECD, 2002a; Pickford et al., 2003; Ankley et al., 2004; Du et al., 2009; Mihaich et al., 2009; Han and Fang, 2010). In the present study, PFOS and BPA were evaluated at nominal concentrations of 0.6 - 300 µg/L and 10 - 400 µg/L, respectively. Following exposure to the maximum PFOS concentration (300 µg/L), malformations such as body flexure followed by 100 % mortality was observed within 14 dpf in F2 generation. Identical observations in all highest PFOS (300 µg/L) binary mixtures, indicate that the effects seen were PFOS-related. These results are in agreement with two recent studies, where malformations followed by 100 % mortality after 96 h post-hatch (Du et al., 2009) and 7 dpf (Wang et al., 2011), were reported for embryos and larvae derived from maternal exposure to 250 µg/L PFOS. Decreased offspring survival following maternal PFOS exposure has also been reported for other test organisms, e.g., swordtail fish (*Xiphophorus helleri;* Han and Fang, 2010), Northern bobwhite quail (*Colinus virginianus*; Newsted et al., 2007), mouse (*Mus musculus*; Lau et al., 2003) and rat (*Rattus norvegicus*; Lau et al., 2003), thus further supporting the results obtained in the present study. In contrast, Ankley et al. (2005) found no significant effects on offspring survival (≤ 300 µg/L PFOS) in a partial life-

cycle study with the fathead minnow (*Pimephales promelas*). However, the survival rate following exposure to 300 µg/L PFOS represented the lowest among all treatments. In addition to inter-species differences in sensitivity, the diverging rates in offspring survival reported in the literature could possibly be related to the duration of the parental exposure; 180 d in this present study, 70 and 42 d in the study by Du et al. (2009) and Han and Fang (2010), respectively, and finally, 21 d in the study of (Ankley et al., 2005). The present study also revealed significant mortality in the F2 generation after exposure to 200 µg/L BPA. However, since no significant mortality was detected in the lowest or highest BPA exposure groups (10 and 400 µg/L, respectively), nor in any of the lowest PFOS (0.6 µg/L) binary exposures, the observed mortality is not believed to reflect a BPA-derived toxicity.

BPA- and PFOS-exposure have been associated with reduced mean body weights and body lengths in different organisms such as crustaceans (Lemos et al., 2010), fish (Sohoni et al., 2001; Han and Fang, 2010; Wang et al., 2011), amphibians (Ankley et al., 2004) and mammals (Seacat et al., 2002). In the present study, suppressed growth was seen in both F1 and F2 adults following 180 d PFOS exposure. Exposure to BPA had no consistent effects on growth in the F1 generation; however, in adult F2 fish at 180 dpf, the highest tested BPA concentration of 400 µg/L significantly decreased growth in both males and females.

Hepatotoxic effects as predicted by histological diagnosis

The liver is well known to be one of the target organs following PFOS exposure (Hagenaars et al., 2008; Ivan et al., 2008; Cui et al., 2009). In the present study, histological evaluation of the liver revealed vacuolization in fish exposed to PFOS (100 and 300 µg/L), both alone and in combination with BPA. For both F1 and F2 generation males, the prevalence of hepatocellular vacuolization was found to be more severe at 90 dpf than at 180 dpf, suggesting an adaptive response over time. This is in contrast to the findings by Du et al. (2009) showing that the severity of vacuolization in 250 µg/L PFOS-exposed zebrafish males was unchanged after 30 d in clean water. In accordance with findings by Du et al. (2009), effects were more pronounced in males in terms of severity and amount of individuals affected, thus, pointing towards a gender-specific toxicity. Another histopathological finding with a dominating prevalence in PFOS-exposed males was an increased occurrence of distinct spherical granulomas. Granulomas were observed both in isolated PFOS-exposures as well as in binary mixtures, thus, strongly indicating a PFOS-related effect. In a study by Novotny et al. (2010), identical granulomas were detected in the liver of the freshwater fish *Aphyosemion*

gardneri and diagnosed as generalized mycobacteriosis, a chronic and progressive bacterial disease commonly seen in wild and cultured fish (Chinabut, 1999). Although we were unable to trace *Mycobacterium* spp. in lesions, previous studies have demonstrated that an occurrence of a mycobacterial infection is often not well correlated with the presence of visualized *Mycobacterium* spp. (Watral and Kent, 2007). Mycobacteriosis is considered to be precipitated by stress (Gauthier and Rhodes, 2009). Chemical stress is generally supposed to act immunosuppressive (Prosser et al., 2011), and it may be speculated that the granulomas seen in PFOS-exposed fish occurred as a result of a suppressed immune system. Since PFOS has previously been reported to exert immunotoxic effects (Peden-Adams et al., 2008), an immunosuppressive action of PFOS could help to explain the observations in the present study. Further support is provided by the study of Jacobson et al. (2010) showing a correlation between PFOS-exposure and an increased incidence of a parasitic infection in amphipods (*Monoporeia affinis*).

Altered Vtg pattern

Vtg levels were significantly elevated in F1 males after 90 d exposure to the highest BPA concentration tested (400 µg/L). Induced Vtg synthesis in fish following BPA-exposure has previously been reported for fathead minnows with significant Vtg inductions in males already at 160 µg/L (Sohoni et al., 2001). The measured Vtg concentrations of approximately 1 and 2 µg/g in F1 control males at 90 and 180 dpf are rather high; however, Vtg levels in the same order of magnitude have previously been reported for unexposed males of zebrafish (Holbech et al., 2001; Christianson-Heiska et al., 2008) and rainbow trout (*Oncorhynchus mykiss*; Copeland et al., 1986). No significant increase in Vtg concentration was seen for F1 adults; however; overall, Vtg levels in the BPA exposure groups increased in a concentration-dependent manner at both sampling times. As in the F1 generation, at 90 dpf, Vtg levels in F2 males were significantly elevated in the highest BPA 400 µg/L exposure group. Given the unexplained low Vtg concentrations in adult control males in the F2 generation (180 dpf; approximately 11 ng/g), all BPA concentrations tested (10, 200 and 400 µg/L) were significantly higher.

PFOS and other PFCs such as PFOA have previously been demonstrated to have an estrogenic potential in fish both *in vivo* (Oakes et al., 2005; Wei et al., 2007; Du et al., 2009; Benninghoff et al., 2011) and *in vitro (*Liu et al., 2007*)*. In the present study, the lowest tested PFOS concentration of 0.6 µg/L was shown to significantly increase Vtg levels in F1 and F2

males. In line with our findings, Du et al. (2009) reported a significant up-regulation of Vtg mRNA in zebrafish males exposed to PFOS in a similar concentration range (10 µg/L). Surprisingly, we discovered that in contrast to the Vtg response induced by 0.6 µg/L PFOS, the estrogenic potential tended to decrease with an increasing PFOS-exposure. Similar observations were made in a study with fathead minnow (Ankley et al., 2005), where lower PFOS concentrations were shown to stimulate steroidogenesis whereas higher concentrations had a suppressing effect. In the present study, binary mixture exposures with the highest PFOS (300 µg/L) concentration were observed to significantly suppress Vtg levels in F1 males at 90 dpf if compared with single exposures of BPA. In adult F1 males at 180 dpf, the addition of PFOS 0.6 µg/L to the highest BPA (400 µg/L) exposure significantly induced Vtg synthesis compared with the two chemicals alone as well as compared to controls. The induction was higher than would have been expected based on an additive toxicity assumption; however, since this trend was not seen at the other sampling times in F1 and F2 generations, this observation is not believed to reflect a toxicity-increasing effect by PFOS.

Vtg in relation to histological findings

Previous studies have reported suppressed Vtg levels in pathogen-infected (Hecker and Karbe, 2005; Burki et al., 2010), thus offering a possible explanation to the observations in the present study. As already discussed, the prevalence of granulomas in liver of fish exposed to 300 µg/L PFOS could be indicative of a bacterial infection following suppression of the immune system. The mechanisms underlying reduced Vtg inductions in diseased fish are unknown; however, given the energy requiring process of Vtg synthesis, it is believed that this metabolic cost is saved in favor for immunological defense against the disease (Rushbrook et al., 2007; Burki et al., 2010). However, since observations in the present study indicated an increased severity of granulomas with age, the stronger Vtg suppressive effect of PFOS in younger F1 males compared with F1 adults contradicts this theory. An additional explanation could be related to the hepatotoxic effects seen in all treatments with the highest PFOS concentration (300 µg/L) as indicated by vacuolization of hepatocytes. As previously mentioned, the grade of vacuolization was found to be more severe at 90 dpf than at 180 dpf, thus, paralleling the Vtg-suppressive response seen by PFOS. It appears reasonable that an overall decreased fitness of the liver would lower the Vtg synthesizing capacity of the hepatocytes, thus potentially helping to explain the decreased estrogenic potential of PFOS at higher concentrations. In any case, in agreement with Van der Ven et al. (2003), the present

study further highlights the importance of histopathology as a tool when evaluating endocrine disruptors in zebrafish.

Reproductive success

Reproductive success is considered to be one of the most ecologically relevant endpoints in fish life-cycle exposures (Arcand-Hoy and Benson, 1998). In the F1 generation, spawning in the lowest PFOS (0.6 µg/L) binary exposures decreased in a BPA concentration-dependent manner with a significantly lower fecundity in the highest binary mixture (PFOS 0.6 µg/L + BPA 400 µg/L), both compared with the single exposures of the two chemicals and compared with controls. However, no further significance was found in the breeding study with the F1 generation fish. The low fecundity documented in the F2 control group is believed to reflect stress induced by technical problems temporarily experienced within the test facility; thus, no further statistical or comparative analyzes were carried out for female fecundity in the F2 generation. The variation in the total amount of eggs spawned per female seen in both generations is commonly seen in zebrafish and has previously been reported in the literature (Brion et al., 2004; Christianson-Heiska et al., 2008). One could ask if the present test design had sufficient power to detect the effects of PFOS and BPA: The numbers of eggs transferred from F1 to F2 and from F2 to F3 were between 160 and 170 (with one outlier of 145 eggs in the F3 400 µg/L BPA group) per treatment. These numbers are higher than the numbers of eggs used to start the OECD TG 234 (2011), where comprehensive power analysis and statistics have been included in the validation of the test. In TG 234, the groups are started with 120 fertilized eggs and the endpoints also include Vtg and sex determination. Thus, the present design should indeed be valid in relation to sample size.

5. General conclusions

With reference to the overall aim of this thesis, to evaluate the toxicity increasing potential and long-term effects of selected PFCs with focus on perfluorooctane sulfonate (PFOS), the following conclusions can be drawn:

PFOS was found to be non-genotoxic in the micronucleus test with hamster V79 cells. In binary mixtures with the genotoxicant cyclophosphamide monohydrate (CPP), micronuclei induction increased if compared with single exposure treatments. These results indicate a synergistic action of PFOS which according to previously published findings, could be associated with an increased cell membrane permeability following PFOS-exposure.

All PFCs tested increased accumulation of the standard P-gp substrate rhodamine B (RhB) in zebrafish embryos. The strongest chemosensitizing effect was shown for PFOS causing an up to 14-fold higher RhB uptake than in controls after only 1 h of exposure. The remarkable effect exerted by PFOS in zebrafish embryos, if compared with some standard MXR inhibitors, implies that a direct P-gp blockage is less likely as main reason. An additional mechanism of action could be related to the cell membrane-altering behavior of PFOS which, according to previous research, could lead to an indirectly blocked pump activity *via* changed P-gp conformity. With respect to the surprisingly low transporter inhibition observed by the investigated transporter reversers, their suitability as positive controls in efflux transporter activity assays with zebrafish is questionable. The strongest effect was observed for MK571 sodium salt; however, the lack of reproducibility further hampers its role as a control compound in the chosen test system.

In the long-term experiment, tested concentrations of PFOS did not increase the Vtg inducing potential of bisphenol A (BPA) when combined in a binary mixture. In contrast, binary mixtures with the highest tested PFOS concentration (300 µg/L) tended to suppress the Vtg induction in F1 males at 90 dpf when compared with the single exposures of BPA. Whereas the lowest tested PFOS concentration (0.6 µg/L) showed estrogenic potential in terms of Vtg induction, Vtg levels were decreased with increasing PFOS exposure. PFOS-induced hepatotoxicity at higher concentrations may be a possible explanation for the Vtg suppression observed with increasing PFOS concentrations. Vtg levels in the F2 generation generally followed a similar pattern as previously seen in the F1 generation after exposure to BPA and PFOS. Survival in the F2 generation was severely reduced at the highest tested

PFOS exposure (300 µg/L) within 14 dpf. Since adverse effects on hepatotoxicity and survival were only observed at concentrations of BPA and PFOS well above ecologically relevant concentrations, these results suggest a low environmental risk of a combined exposure to PFOS and BPA. However, while this study was limited to a binary mixture of chemicals, more complex mixtures of pollutants in the environment and additional stressors might well interfere with the outcome.

6. Future perspectives

This thesis further demonstrates the potential of PFCs to increase the cellular and organismal accessibility of other compounds. However, this work failed to provide information regarding the exact mechanism of action underlying these observations. As previous work on PFOS presented hints to the involvement of cellular membrane alterations, one option to gain deeper insights is to conduct theoretical modelling of the structure of cellular membranes when in contact with PFCs. By studying computer models of lipid bilayers using Molecular Dynamics simulations, questions such as how PFCs may increase the permeability of the membrane bilayer could be elucidated. Moreover, this technique would offer the prospect of revealing the most thermodynamically favorable location of the PFCs in the cellular membrane along with key interactions between PFCs and membrane constituents such as efflux transporter proteins. The great advantage of such a modeling approach is its non-invasive nature allowing directed monitoring of presumed key mechanistic actions related to membrane modulations.

With respect to the evaluated end points in the long-term study, no crucial effects were documented in the lower concentration range tested. However, as prime focus was given endocrine disruption, reproduction and cellular alterations, effects on other endpoints cannot be ruled out. Recent publications have indicated the potential of PFOS and other PFCs to act as neurotoxicants. As the nervous system is considered particularly vulnerable during early development, neurotoxic monitoring in life-cycle assessments would be of particular interest for future PFC studies. Combination effects with other neurotoxic contaminants such as heavy metals represent another topic of relevance for the aquatic environment.

7. References

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