Common carp *Cyprinus carpio* responses to sub-chronic exposure to perfluorooctanoic acid

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Abstract

Perfluorooctanoic acid (PFOA) is an important and diffuse perfluorinated alkylated substance, but knowledge of the toxicological effects of this endocrine disrupter in fish is limited. Adult common carp *Cyprinus carpio*, L. were exposed to 200 ng/l (a concentration reported in impacted aquatic ecosystems) and 2 mg/l PFOA solutions in a flow-through system for 56 days to determine tissue accumulation and histological alterations of the primary target organs. PFOA was extracted from blood, gill, liver, muscle, kidney, gonad, and brain by an ion-pairing liquid extraction procedure and quantified using high performance liquid chromatography with electrospray ionization tandem mass spectrometry. The limit of detection (LOD) was 0.4 ng/g wet weight (ww). PFOA was not detectable in unexposed fish or in fish exposed to 200 ng/l, but was >LOD in most samples of carp exposed to 2 mg/l. Mean PFOA concentration ranged from 0.5 to 65 ng/g ww, depending on the tissue, with highest levels in blood and liver. There were no significant differences in condition factor, hepato-somatic index, or gonado-somatic index among the fish of the three groups. Histological, histochemical, and immunohistochemical staining was performed on sections of liver and gonad. Occurrence of atretic oocytes and a paucity of spermatozoa were documented in carp treated with 2 mg/l PFOA. Exposed fish did not show gross hepatic anomalies, but there was enhancement of hepatocytes in proliferation (positive to anti-PCNA antibody) compared to controls.

Keywords: perfluorinated alkylated substances; endocrine disruptors, bioaccumulation, hepatocyte proliferation, oocyte atresia; spermatozoa suppression

Introduction

Since fish are common components of aquatic ecosystems and an important human food source (Nania et al. 2009), knowledge of tissue accumulation and consequences of their exposure to emergent pollutants such as perfluorooctanoic acid (PFOA) is essential to understanding the potential risks for the environment, wildlife, and human health.

PFOA has the chemical formula $CF_3(CF_2)_6COOH$ and belongs to perfluorinated alkylated substances (PFAS), a class of organic contaminants of global concern (EFSA 2008; Suja et al. 2009; Lindstrom et al. 2011; Post et al. 2012). Due to their stability and resistance to degradation, PFAS have been, and are currently, used in a wide range of applications (Prevedouros et al. 2006; Suja et al. 2009; Ahrens and Bundschuh 2014). In particular, PFOA is employed as an adjuvant/emulsifier in the production of fluoropolymers such as polytetrafluoroethylene and Teflon for coatings on cooking pans and surfactants in aqueous film-forming foams (AFFFs) and personal care products such as soap and shampoos (Prevedouros et al. 2006; Davis et al. 2007; Suja et al. 2009; Post et al. 2012). PFOA and other PFAS are widely diffused and persistent in the environment and in living organisms (EFSA 2008; Houde et al. 2011; Ahrens and Bundschuh 2014). PFOA has been found in relatively high concentrations in rivers in the USA (Hansen et al. 2002; Davis et al. 2007) and in Europe (Loos et al. 2009). The Po River in Northern Italy was identified as the dominant source of PFOA on the European continent (McLachlan et al. 2007; Loos et al. 2008).

PFOA has carcinogenic (ECHA 2012) and endocrine disrupting properties (White et al. 2011). PFOA's estrogen-like activity has been documented in rare minnow *Gobiocypris rarus* and rainbow trout *Oncorhynchus mykiss* (Wei et al. 2007, 2008; Tilton et al. 2008; Benninghoff et al. 2011). The present research used the common carp *Cyprinus carpio* L. as an animal model. Data on PFAS in carp are available from both field (Ye et al. 2008a, b) and experimental studies (Kim et al. 2010; Inoue et al. 2012).

Histology of model fish species has proven useful in many areas of toxicology, including the study of endocrine disruptors (ED) (Wester et al. 2002; Leino et al. 2005). In particular, fish gonad histology is pivotal in understanding the estrogenic contaminant effects (Sumi et al. 2007; Tanna et al. 2013). Histopathological changes do not appear as early as, and are less sensitive than, biochemical or genome level markers but have a greater ecological relevance compared to other biomarker responses at lower levels of organization (van der Oost et al. 2003).

The primary goal of this study was to contribute to the knowledge of accumulation, tissue distribution, and toxicological effects of PFOA. Accumulation and impact of PFOA on fish tissues was examined following sub-chronic exposure (56 days) at 200 ng/l, a level found in impacted aquatic ecosystems and thus of environmental relevance, and at 2 mg/l, for comparison with published experimental data.

Materials and Methods

Fish and exposure test

Thirty-one two-year-old common carp *Cyprinus carpio* L. were purchased from a local fish farm in September 2012 and acclimated according to the OECD (1996) test guidelines 305. Four weeks prior to the start of the experiment, fish were transferred into the test tanks to adapt to the exposure environmental conditions. The fish were fed pelleted feed three times per week with feeding stopped two days before fish were killed.

Feeding was carried out manually to ensure rapid and complete consumption. Uneaten feed and feces were removed from the tank to minimize PFOA sequestration by these organic substances.

Fish were placed into each of three 120 l glass aquaria filled with tap water, with a continuous supply of fresh water provided at a flow-through rate of 500 ml/min. Stock solutions of PFOA were continuously dispensed and diluted to deliver the tested concentrations to the tanks over a period of 56 days. Exposures were conducted at 200 ng/l (n=10) and 2 mg/l (n=11), levels based on environmental reports (Loos et al. 2008, 2009) and on reported experimental data, respectively (Oakes et al. 2004; Wei et al. 2007). A control group of 10 carp were held in tap water only. The stock solutions were prepared by dissolving perfluorooctanoic acid (PFOA standard, chemical purity 96%, Sigma-Aldrich) in distilled water and delivered into the treatments tanks by a peristaltic pump at a flow rate of 0.42 ml/min. At time 0 of exposure, an initial volume of the stock solution was added to the treatments tanks to immediately achieve the desired exposure concentration. Water parameters were monitored and recorded three times weekly for temperature (10-15 °C), pH (6.70-8.00), and oxygen saturation (>80%). PFOA concentration in the test water from each test tank was measured by liquid chromatography-mass spectrometry three times during the exposure period. The results of these analyses indicate that PFOA concentration was maintained at >80% of each nominal concentration.

At the end of the 56 day exposure, the fish were anesthetized with MS-222, killed by a deep cut through the neck, and dissected. Sex was recorded, and animals were measured for total length (TL), body wet weight, and liver and gonad weight. Condition factor (K), hepato-somatic index (HSI) and gonado-somatic index were calculated respectively according to the following formulae:

K = body weight (g)/TL(cm)^3 x 100

HSI = liver weight (g)/body weight (g) x 100

GSI = gonad weight (g)/body weight (g) x 100

Samples of whole blood, gill, liver, skinned white muscle, kidney, gonad, and brain were flash-frozen and stored at -20 °C until processing for PFOA analysis.

Histology

Fragments of liver and gonad were fixed in 10% neutral buffered formalin for 24 h and subsequently stored in 70% ethanol processing for histology. The fixed tissues were dehydrated through an alcohol series and paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor. Five µm sections were cut from each tissue block, stained with hematoxylin and eosin (H&E) and/or Alcian blue 8 GX, pH 2.5, combined with periodic acid Schiff's reagent (AB/PAS).

Histological sections of liver were subjected to indirect immunohistochemistry (peroxidase-anti-peroxidase immunocomplex) using a commercially available antibody anti-PCNA (PC10 sc-56 mouse monoclonal antibody, Santa Cruz Biotechonology, Inc.). The method followed was reported in Dezfuli et al. (2012). For each fish, the ratio of PCNA positive nuclei was determined by scoring 1,000 hepatocytes per individual in 2 randomly selected fields examined via light microscopy at × 400 magnification using computerized image analyzer software (Nis Elements AR 3.0).

Sections were examined by light microscopy using a Nikon Microscope ECLIPSE 80i, without knowledge of experimental group.

Analysis of PFOA in tissue

Concentrations of PFOA in whole blood, gill, liver, kidney, gonad, muscle, and brain were analyzed to determine the accumulation and distribution patterns after 56 days of waterborne exposure. PFOA was extracted using an ion-pairing extraction procedure and measured using high performance liquid chromatography with electrospray ionization tandem mass spectrometry. The method used was detailed in Guerranti et al. (2013) and Giari et al. (2015).

Quality assurance and Quality control (QA/QC) of PFOA determination

Laboratories complied with ISO 9001:2000 and ISO 14001 standards for ecotoxicological analysis. Chemicals and reagents were analytical grade, and Teflon coated labware were avoided during the whole process of sampling, pre-treatment and analysis to minimize contamination of the samples. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analysed with each set of five tissue samples as a check for possible laboratory contamination and interferences; recoveries, assessed using spiked matrix with a concentration of 5 ng/g of the analyte, were over 93% in blood, and over 89% in tissues. LOD, determined as three times the signal-to-noise (S/N) ratio, was 0.4 ng/g wet weight (ww).

Statistics

The statistical package Statistica v 7.1 (StatSoft Inc., Tulsa, OK) was used to analyze data, with significance set at $p < 0.05$. The mean, standard deviation, and range were determined for PFOA in tissues/organs of each carp from the three experimental groups. To allow inclusion of all samples in the statistical analyses, those in which PFOA was not detected were assigned a value of one-half the LOD of 0.4 ng/g ww.

The Shapiro–Wilk's and Levene's Tests were used to check the normality and the homogeneity of variance of the data. Kruskal–Wallis test was applied to assess difference in tissue PFOA concentrations among the three experimental groups. One-way analysis of variance (ANOVA) was used to compare K, HSI, and GSI among treatment groups. The number of PCNA positive hepatocytes (comparing unexposed vs. exposed, unexposed vs. 200 ng/l; unexposed vs. 2 mg/l, 200 ng/l vs. to 2 mg/l) and PFOA concentration in tissue/organs were assessed by contrast analysis. Correlation of the measured PFOA with fish size was evaluated using the Pearson coefficient.

Results

No mortality was observed during the acclimation or test in either control or treatment fish. In each group of carp the sex ratio was approximately 1:1 (unexposed 5♀:5♂; 200 ng/l 4♀:6♂; 2 mg/l 6♀:5♂). Following 56 day exposure, no significant differences were observed in K, HSI, or GSI (compared between sexes), among the three groups (Table 1).

Analysis of PFOA in tissues

In unexposed carp and in carp exposed to 200 ng/l PFOA, tissue concentrations were below the LOD and were significantly lower than those in carp exposed to 2 mg/l in all examined tissues (Kruskal Wallis, $p < 0.05$). In fish treated with 2 mg/l, all samples, with the exception of some brain tissue, showed detectable PFOA. Mean

concentrations ranged from 0.45 to 64.87 ng/g ww, with organs exhibiting differing capacities for accumulating PFOA (Table 2). The mean PFOA level was highest in blood, followed by liver and then, muscle, gill, gonad, kidney, and finally brain (Table 2). The mean PFOA concentration in blood was significantly higher than that in the other organs examined (ANOVA, $p < 0.05$). PFOA levels in muscle, gill, gonad, kidney, and brain showed no significant differences (ANOVA, $p > 0.05$), and all were lower than that in liver (ANOVA, $p < 0.05$).

There were no significant differences in mean tissue PFOA concentrations between the sexes in any tissues/organs (*t* test, $p > 0.05$), with the exception of kidney, which exhibited higher level in females (*t* test, $p < 0.05$). No significant correlations were found between PFOA concentration in organs and total length or weight (Pearson correlation, $p > 0.05$) of carp.

Histology

Histological examination of the ovaries revealed the pre-vitellogenic oocytes to be dominant. Oocytes were characterized by homogenous eosinophilic cytoplasm and numerous nucleoli at the periphery of an enlarged nucleus (Fig. 1a,b). Oogonia were present and, in many ovaries, the adipose component of the organ was abundant (Fig. 1a,c). In the testes, seminiferous tubules showed reproductive cells at various stages of the spermatogenic cycle: spermatogonia, spermatocytes, spermatids, and spermatozoa (Fig. 2a, b, c). Ovaries and testes in both controls and carp exposed to 200 ng/l PFOA appeared normal (Figs. 1a, b and 2b, c), while pathological changes were found in the gonads of carp treated with 2 mg/l PFOA. Females exposed to 2 mg/l had an elevated incidence of degenerating oocytes (atresia) characterized by disorganization of the ooplasm (Fig. 1d, e). In males exposed to 2 mg/l PFOA, spermatogonia and spermatocytes were the dominant germ cells with no or rare spermatids and spermatozoa, suggesting a possible delay of spermatogenesis (Fig. 2d). In some cases, an increased amount of interstitial tissue and proliferation of Sertoli cells in the testes was observed in fish exposed to the highest concentration compared to other two groups (Fig. 2d). No occurrences of macrophage aggregates (MA) or intersex were observed in gonad tissues of any carp.

The hepatic sections of exposed carp appeared similar to those of the control, with no evidence of degeneration. In all groups, the hepatocyte cytoplasm was vacuolated with a foamy appearance (Fig. 3a, b, c). Glycogen deposits, revealed by PAS staining, were present in both control and treated fish (Fig. 3d, e). In liver of fish exposed to PFOA there was a significantly higher number of hepatocytes immunoreactive to PCNA (*i.e.* in proliferation) compared to that of controls (ANOVA, $p < 0.05$; Fig. 3f, g). The PCNA-positive hepatocytes were more abundant in carp exposed to 2 mg/l PFOA than in unexposed carp (ANOVA, $p < 0.05$; Fig. 3f, g), while there was no significant difference between carp treated with 200 ng/l and control (ANOVA, $p > 0.05$) or between carp exposed to 200 ng/l and those exposed to 2 mg/l (ANOVA, $p > 0.05$).

Discussion

PFAS are ubiquitous pollutants of waters worldwide (Falk et al. 2014). Together with perfluorooctane sulfonate (PFOS), PFOA is one of the primary PFAS found as residues in the environment (Giesy et al. 2001). Several field and laboratory studies on vertebrates have identified blood, liver, and kidney as key sites of PFAS accumulation (Ahrens et al. 2009; Yoo et al. 2009; Peng et al. 2010; Ng and Hungerbuhler 2013; Falk et al. 2014). Our results showed marked differences in PFOA bioaccumulation among the tissues/organs, with high hematic and hepatic levels. Due to PFAS affinity to specific proteins, including serum albumin (Han et al. 2003;

196 Jones et al. 2003), PFOA accumulates particularly in plasma and in highly vascularized areas such as liver
197 (Martin et al. 2003a, b; Ng and Hungerbuhler 2013; Consoer et al. 2014). PFAS in the liver also bind to fatty-
198 acid binding protein (Ng and Hungerbuhler 2013). The gonad is a main target organ of PFOA, which is known
199 to exert estrogenic effects; however research quantifying PFOA in ovaries and testes of experimentally exposed
200 fish is scarce (Martin et al. 2003a; Lee and Schultz 2010). In rainbow trout after 12 days aqueous exposure,
201 PFOA concentrations were highest in blood > kidney > liver > gall bladder and lowest in gonads > muscle > gill
202 > adipose tissue (Lee and Schultz 2010). Martin et al. (2003a, b) and Consoer et al. (2014), conducting
203 waterborne, dietary, and intra-arterial injection exposure tests, demonstrated that PFOA has little affinity for fish
204 muscle. Our results on tissue distribution in carp after 56 days of waterborne exposure confirm that gonad and
205 muscle are not key organs for PFOA accumulation, exhibiting mean concentrations markedly lower than those
206 found in blood and liver.
207 Sex differences in concentration profiles, with higher PFOA levels in males than in females, have been
208 observed in fathead minnows *Pimephales promelas* and zebrafish *Danio rerio* (Lee and Schultz 2010; Hagenaars
209 et al. 2013). Sex differences are associated with different excretion rates (females eliminate PFOA more rapidly
210 than males) and have also been reported in rats (Kudo et al. 2002; Vandel Heuven et al. 2006). Results of the
211 present study did not agree with these data; male and female carp showed similar levels of accumulation in most
212 organs. It is reasonable that not all animals will exhibit sex differences in PFOA elimination (Lee and Schultz
213 2010). Besides gender, numerous other factors including age and size, reproductive stage, diet, type of organisms
214 (water-breathing or air-breathing; poikilotherms or homeotherms) could affect the PFOA accumulation pattern
215 (Sijm and van der Linde 1995; Martin et al. 2003b, 2004; Ye et al. 2008). The data reported here on distribution
216 and concentration of PFOA in tissues refer to two-year-old carp and extrapolation to other fish species or even to
217 smaller (juveniles) or larger size carp should be exercised with caution. Indeed Hoff et al. (2005) and Ye et al.
218 (2008a) noted the lack of correlation of perfluoroalckyl acids concentration with weight in *Cyprinus carpio*.
219 The absence of significant differences in K, HSI, and GSI between unexposed and exposed carp suggests
220 that the PFOA concentrations and duration of exposure used in this study did not affect overall fish health and
221 the mass of liver or gonad. Similar findings were reported by Yang (2010) in male Japanese medaka *Oryzias*
222 *latipes* exposed to 10, 50, 100 mg/l PFOA for 7 days. No significant change of HSI was found in common carp
223 after 4 days of exposure to PFOA and PFOS (Kim et al. 2010) or in rainbow trout exposed 12 days to
224 perfluorinated acids (Martin et al. 2003a), suggesting that these treatments did not cause hepatomegaly.
225 Swordtail fish *Xiphophorus helleri* exposed to PFOS had similar K values but increased HSI in both sexes and
226 lower GSI in females, compared to controls (Han and Fang 2010). HSI and GSI increased in rats following 28
227 days administration of PFOS or PFOA (Cui et al. 2009). Although liver and gonad indices did not show gross
228 alteration in either group of PFOA-exposed carp, histological changes were observed.
229 Since several chemicals, including PFAS, may act as EDs, there is interest in assessing fish reproductive
230 health, and evaluation is frequently based on gonad histology (Blazer 2002). Information on the histological
231 effects of PFOA in fish gonads is limited, and most studies have involved exposure to water concentration many
232 times the highest environmental PFOA. The gonads of carp exposed 56 days to 200 ng/l PFOA in this study
233 appeared normal, suggesting a lack of reproductive impairment at the histological level. This is in agreement
234 with results of Hagenaars et al. (2013), who reported no effects on reproduction in zebrafish exposed to 0.1, 0.5,
235 or 1 mg/l PFOA for one month. No histological alterations were documented in gonads of female eels *Anguilla*

anguilla inhabiting the Po River and naturally exposed to PFOA (Giari et al. 2015). In female rare minnows exposed for 28 days to 3, 10, and 30 mg/l PFOA, ovaries underwent atresia of vitellogenic oocytes, and, in some males treated at 10 and 30 mg/l, occurrence of testes/ova and reduced numbers of sperm cells were observed (Wei et al. 2007). In the present study, atretic oocytes and a paucity of spermatozoa were observed in carp exposed to a similar PFOA concentration (2 mg/l). In sexually mature fathead minnows exposed to 0.3 and 1 mg/l PFOS for 21 days, an elevated incidence of atretic follicles and occurrence of more and/or larger MAs compared to controls were observed (Ankley et al. 2005). Atresia of vitellogenic oocytes is a degenerative and resorptive process that is a natural physiological process, but increased atresia, especially of pre-vitellogenic oocytes, can indicate a pathological condition linked to exposure to contaminants (Blazer 2002). Altered sexual maturation and gamete production, such as disproportional distribution of germ cell types with proliferation or absence of a single cell population, might be a result of endocrine disruption. Delayed spermatogenesis has been reported in fish affected by estrogenic exposure in fresh waters (Jobling et al. 2002; Bjerregaard et al. 2006).

The liver is frequently examined in ecotoxicology studies due to its high sensitivity to contaminants, and because alterations to its structure can be significant in fish health (Myers et al. 1998; Tophon et al. 2004). Studies on mammals have identified the liver as the primary target organ of both acute and chronic exposure to PFAS (Seacat et al. 2003; Cui et al. 2009). Hepatic effects of PFOA are well documented in rodents and comprise cellular hypertrophy, changes in enzyme activity, liver weight increase, hypolipidemia, and peroxisome and smooth endoplasmic reticulum proliferation (EFSA 2008). There are less available data on liver toxicity in fish, with studies reporting different results depending on studied species and focused on enzyme activity and gene expression perturbations (Oakes et al. 2004; Liu et al. 2007, 2009; Yang et al. 2010). Contrary to the present results, depletion of liver glycogen was documented in zebrafish exposed to 0.1, 0.5 and 1 mg/l PFOA for 4 and 28 days (Hagenaars et al. 2013). Recent investigations on fish and mouse models suggest that PFOA affects the metabolism of carbohydrates and lipids by altering the gene expression of proteins involved in their biosynthesis and transport (Wei et al. 2008b; Fang et al. 2010; Haagenars et al. 2013; Zhang et al. 2014; Yan et al. 2015). Specimens of rare minnows exposed to PFOA showed significant changes in the expression of apolipoprotein genes, genes involved in fatty acid biosynthesis and transport, and genes associated with transport of cholesterol (Wei et al. 2008b; Fang et al. 2010). These modifications may influence lipid metabolism and related fish physiological functions**,** including reproduction. Indeed, PFOA can induce testis dysfunction in mouse by modifying steroidogenic gene expression levels (Zhang et al. 2014) and it is presumable that in fish a similar molecular mechanism may also contribute to the reproductive toxicity of PFOA.

A number of experimental studies have examined the relationship between PCNA expression and exposure to toxicants in fish, with particular attention to liver (Ortego et al. 1995; Blas-Machado et al. 2000; Kong et al. 2008). Altered expression of PCNA, a 36 kd protein directly involved in DNA synthesis, can be detectable through immunohistochemical techniques, and can serve in toxicity bioassays as a warning of changes in cell proliferation (Ortego et al. 1994, 1995). Our immunohistochemical results demonstrated a significantly higher number of PCNA-positive hepatocytes in the liver of carp exposed to PFOA than in unexposed specimens. An enhanced expression of PCNA is widely accepted as a marker associated with the development of neoplastic tissue (Ortego et al. 1994; Haramis et al. 2006). The increase in hepatocyte proliferation reported here indicates a deviation from normal function that may be elicited by PFOA exposure. PFOA is known to promote hepatic

tumors in mammals and fish via peroxisome proliferation or through mechanisms involving estrogenic signals (Abdellatif et al. 1991; EFSA 2008; Tilton et al. 2008; Yang et al. 2010).

Conclusions

There was a clear difference in the concentrations of PFOA among organs and experimental groups. Carp exposed to 200 ng/l, a concentration possible in the field, exhibited PFOA levels similar to control fish and significantly lower than those exposed to 2 mg/l. Fish exposed to 2 mg/l PFOA, well above reported environmental levels, responded with signs of alteration in liver and gonads. Based on the species/endpoint evaluated, the lowest PFOA concentration used would not appear to represent potential risk to fish fecundity. Carp of this age and at this reproductive stage appeared to tolerate exposure to 200 ng/l PFOA over the two-month experimental period. However, effects at the molecular/transcriptional level, which responds more rapidly, and the responses to chronic exposure have yet to be explored.

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Conflict of Interest

The authors declare that they have no conflict of interest.

296 **REFERENCES**

297 Abdellatif AG, Preat V, Taper HS, Robertfroid M (1991) The modulation of rat liver carcinogenesis by
298 perfluorooctanoic acid, a peroxisome proliferator. Toxicol Appl Pharmacol 111:530-537.

299 Ahrens L, Bundschuh M (2014) Fate and effects of poly- and perfluoroalkyl substances in the aquatic
300 environment: a review. Environ Toxicol Chem 33:1921-1929.

301 Ahrens L, Siebert U, Ebinghaus R (2009) Total body burden and tissue distribution of polyfluorinated
302 compounds in harbor seals (*Phoca vitulina*) from the German Bight. Mar Pollut Bull 58:520-525.

303 Ankley GT, Kuehl DW, Kahl MD, Jensen KM, Linnum A, Leino RL, Villeneuvet DA (2005) Reproductive and
304 developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the
305 fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 24:2316-2324.

306 Benninghoff AD, Bisson WH, Koch DC, Ehresman DJ, Kolluri SK, Williams DE (2011) Estrogen-like activity
307 of perfluoroalkyl acids in vivo and interaction with human and rainbow trout estrogen receptors in vitro.
308 Toxicol Sci 120:42-58.

309 Bjerregaard LB, Madsen AH, Korsgaard B, Bjerregaard P (2006) Gonad histology and vitellogenin
310 concentrations in brown trout (*Salmo trutta*) from Danish streams impacted by sewage effluent.
311 Ecotoxicology 15:315-327.

312 Blas-Machado U, Taylor HW, Means JC (2000) Apoptosis, PCNA, and p53 in *Fundulus grandis* fish liver after
313 in vivo exposure to N-methyl-N'-nitro-N-nitrosoguanidine and 2-aminofluorene. Toxicol Pathol 28:601-
314 609.

315 Blazer VS (2002) Histopathological assessment of gonadal tissue in wild fishes. Fish Physiol Biochem 26:85-
316 101.

317 Consoer DM, Hoffman AD, Fitzsimmons PN, Kosian PA, Nichols JW (2014) Toxicokinetics of
318 perfluorooctanoate (PFOA) in rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 156, 65-73.

319 Cui L, Zhou QF, Liao CY, Fu JJ, Jiang GB (2009) Studies on the toxicological effects of PFOA and PFOS on
320 rats using histological observation and chemical analysis. Arch Environ Contam Toxicol 56:338-349.

321 Davis KL, Aucoin MD, Larsen BS, Kaiser MA, Hartten AS (2007) Transport of ammonium perfluorooctanoate
322 in environmental media near a fluoropolymer manufacturing facility. Chemosphere 67:2011-2019.

323 Dezfuli BS, Giari L, Lui A, Squerzanti S, Castaldelli G, Shinn AP, Manera M, Lorenzoni M (2012) Proliferative
324 cell nuclear antigen (PCNA) expression in the intestine of *Salmo trutta trutta* naturally infected with an
325 acanthocephalan. Parasites & Vectors 5:198.

326 ECHA–European Chemicals Agency (2012) Opinions of the Committee for Risk Assessment on proposals for
327 harmonised classification and labelling. [http://echa.europa.eu/web/guest/opinions-of-the-committee-for-
328 riskassessment-on-proposals-for-harmonised-classification-and-labelling].

329 EFSA–European Food Safety Authority (2008) Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid
330 (PFOA) and their salts. Scientific Opinion of the Panel on Contaminants in the Food chain (Question N,
331 EFSA-Q-2004-163), adopted on 21.02.008. EFSA Journal 653, 1–131.

332 Falk S, Failing K, Georgii S, Brunn H, Stahl T (2015) Tissue specific uptake and elimination of perfluoroalkyl
333 acids (PFAAs) in adult rainbow trout (*Oncorhynchus mykiss*) after dietary exposure. Chemosphere 129:150-
334 156.

335 Fang X, Wei Y, Liu Y, Wang J, Dai J (2010) The identification of apolipoprotein genes in rare minnow
336 (*Gobiocypris rarus*) and their expression following perfluorooctanoic acid exposure. Comp Biochem
337 Physiol C Toxicol Pharmacol 151:152-159.
338 Giari L, Guerranti C, Perra G, Lanzoni M, Fano EA, Castaldelli G (2015) Occurrence of
339 perfluorooctanesulfonate and perfluorooctanoic acid and histopathology in eels from north Italian waters.
340 Chemosphere 118:117–123.
341 Giesy JP, Kannan K, Jones PD (2001) Global biomonitoring of perfluorinated organics. Scient World 1:627-629.
342 Guerranti C, Perra G, Corsolini S, Focardi SE (2013) Pilot study on levels of perfluorooctane sulfonic acid
343 (PFOS) and perfluorooctanoic acid (PFOA) in selected foodstuffs and human milk from Italy. Food Chem
344 140:197-203.
345 Hagenaars A, Vergauwen L, Benoot D, Laukens K, Knapen D (2013) Mechanistic toxicity study of
346 perfluorooctanoic acid in zebrafish suggests mitochondrial dysfunction to play a key role in PFOA toxicity.
347 Chemosphere 91:844-856.
348 Han J, Fang Z (2010) Estrogenic effects, reproductive impairment and developmental toxicity in ovoviparous
349 swordtail fish (*Xiphophorus helleri*) exposed to perfluorooctane sulfonate (PFOS). Aquat Toxicol 99:281-
350 290.
351 Han X, Snow T, Kemper R, Jepson G (2003) Binding of perfluorooctanoic acid to rat and human plasma
352 proteins. Chemic Res Toxicol 16:775-781.
353 Hansen KJ, Johnson HO, Eldridge JS, Butenhoff JL, Dick LA (2002) Quantitative characterization of trace
354 levels of PFOS and PFOA in the Tennessee River. Environ Sci Technol 36:1681-1685.
355 Haramis APG, Hurlstone A, van der Velden Y, Begthel H, van den Born M, Offerhaus GJA, Clevers HC (2006)
356 Adenomatous polyposis coli-deficient zebrafish are susceptible to digestive tract neoplasia. EMBO Rep
357 7:444-449.
358 Hoff PT, Van Campenhout K, Van de Vijver K, Covaci A, Bervoets L, Moens L, Huyskens G, Goemans G,
359 Belpaire C, Blust R, De Coen W (2005) Perfluorooctane sulfonic acid and organohalogen pollutants in liver
360 of three freshwater fish species in Flanders (Belgium): relationships with biochemical and organismal
361 effects. Environ Pollut137:324–333.
362 Houde M, De Silva AO, Muir DCG, Letcher RJ (2011) Monitoring of perfluorinated compounds in aquatic
363 biota: An updated review. Environ Sci Technol 45:7962-7973.
364 Inoue Y, Hashizume N, Yakata N, Murakami H, Suzuki Y, Kikushima E, Otsuka M (2012) Unique
365 physicochemical properties of perfluorinated compounds and their bioconcentration in common carp
366 *Cyprinus carpio* L. Arch Environ Contam Toxicol 62:672-680.
367 Jobling S, Beresford N, Nolan M, Rodgers-Gray TP, Brighty G, Sumpter JP, Tyler CR (2002) Altered sexual
368 maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage
369 effluents. Biol Reprod 66:272-281.
370 Jones PD, Hu WY, De Coen W. Newsted JL, Giesy JP (2003) Binding of perfluorinated fatty acids to serum
371 proteins. Environ Toxicol Chem 22:2639-2649.
372 Kim W-K, Lee S-K, Jung J (2010) Integrated assessment of biomarker responses in common carp (*Cyprinus*
373 *carpio*) exposed to perfluorinated organic compounds. J Haz Mat 180:395-400.

Kong RYC, Giesy JP, Wu RSS, Chen EXH, Chiang MWL, Lim PL, Yuen BBH, Yip BWP, Mok HOL, Au DWT (2008) Development of a marine fish model for studying in vivo molecular responses in ecotoxicology. Aquat Toxicol 86:131-141.

Kudo N, Katakura M, Sato Y, Kawashima Y (2002) Sex hormone-regulated renal transport of perfluorooctanoic acid. Chem Biol Interact 139:301-316.

Lee JJ, Schultz IR (2010) Sex Differences in the Uptake and Disposition of Perfluorooctanoic Acid in Fathead Minnows after Oral Dosing. Environ Sci Technol 44:491-496.

Leino RL, Jensen KM, Ankley GT (2005) Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). Environ Toxicol Pharmacol 19:85-98.

Lindstrom AB, Strynar MJ, Libelo EL (2011) Polyfluorinated compounds: Past, present, and future. Environ Sci Technol 45:7954-7961.

Liu C, Du Y, Zhou B (2007) Evaluation of estrogenic activities and mechanism of action of perfluorinated chemicals determined by vitellogenin induction in primary cultured tilapia hepatocytes. Aquat Toxicol 85:267-277.

Liu Y, Wang J, Liu Y, Zhang H, Xu M, Dai J (2009) Expression of a novel cytochrome P450 4T gene in rare minnow (*Gobiocypris rarus*) following perfluorooctanoic acid exposure. Comp Biochem Physiol, part C 150:57-64.

Loos R, Locoro G, Huber T, Wollgast J, Christoph EH, de Jager A, Gawlik BM, Hanke G, Umlauf G, Zaldívar J-M (2008) Analysis of perfluorooctanoate (PFOA) and other perfluorinated compounds (PFCs) in the river Po watershed in N-Italy. Chemosphere 71:306-313.

Loos R, Gawlik BM, Locoro G, Rimaviciute E, Contini S, Bidoglio G (2009) EU-wide survey of polar organic persistent pollutants in European river waters. Environ Pollut 157:561-568.

Martin JW, Mabury SA, Solomon KR, Muir DCG (2003a) Bioconcentration and tissue distribution of perfluorinated acids in rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 22:196-204.

Martin JW, Mabury SA, Solomon KR, Muir DCG (2003b) Dietary accumulation of perfluorinated acids in juvenile rainbow trout (*Oncorhynchus mykiss*). Environ Toxicol Chem 22:189-195.

Martin JW, Whittle DM, Muir DC, Mabury SA (2004) Perfluoroalkyl contaminants in a food web from Lake Ontario. Environ Sci Technol 38:5379-5385.

McLachlan MS, Holmstrom KE, Reth M, Berger U (2007) Riverine discharge of perfluorinated carboxylates from the European continent. Environ Sci Technol 41:7260-7265.

Myers MS, Johnson LL, Hom T, Collier TK, Stein JE, Varanasi U (1998) Toxicopathic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: relationships with other biomarkers of contaminant exposure. Mar Environ Res 45:47-67.

Nania V, Pellegrini GE, Fabrizi L, Sesta G, De Sanctis P, Lucchetti D, Di Pasquale M, Coni E (2009) Monitoring of perfluorinated compounds in edible fish from the Mediterranean Sea. Food Chem 115:951-957.

Ng CA, Hungerbuhler K (2013) Bioconcentration of Perfluorinated Alkyl Acids: How Important Is Specific Binding? Environ Sci Technol 47:7214-7223.

Oakes KD, Sibley PK, Martin JW, Solomon KR, Mabury SA, Kraak GLVD (2004) Impact of perfluorooctanoic acid on fathead minnow (*Pimephales promelas*) fatty acyl-CoA oxidase activity, circulating steroids, and reproduction in outdoor microcosms. Environ Toxicol Chem 23:1912-1919.

OECD-Organization for Economic Cooperation, Development (1996) Guidelines for Testing of Chemicals, Guideline 305, Bioconcentration: Flow-through Fish Test. Organization for Economic Cooperation and Development, Paris.

Ortego LS, Hawkins WE, Walker WW, Krol RM, Benson WH (1994) Detection of proliferating cell nuclear antigen in tissues of three small fish species. Biotech Histochem 69:317-323.

Ortego LS, Hawkins WE, Walker WW, Krol RM, Benson WH (1995) Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) in tissues of aquatic animals utilized in toxicity bioassays. Mar Environ Res 39:271-273.

Peng H, Wei Q, Wan Y, Giesy JP, Li L, Hu J (2010) Tissue distribution and maternal transfer of poly- and perfluorinated compounds in Chinese sturgeon (*Acipenser sinensis*): implications for reproductive risk. Environ Sci Technol 44:1868-1874.

Post GB, Cohn PD, Cooper KR (2012) Perfluorooctanoic acid (PFOA), an emerging drinking water contaminant: A critical review of recent literature. Environ Res 116:93-117.

Prevedouros K, Cousins IT, Buck RC, Korzeniowski SH (2006) Sources, fate and transport of perfluorocarboxylates. Environ Sci Technol 40:32–44.

Rumsby PC, McLaughlin CL, Hall T (2009) Perfluorooctane sulphonate and perfluorooctanoic acid in drinking and environmental waters. Philos Transact A Math Phys Eng Sci 367:4119-4136.

Seacat AM, Thomford PJ, Hansen KJ, Clemen LA, Eldridge SR, Elcombe CR, Butenhoff JL (2003) Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. Toxicology 183:117–131.

Sijm D, van der Linde A (1995) Size-dependent bioconcentration kinetics of hydrophobic organic chemicals in fish based on diffusive mass transfer and allometric relationships. Environ Sci Technol 29:2769–2777.

Suja F, Pramanik BK, Zain SM (2009) Contamination, bioaccumulation and toxic effects of perfluorinated chemicals (PFCs) in the water environment: a review paper. Water Sci Technol 60:1533–1544.

Sumi M, Kawashima Y, Fukumaki T, Ishibashi H, Arizono K, Iguchi T, Shimizu M (2007) Comparison of serum vitellogenin, steroid hormone, gonad histopathology and bioaccumulation in common carp (*Cyprinus carpio*) of two rivers and a lake in Japan: potential for endocrine disruption. Environ Sci 14, 41-54.

Tanna RN, Tetreault GR, Bennett CJ, Smith BM, Bragg LM, Oakes KD, McMaster ME, Servos MR (2013) Occurrence and degree of intersex (testis-ova) in darters (*Etheostoma* SPP.) across an urban gradient in the Grand River, Ontario, Canada. Environ Toxicol Chem 32:1981-1991.

Thophon S, Pokethitiyook P, Chalermwat K, Upatham E, Sahaphong S (2004) Ultrastructural alterations in the liver and kidney of white seabass, in acute and subchronic cadmium exposure. Environ Toxicol 19:11–19.

Tilton SC, Orner GA, Benninghoff AD, Carpenter HM, Hendricks JD, Pereira CB, Williams DE (2008) Genomic profiling reveals an alternate mechanism for hepatic tumor promotion by perfluorooctanoic acid in rainbow trout. Environ Health Persp 116:1047-1055.

van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57-149.

Vanden Heuvel JP, Davis JW, Sommers R, Peterson RE (2006) Renal excretion of perfluorooctanoic acid in male rats: inhibitory effect of testosterone. J Biochem Toxicol 7:31-36.

Wei Y, Dai J, Liu M, Wang J, Xu M, Zha J, Wang Z (2007) Estrogen-like properties of perfluorooctanoic acid as revealed by expressing hepatic estrogen-responsive genes in rare minnows (*Gobiocypris rarus*). Environ Toxicol Chem 26:2440-2447.

Wei Y, Chan LL, Wang D, Zhang H, Wang J, Dai J (2008a) Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. J Proteome Res 7:1729-1739.

Wei Y, Liu Y, Wang J, Tao Y, Dai J (2008b) Toxicogenomic analysis of the hepatic effects of perfluorooctanoic acid on rare minnows (*Gobiocypris rarus*). Toxicol Appl Pharmacol 226:285-297.

Wester PW, van der Ven LTM, Vethaak AD, Grinwis GCM, Vos JG (2002) Aquatic toxicology: opportunities for enhancement through histopathology. Environ Toxicol Pharmacol 11:289-295.

White SS, Fenton SE, Hines EP (2011) Endocrine disrupting properties of perfluorooctanoic acid. J Steroid Biochem 127:16–26.

Yan S, Zhang H, Zheng F, Sheng N, Guo X, Dai J (2015) Perfluorooctanoic acid exposure for 28 days affects glucose homeostasis and induces insulin hypersensitivity in mice. Scien Rep 5, Article number 11029.

Yang J-H (2010) Perfluorooctanoic acid induces peroxisomal fatty acid oxidation and cytokine expression in the liver of male Japanese medaka (*Oryzias latipes*). Chemosphere 81:548-552.

Ye X, Schoenfuss HL, Jahns ND, Delinsky AD, Strynar MJ, Varns J, Nakayama SF, Helfant L, Lindstrom AB (2008a) Perfluorinated compounds in common carp (*Cyprinus carpio*) fillets from the Upper Mississippi River. Environ Int 34:932-938.

Ye X, Strynar MJ, Nakayama SF, Varns J, Helfant L, Lazorchak J, Lindstrom AB (2008b) Perfluorinated compounds in whole fish homogenates from the Ohio, Missouri, and Upper Mississippi Rivers. USA. Environ Pollut 156:1227–1232.

Yoo H, Guruge KS, Yamanaka N, Sato C, Mikami O, Miyazaki S, Yamashita N, Giesy JP (2009) Depuration kinetics and tissue disposition of PFOA and PFOS in white leghorn chickens (*Gallus gallus*) administered by subcutaneous implantation. Ecotox Environ Safe 72:26-36.

Zhang H, Lu J, Luo B, Yan S, Guo X, Dai J (2014) Proteomic analysis of mouse testis reveals mice Perfluorooctanoic acid-induced reproductive dysfunction via direct disturbance of testicular steroidogenic machinery. J Proteome Res 13:3370-3385.

Figure legends

Fig. 1. Histological sections of carp ovaries stained with H&E. (**a**) Numerous pre-vitellogenic oocytes (arrows) and some oogonia (arrowheads) in a control carp; bar = 20 μm. (**b**) Carp exposed to 200 ng/l PFOA: normal pre-vitellogenic oocytes (arrows) with homogenous eosinophilic cytoplasm and nucleoli (arrowheads) at the periphery of the nucleus (N); bar = 20 μm. (**c**) Gonad of a control carp: presence of abundant adipose tissue (asterisk) can be seen; bar = 100 μm. (**d, e**) Ovaries of fish exposed to 2 mg/l PFOA showing normal oocytes (arrowheads) and some oocytes in degeneration (arrows); bar = 20 μm.

Fig. 2. Histological sections of carp testes stained with H&E. (**a, b**) Mature gonad of control carp: numerous seminiferous tubules (arrows) filled with spermatogonia (arrowheads), spermatocytes (black asterisk), spermatids (white asterisk), and spermatozoa (arrows); bars = 50 and 20 μm respectively. (**c**) Carp exposed to 200 ng/l PFOA: testes similar to control fish showing reproductive cells at various stages of the spermatogenic cycle; bar = 20 μm. (**d**) Gonad of fish exposed to 2 mg/l PFOA lacking in spermatozoa and rich in spermatogonia, interstitial and Sertoli cells (asterisks); bar = 20 μm.

Fig. 3. Histological sections of carp liver. (**a**) Hepatocytes (arrowheads) in a control carp: note the foamy appearance of the cytoplasms. H&E, bar = 20 μm (**b, c**) Liver of carp exposed to 200 ng/l PFOA (b) and to 2 mg/l PFOA (c): hepatocytes (arrowheads) similar in appearance to those of unexposed fish. Note the occurrence of rodlet cells (arrows) in the epithelium of the biliary ducts. H&E, bars = 20 μm. (**d, e**) Hepatocytes in control (d) and exposed fish (e) displaying similar presence of glycogen granules. AB/PAS, bars = 20 μm. (**f, g**) Liver sections treated with the anti-PCNA antibody: few hepatocytes (arrows) immunopositive (i.e. in proliferation) in control fish (f), and several immunopositive hepatocytes (arrows) in a carp exposed to 2 mg/l PFOA (g); bars = 20 μm.