A Mechanistic Investigation of Perfluoroalkyl Acid Kinetics in Rainbow Trout (*Oncorhynchus mykiss*)

A DISSERTATION SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Daniel M. Consoer

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Advisors Dr. Kendall Wallace, Dr. John Nichols

May 2017

Acknowledgements

I would like to acknowledge the help of my patient advisors Dr. Ken Wallace and Dr. John Nichols, without whom this would have long fallen by the wayside. I want to also thank Pat Fitzsimmons, Alex Hoffman, and Pat Kosian for all the help in the lab, helping to set everything up, navigating the ordering process, teaching me to run the instruments, and how not to run bile samples. I also want to thank Dale Hoff for all his help and support, especially in finding a way to get this work funded. Particular thanks goes to Dr. Nichols, my mentor and primary advisor, for doing so much work above and well beyond the call of duty to help me get everything done and to finally whip this work into shape. Finally, I would like to thank my family, Heather and Johann, for all the love, support, and forbearance.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

PFOS and PFOA as Representative PFAAs

Perfluoroalkyl acids (PFAAs) are synthetic organic compounds which consist in part of a hydrocarbon-like carbon backbone in which all carbon-hydrogen bonds have been replaced with carbon-fluorine bonds. Each molecule also contains an acidic moiety, typically a carboxylate, sulfonate, or phosphonate group (Lau et al., 2007, 2009). Measured p*K*a values for PFAAs are extremely low (≤ 1.0; Moroi et al., 2001; Goss et al., 2008; Cheng et al., 2009). As such, they are almost entirely ionized at typical environmental pH values. The fluorinated carbon backbone gives these compounds some hydrophobic character, while the charged group confers a degree of water solubility, lipophobicity, and surfactant activity (Lau et al., 2007; Lau, 2009; DeWitt, 2015). Carbon-fluorine bonds are extremely resistant to cleavage (Key et al., 1997) even more so than the carbon-chlorine bonds found in other stable chemicals such as the polychlorinated biphenyls (PCBs). While biologically-mediated dehalogenation reactions involving carbon-fluorine compounds are possible, this typically requires the presence of at least one hydrogen bonded to a carbon, and is unable to proceed against a fully fluorinated carbon backbone (Key et al., 1998).

PFAAs and chemical precursors have been used extensively as a surface treatment for carpets, fabric, leather, paper, and direct-contact food packaging, largely because of their grease, dirt, and stain-resistant properties (3M, 1999; Lau et al., 2007; Buck et al., 2011). They also have been used in foams designed to fight fires involving flammable liquids. This application takes advantage of the surfactant properties of these compounds as well as their extreme resistance to heat-mediated breakdown (Buck et al., 2011). In addition, PFAAs and precursors have been used as surfactants for mining

and well production, as mist-suppressants in electroplating applications, and in cleaners, floor polishes, shampoos, and insecticide formulations (Renner, 2001; Seacat, 2002).

The studies described in this dissertation focus on two well-known PFAAs, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). PFOS consists of a fluorinated eight-carbon chain bonded to a terminal sulfonate group, while PFOA contains a fluorinated seven-carbon chain bonded to a carboxylate group. These compounds are noteworthy for several reasons. Both compounds possess a high degree of surfactant activity (Lau et al., 2007; Kissa et al., 2001). Both have been produced in large quantities. For example, estimated global production of POSF, a PFOS precursor, reached 4.5 thousand tons prior to 2002 (Paul et al., 2009). Both compounds have been detected in tissues of humans and wildlife as well as in environmental matrices (e.g. soils, sediments, ground and surface water). Importantly, both of these eight-carbon PFAAs appear to be close to an apparent threshold (relative to shorter chain-length PFAAs) for significant accumulation in wildlife (Lau et al., 2007). Though they both would be expected to possess similar kinetic characteristics, based on the similar number of perfluorinated carbon atoms in each, large differences have been noted in their tendency to accumulate.

PFOS and some precursors (compounds which environmentally degrade to PFOS) were produced by the 3M Corporation (Seacat et al., 2002) using an electrofluorination process which results in a mixture of approximately 70% linear and 30% branched products (Lau et al., 2007; De Silva et al., 2010). Prior to 2002, 3M was also the largest domestic producer of PFOA, resulting in a mix of linear and branched products. After 2002, DuPont became the largest domestic producer of PFOA, using a telomerization process that resulted in a straight-chain product.

As of this writing, domestic production of PFOS and PFOA has been largely phased out. This began with a voluntary phase-out of PFOS and related products by the 3M Corporation in 2002 (Lau et al., 2007). In 2006, US EPA instituted the PFOA Stewardship Program. Under this program, eight major manufacturers of PFOA agreed to reduce emissions of PFOA, PFOA precursors, and longer-chain carboxylate PFAAs by 2010, and eliminate all such emissions by 2015. As of this writing, all eight manufacturers of PFOA had met the goals of the program (US EPA, 2017; DuPont, 2017). This progress may be contrasted with a 2006 EPA estimate of twenty million pounds of global PFOA production, of which more than half was produced in the United States (US EPA, 2009). While the domestic chemical industry has phased out production of PFOS, PFOA, and longer chain PFAAs, it is not known how much the production of these compounds has increased elsewhere in the world as compensation. Moreover, shorter-chain PFAAs continue to be produced in high volumes (Buck et al., 2011). More recent regulatory action was taken in May 2016, when US EPA established health advisories for PFOA and PFOS in drinking water, setting the advisory level at 70 parts per trillion (EPA, 2016).

In 2001, Giesy and Kannan published their findings on the global distribution of PFOS in wildlife, which raised concerns about the possible importance of PFAAs as persistent environmental pollutants (Giesy and Kannan, 2001). Since then, PFAAs have been measured in a wide range of species as well as environmental matrices far from any known sources (Prevedouros et al., 2006). In virtually all cases, PFOS has been found at the highest concentrations (Giesy and Kannan, 2001; Kannan, 2001, 2005; Houde et al., 2006), although PFOA is often present at detectable levels. Houde et al. (2008) measured PFOS and other PFAAs in several invertebrates, though typically at

low concentrations. Other investigators have measured PFAAs in birds, reptiles, and amphibians (Giesy and Kannan, 2001; Kannan et al., 2001; Kannan et al., 2005). Zhang et al. (2010) studied PFAAs in chickens and eggs as a potential source for human exposure.

As with several other persistent organic pollutants, PFAAs have been widely studied in mammals that feed at the top of aquatic food webs, including seals (Ishibashi et al., 2008), whales (Reiner et al., 2011), and polar bears (Giesy and Kannan, 2001). These studies have provided evidence for very high levels of environmental exposure, as indicated by measured PFOS concentrations in blood exceeding 100 ppm (Giesy and Kannan, 2001; Moody et al., 2001). PFAAs also have been measured in terrestrial mammals such as caribou (Ostertag et al., 2009) and wolves (Muller et al., 2011) to better understand dietary sources of PFAA exposure. Of most significance to the studies which make up this dissertation, there are numerous reports of PFAAs in fish. PFOS and PFOA have been measured in fish collected across North America (Giesy and Kannan, 2001; Martin et al., 2004; Kannan et al., 2005) and in Asia (Murakami et al., 2011).

Toxicity of PFAAs to Mammals and Fish

PFAAs and other Perfluoroalkyl Substances (PFASs) do not appear to form DNA adducts or react with proteins in ways that would cause direct cellular toxicity. Initially, therefore, these compounds were considered biologically inert. It has been widely noted, however, that PFAAs are structurally and (to a limited extent) chemically similar to fatty acids. While they are not thought to enter into fatty-acid reaction pathways, they may exhibit some behaviors similar to those of ionized fatty acids, including binding to albumin and fatty-acid binding proteins in plasma and liver (Han et al., 2003; Lau et al.,

2007; Dewitt, 2015). PFAAs have been shown to interact with cell membranes (Han et al., 2009) and specific membrane transporters (Nakagawa et al., 2009; Zhao et al., 2017), potentially interfering with normal membrane transport processes. They are also likely to interact with nuclear receptors involved in cellular metabolism which would otherwise be responsive to structurally similar fatty acids (Bjork et al., 2009, 2011; Bijland et al., 2011; Elcombe et al., 2012).

A number of health effects caused by PFAAs have been noted in the literature, typically associated with repeat-dose, sub-chronic, or chronic exposures. Experiments performed using rodents and non-human primates have demonstrated that PFAAs can cause weight-loss, hepatomegaly (typically hypertrophy rather than hyperplasia) with vacuolization, reduced serum cholesterol, and mortality (Lau et al., 2007; Seacat et al., 2002). Rats exposed to a high dose of PFOS developed hepatocellular adenomas (Seacat et al., 2003), while mice dosed with PFOA exhibited a decrease in adaptive immune response (Yang et al., 2002, DeWitt et al., 2008). In a number of studies in which mice were dosed with PFOA during gestation, mammary tissue development was impaired in both dams and offspring (Macon et al., 2011; White et al., 2007, 2008, 2011).

Several PFAAs have been shown to exhibit endocrine activity (Kjeldsen and Bonefeld-Jorgensen, 2013). For example, pregnant rats dosed with perfluorodecanoic acid (PFDA; Langley et al., 1985) or PFOS (Thibodeaux et al., 2003) exhibited decreased levels of serum T3 and T4. Fluorotelomer alcohols, precursors which may degrade to PFAAs, were shown to induce MCF-7 human breast cancer cells, thereby providing evidence for an estrogenic effect (Maras et al., 2006).

The potential for PFAA toxicity mediated by peroxisome proliferator-activated receptor alpha (PPARα) has been extensively investigated (Rosen et al., 2008; Bjork

and Wallace 2009, Bjork et al., 2011). Studies of liver tumors in rodents demonstrated compatibility with PPARα receptor-mediated toxic effects (Klaunig et al., 2003). However, this toxicity pathway is not considered to be relevant to human disease (Lau et al., 2007; Corton et al., 2014). Other studies have found that hepatotoxic effects in liver were due, in part, to activation of CAR/PXR as well as PPARα (Bijland et al., 2011; Elcombe et al., 2012). PFAAs were also shown to interact with the peroxisome proliferator-activated receptor gamma (PPARγ) in mouse and human cells (Takacs and Abbott, 2007).

Toxicity studies with PFAAs in fish have largely focused on the effects of PFOS. These investigations have demonstrated reproductive impairment in adult swordtails (*Xiphoophorous hellerii)* and developmental toxicity in swordtail embryos (Han and Fang, 2010). Other work provided evidence for hepatotoxicity in common carp (*Cyprinus carpio*; Hoff et al., 2003) and effects on zebrafish (*Danio rerio*) embryo behavior (Huang et al., 2010). Shi et al. (2008) found that PFOS exposure during early development produced developmental effects on zebrafish larvae, including spine curvature and heart malformations. Another study using zebrafish found that organogenesis was impaired in zebrafish larva exposed to PFOS during early development (Chen et al., 2014). These effects were correlated with numerous changes in gene expression, including changes in expression of several genes known to be involved in organ development. Wei et al. (2008) used cDNA microarray analysis to study effects on gene transcription in rare minnows (*Gobiocypris rarus*) exposed to PFOA. The observed effects were similar to those associated with PPARα activation in mammals. In addition, the authors noted a downregulation of genes involved in biosynthesis of thyroid hormone, and liver histopathology similar to that seen in mammals. A number of PFAAs and precursor

compounds were shown to induce vitellogenin production in Nile tilapia (*Oreochromis nilotichus*) hepatocytes (PFOS 48 h EC50: $1.5*10^{-5}$M; PFOA: $2.9*10^{-5}$M) which is a marker of estrogenic activity (Liu et al., 2007).

PFAA Kinetics in Mammals

A standard approach for describing chemical kinetics in biological systems is to independently consider the processes of absorption, distribution, metabolism, and elimination (ADME). Each of these processes impacts the rate and extent of chemical accumulation. They also determine, for a given exposure scenario, the chemical concentration-time course in target tissues, which may be associated in turn with observed toxic effects.

Dietary uptake of PFAAs has been studied extensively. PFOA was shown to be more than 90% absorbed in feeding studies with rats (Johnson et al., 1979). Similar high dietary absorption efficiencies were observed in feeding studies with rabbits, mice, and hamsters (Hundley et al., 2006; Jandacek et al., 2010). Carboxylate PFAAs other than PFOA are also taken up from the diet with high efficiency (Kudo et al., 2001, Gannon et al., 2011). PFOS was at least 95% absorbed in rats following oral exposure (Chang et al., 2012).

Most of the information concerning dermal absorption of PFAAs has come from studies of PFOA. Using diffusion cells, Fasano et al. (2005) found that 1.4% of PFOA penetrated rat skin samples after a 48-hour exposure. During the same time period, only 0.05% of PFOA penetrated samples of human skin. In a second *in vitro* study, 39% and 23.4% of PFOA contained in acetone (0.5 mg in 50 µL) penetrated mouse and human skin samples respectively during a 24-hour exposure (Franco et al., 2012). Kudo

(2015) speculated that differences between these studies may have been due to acetone facilitating PFOA uptake in the more recent study. Franco et al. (2012) also studied absorption of PFOA in mice *in vivo*, and found a serum concentration of 150-226 ppm after a 4-day exposure to PFOA applied to the dorsal side of the ear. Earlier *in vivo* studies conducted using rabbits provided evidence for dose-dependent dermal absorption of PFOA (Kennedy, 1985).

Radiolabeled PFOA administered to rats by i.p. injection was shown to accumulate in blood, liver, and kidney; however, distribution to the liver and kidney was sex-dependent, with female rats showing relatively greater distribution to the kidney (Vanden Huevel et al., 1991; Kudo et al., 2007). These and other studies have shown that the distribution of PFOA is driven by strong interactions with proteins, and that PFOA in plasma is bound almost entirely by serum albumin (Han et al., 2003). Measured PFOA liver/plasma concentration ratios were shown to increase in rats at lower doses (Kudo et al., 2007), possibly due to saturation of membrane transporters in the liver (Han et al., 2008). Similar tissue distribution patterns have been observed for PFOS and other sulfonate PFAAs, with a notable exception being that PFOS liver/plasma ratios in rats tend to be higher (approximately 10:1) than those determined for PFOA (Chang et al., 2012). Because of concerns for human developmental toxicity, PFOS distribution to breast milk and fetal tissue has also been studied (Hinderliter et al., 2005). The steady-state breast milk/plasma concentration ratio was approximately 0.1. A slightly higher ratio was reported for fetal tissue.

Studies have shown PFAAs are not metabolized in mammals. An early study by Ophaug and Singer (1980) showed that PFOA administration by oral intubation had no effect on ionic fluoride concentration in serum or urine of female rats, demonstrating that

no dehalogenation reactions were occurring. In another earlier study, male and female rats were given an i.p. dose of [^{14}C] PFOA (Vanden Huevel et al., 1991). Radiolabeled carbon in urine and bile was present in a single peak chromatographically identifiable with parent PFOA. Other studies have failed to find phase I or phase II metabolic products for PFOA (Johnson et al., 1984; Ylinen et al., 1989; Kemper et al., 2005). It is noteworthy, however, that while PFAAs do not appear to be metabolized in mammals, one of the sources of PFAAs in the environment is the degradation of precursor compounds (Young et al., 2010). PFOS and PFOA may be formed by the breakdown of a number of precursors through both abiotic and biotic processes (Dinglasan et al., 2004; Lau et al. 2007).

One of the interesting features of the PFAAs is that way that their toxicological effects vary, sometimes greatly, between species and between the sexes within a species. In an early study with rats, Kawashima et al. (1989) showed that there were sex-linked differences in acute toxicity of PFOA associated with disturbances in lipid metabolism. This difference was later found to be correlated with sex-linked differences in biological half-life ($T_{½}$; Ylinen et al., 1990; Vanden Huevel et al., 1992; Kudo et al., 2000). Subsequent studies have shown that species and sex differences in elimination of PFOA can be quite large. For example, the $T_{½}$ of PFOA is a matter of hours in female rats (Ohmori et al., 2003) and both male and female rabbits (Kudo, 2003). Half-lives on the order of days have been noted for male rats (Ohmori et al., 2003) and mice of both sexes (Lou et al., 2009). In monkeys, the $T_{½}$ of PFOA ranges from days to months (Harada et al., 2005), while in humans the $T_{½}$ is several years (Olsen et al., 2007). Similar patterns have been observed for PFOS (Kudo, 2015) and some other PFAAs, though sex differences are not observed in all species. Within a species, the $T_{½}$ of a

PFAA tends to increase as the number of perfluorinated carbons increases (e.g., PFDA > PFOA > perfluorobutanoic acid; PFBA), and is generally greater for sulfonate compounds than for carboxylates of similar carbon chain length (e.g., PFOS > PFOA; Ohmori et al., 2003).

Because PFAAs are not metabolized, differences in $T_{½}$ among species and sexes are largely driven by differences in elimination of the parent compound. For non-volatile substances, the principal routes of elimination are biliary/fecal and urinary. In rats dosed with several carboxylate PFAAs, less than 5% of total elimination was due to elimination in feces (Kudo et al., 2005). Administration of cholestyramine substantially increased fecal elimination of PFOA and PFOS in rats, suggesting that both compounds undergo enterohepatic circulation (Johnson et al., 1984). Nevertheless, the feces may still represent an important route of elimination in cases where renal clearance is especially slow (e.g. PFOS and PFOA in humans, see below).

For most species of mammals, PFAAs are eliminated primarily in urine; however, measured renal clearance rates (CL_R; ml/kg/day) vary widely. For example, the CL_R for PFOA may exceed 600 ml/kg/day in rabbits (Kudo et al., 2003) and female rats (Kemper and Nabb, 2003), while in male rats it tends to be substantially lower (10-15 ml/kg/day; Kemper and Nabb, 2003). Renal clearance rates in humans are so low that they are difficult for measure. In a study conducted by Zhang et al. (2013), the reported CL_R for PFOA in humans was 0.03 ml/kg/day. Renal clearance has been shown to vary with both carbon chain length and the identity of the acidic moiety (Lau et al., 2007). With a few idiosyncratic exceptions, PFAA elimination in urine is more rapid for shorter length PFAAs and PFAAs containing a carboxylate group. Slower elimination rates are reported for longer chain compounds and sulfonates (Ohmori et al., 2003). Sex

differences in renal clearance of PFOA in rats completely resolved in castrated male rats treated with 17β-estradiol (Ylinen et al., 1989). This finding demonstrated that gender differences in renal clearance in adult animals are under the control of sex hormones, and are not due to early developmental differences.

Renal clearance rates are determined by the processes of glomerular filtration, passive reabsorption, and membrane transport, operating simultaneously. Glomerular filtration occurs in a capsule-enclosed capillary structure sandwiched between afferent and efferent arterioles (the glomerulus), and results in formation of a glomerular filtrate which is delivered to the renal proximal tubule. The efferent arterioles are smaller in diameter than the afferent arterioles, and are able to constrict or relax in order to maintain hydrostatic pressure across the glomerulus. The molecular weight cutoff for glomerular filtration is about 60,000 (Lote, 2001), which excludes most plasma proteins along with the blood cells. As noted above, PFAAs in plasma are highly bound to serum albumin, leaving only a small fraction available for glomerular filtration. For highly bound chemicals, the glomerular filtration rate (GFR; ml/kg/day) may be orders of magnitude less than renal blood flow rate.

Passive reabsorption occurs in response to the activity gradient defined by unbound chemical concentrations in the tubular fluid and in plasma. For neutral chemicals that diffuse easily across biological membranes, there may be substantial reabsorption driven by reabsorption of filtered plasma (including water and small ionic constituents). In such cases, the CL_R may be much less than the GFR. Because they are largely ionized, PFAAs do not diffuse easily across membranes. We would therefore expect little passive reabsorption once they appear in the proximal tubule.

Membrane transporters are expressed on both the basolateral (lumen) and apical (plasma) side of cells that form the proximal tubule. These proteins may facilitate the movement of chemicals down their electrochemical gradient (facilitated transport) or promote their movement against an electrochemical gradient (active transport). There are two basic mechanisms of active transport. The first derives energy from the hydrolysis of ATP (primary mechanism) while the second relies on energy-requiring processes to produce a chemical gradient that is coupled to the movement of another compound (secondary mechanism). Renal transporters may actively secrete chemicals into the tubular fluid or promote their reabsorption into plasma. Both processes appear to be important in understanding species and sex differences in renal elimination of PFAAs, especially for those differences noted to be under the control of sex hormones.

In vivo evidence for the importance of renal transport as a determinant of PFAA kinetics developed out of the observations of marked sex and species differences in renal elimination. Vanden Huevel et al. (1992) noted that treatment of both female and castrated male rats with probenecid, a uric acid reuptake inhibitor believed to block organic anion transport (OAT) proteins in the kidney, decreased renal elimination of PFOA. In contrast to these findings, there were no significant effects of probenecid on PFOA elimination in intact male rats (which eliminate PFOA more slowly than female rats) treated with sham surgery. These results led to the conclusion that expression of transporters responsible for sex-specific differences in PFOA elimination in rats was likely to involve OATs and was at least partially controlled by testosterone. Other *in vivo* studies of PFOA transport include a rat knockout model used to rule out a significant role for Mrp2 in PFOA transport in rats (Katakura et al., 2007). This study also determined that a low-phosphate diet, which increased the expression of an organic phosphate

transporter, reduced PFOA clearance in rats. This was presumed to be due to the promotion of reuptake. Han et al. (2012) analyzed a number of whole-animal studies to characterize net transport of PFOA in the renal tubule. Net transport, either positive (dominated by secretion) or negative (dominated by reuptake) was determined by the relationship: $CL_S - CL_{ABS} = CL_R - f_u * GFR$, where CL_S is the tubular secretion rate (ml/kg/d), CL_{ABS} is tubular reabsorption rate (ml/kg/d), and f_u is the unbound fraction of PFOA in plasma (unitless). Net secretion was found to occur in rabbits and female rats. In other species, including male rats, dogs, mice, humans, and nonhuman primates, reabsorption predominated.

Based on earlier observations of sex-related differences in PFOA elimination in rats, Kudo et al. (2002) conducted a study in which reverse-transcription polymerase chain reaction (RT-PCR) was used to quantify the mRNA of a number of candidate transporters for PFOA in male, female, ovariectomized female, and castrated male rat kidney tissue. The results showed that Oat2 and Oat3 were both likely candidates. A number of *in vitro* and cell based studies have been performed to identify proteins that that may play a role in membrane transport of PFAAs (Han et al., 2008; Katakura et al., 2007; Nakagawa et al., 2008, 2009; Weaver et al., 2010; Yang et al., 2010; Zhao et al., 2017). Transporters associated with reuptake of PFAAs from urine into kidney proximal tubular cells include OAT4 and URAT (Yang et al., 2010) in human cells. Yang et al. (2009) used a culture of Chinese Hamster Ovary (CHO) cells transfected with rat Oatp1a1 to show that Oatp1a1 increased uptake of PFOA with saturable kinetics and that this uptake was subject to competitive inhibition by other transporter substrates. Several transporters, including human OAT1 and OAT3, as well as their rat orthologs

Oat1 and Oat3, were determined to increase uptake from blood into proximal tubular kidney cells (Han et al., 2012).

Nearly all of the transporters known to be associated with PFAA transport in the kidney are members of the solute carrier family (SLC) of proteins, and operate via a secondary active mechanism (e.g. OATs/Oats). However, ATP binding cassette (ABC) transporters, acting as efflux pumps may play a role as well (Han et al., 2012). This is important, as the transporters that have been associated with PFAA transport thus far have been associated with uptake, from either urine or plasma, into kidney proximal tubular cells (though some of these transporters are potentially bidirectional). Because the kidney is not a site of extensive PFAA accumulation, and because some species and sexes are believed to actively excrete some PFAAs, it is assumed that efflux transporters operate on both the apical (urine side) and basolateral (blood) side cell membranes. Finally, studies of renal transport of PFAAs have been primarily focused on carboxylate PFAAs, however, sulfonates are also believed to be substrates for renal transport, as has been demonstrated in liver and intestine (Zhao et al., 2017).

PFAA Kinetics in Fish

Prior to the work described in this dissertation, the only published studies of PFAA kinetics in fish were those given by Martin et al. (2003a, 2003b), De Silva et al. (2009), and Lee and Schultz (2010). The studies performed by Martin et al. (2003a, 2003b) were published as companion papers. One paper (Martin et al., 2003a) described the dietary accumulation of eleven PFAAs in small (2-5 g) rainbow trout (*Oncorhynchus mykiss*), while the second (Martin et al., 2003b) described the bioconcentration of these same chemicals in somewhat larger trout (5-10 g) following a continuous aqueous exposure. Included among the chemicals tested were PFOS and

PFOA. Both studies employed an experimental design which provided uptake and elimination data, and both sought to collect information needed to estimate relevant metrics of bioaccumulation.

Estimated dietary absorption efficiencies, determined by modeling to measured whole-body PFAA concentrations, ranged from 59% for PFOA to 100% for a number of other PFAAs, including PFOS (Martin et al., 2003a). As such, these findings indicate that PFAAs consumed by fish are taken up with high efficiency. Fitted rate constants (k_U) for branchial uptake in waterborne exposures ranged from 0.53 L/kg/d for PFOA to 700 L/kg/d for perfluorododecanoic acid (PFDoA; Martin et al., 2003b). The distribution of PFAAs to various tissues followed the same general pattern noted previously in studies with mammals (Martin et al., 2003a). Thus, the highest concentrations were found in plasma, liver, and kidney, while substantially lower concentrations were measured in muscle, gonads, gill tissues, and adipose fat.

Both studies performed by Martin et al. (2003, 2003b) demonstrated that the $T_{½}$ of PFAAs in trout varies with carbon chain length and the identity of the acidic moiety. In the bioconcentration study, whole-body $T_{½}$ values ranged from 5.2 days for PFOA to 28 days for perfluorotetradecanoic acid (PFTA; Martin et al., 2003b). When combined with modeled rates of branchial uptake, these measured elimination rates corresponded to bioconcentration factors (BCFs) ranging from 4 (PFOA) to 23,000 (PFDoA). Importantly, the BCF determined for PFOS was 1100, which just meets the regulatory criteria (BCF > 1000) used by US EPA to identify “bioaccumulative” substances (Gobas et al., 2009). When regressed against carbon chain length, $T_{½}$ values for the carboxylate PFAAs increased in a roughly linear manner. Plotted against this linear relationship, $T_{½}$ values for the sulfonate PFAAs were well above the line, indicating a reduced rate of elimination

relative to carboxylate PFAAs for a given carbon chain length. Elimination half-lives measured in the dietary accumulation study were similar to those determined following waterborne exposures, and calculated biomagnification factors (BMFs; referred to by the authors as BAFs) ranged from 0.04 for PFOA to 1.0 for PFDoA (Martin et al., 2003a).

Using a dietary exposure protocol similar to than employed by Martin et al. (2003a), De Silva et al. (2009) dosed juvenile (~30 g) rainbow trout with a mixture of branched and linear PFOA isomers, *n*-perfluorononanoate (*n*-PFNA), and isopropyl PFNA. In general, fish tended to accumulate linear isomers of PFOA and PFNA to a greater extent than branched structures. Consistent with these findings, $T_{½}$ values for the linear structures were longer that $T_{½}$ values for branched structures containing the same number of fluorinated carbons. In a separate study, De Silva et al. (2009) dosed trout with a lower concentration of PFOA than that used in the initial mixture study. Recognizing the potential importance of renal transport as a route of elimination for PFAAs, the authors hypothesized that the elimination kinetics of PFOA may be dose-dependent. However, no such effect was seen. Lee and Schultz (2010) exposed male and female fathead minnows (*Pimephales promelas*) to PFOA in the diet and then allowed the fish to depurate. The $T_{½}$ for males was 68.5 hours, while that for females was 6.3 hours. Treatment of females with an androgen (17-β-trenbolone) increased the $T_{½}$ to 25.3 hours. In contrast, treatment of males with an estrogen (ethinylestradiol, EE) had no apparent effect on PFOA elimination. This study is important because it demonstrated reversible sex differences in PFOA elimination in fathead minnows, analogous to that observed in mammals. In addition, it suggested that elimination of PFOA in fatheads is under androgenic control.

<u>Bioaccumulation Assessment for PFAAs in Fish</u>

In 2001, a coalition of nations organized through the United Nations Environment Program (UNEP) signed a treaty regarding twelve synthetic chemicals, of which nine were to be banned, and a tenth (DDT) restricted to use (though not banned) in malaria control programs (Gobas et al., 2009; UNEP, 2001). Of special concern was the potential for these compounds to distribute in the environment by long-range transport. As of 2016, this agreement, known as the Stockholm Convention on Persistent Organic Pollutants (POPs), had been signed and ratified by 179 individual entities and the European Union, with the most notable exception being the United States. All chemicals on the "dirty dozen" list exhibit a tendency to persist in the environment, high potential for bioaccumulation, and substantial toxicity. The Stockholm Convention was largely motivated by a desire to protect human health; however, the potential for environmental harm is considered. Signatories may nominate chemicals for addition to the Stockholm Convention under three Annexes (A-C), which represent varying degrees of action. PFOS and a precursor were added to the list of restricted compounds (Annex B) at a fourth Conference of the Parties in 2009 (UNEP, 2009). This designation requires that all parties take measures to restrict the production and use of PFOS "in light of any applicable acceptable purposes and/or specific exemptions."

Consistent with the goals of the Stockholm Convention, a number of jurisdictions have (in many cases, previously) developed legislation which mandates the evaluation and classification of commercial chemicals. Examples include the Toxic Substance Control Act (TSCA), which is the major piece of United States legislation that deals with industrial compounds (US EPA, 1976, 2016), the Canadian Environmental Protection Act (CEPA, 1999), and the European Registration, Evaluation and Authorization of Chemicals (REACH) legislation (Council of European Union, 2006). Under each of

piece of legislation, compounds meeting criteria for persistence, bioaccumulation, and toxicity (i.e., “PBT compounds”) are subject to further assessment to determine their potential for causing adverse human and environmental effects.

Chemicals may accumulate in both terrestrial and aquatic organisms. However, the focus of most bioaccumulation assessments is on chemical accumulation in fish. This focus acknowledges the well-documented tendency of hydrophobic organic chemicals to partition out of water and into fish tissues, posing a threat to these animals and the animals that consume them. A number of definitions have been developed to qualify and quantify the tendency for chemicals to accumulate in fish. When used in this context, the term “bioaccumulation” refers to chemical accumulation in an organism occurring by all routes of exposure. This term is best understood in the context of an environmental setting where multiple routes of exposure (e.g., dietary, inhalation, dermal) are likely. Chemical accumulation that occurs during a water-only exposure is termed “bioconcentration.” Dermal uptake may contribute substantially to chemical bioconcentration in very small fish and the young of larger species. For fish that exceed about 1 g in size, dermal uptake becomes negligible and chemical uptake from water occurs primarily at the gills (Lien and McKim, 1993). The concept of bioconcentration may apply to an environmental setting where uptake by non-respiratory routes of exposure is negligible. More commonly, however, it applies to controlled waterborne exposures performed in a laboratory testing environment. Another key concept is chemical “biomagnification.” This term refers to the tendency for chemical concentrations in tissues to increase at successively higher trophic levels.

Bioaccumulation, bioconcentration, and biomagnification in fish are typically quantified in terms of “factors.” The BCF describes the ratio of the chemical

concentration in biota (tissues or whole-body) to that in water, achieved in a water-only exposure (Gobas et al., 2009; Weisbrod et al., 2009). Because it is most often based on controlled laboratory exposures, the BCF is generally assumed to represent steady-state conditions. The bioaccumulation factor (BAF) describes the ratio of the chemical concentration in biota to that in water, where accumulation occurs by all possible routes of exposure. BAFs based on measured residues in field collected animals may or may not represent steady-state conditions, while modeled BAFs generally reflect this assumption. Hydrophilic and moderately hydrophobic compounds (log K_{OW} < 4) are taken up by fish primarily across the gills. For such compounds, the laboratory BCF is expected to be close to the field BAF (measured or modeled). For more hydrophobic compounds, the field BAF may exceed the laboratory BCF due to the contribution of dietary uptake (Gobas and Morrison, 2000). The BMF relates the ratio of the chemical concentration in biota to the concentration in its diet. For hydrophobic compounds, the lipid content of a fish strongly determines its capacity to accumulate chemical. For this reason, BMFs are frequently calculated based on lipid-normalized chemical concentrations in the predator and its prey. Calculated in this manner, a BMF > 1 indicates that the chemical concentration in a predator exceeds that which would have been predicted had the predator and its prey come to thermodynamic equilibrium.

Predictive models for bioaccumulation and bioconcentration assessment

Field and laboratory studies designed to measure chemical accumulation in fish are numerous and ongoing. In general, however, the need to evaluate bioaccumulation potential in support of legislated hazard assessments is much greater than existing testing capabilities. Moreover, there is increasing pressure in Europe and elsewhere to limit these testing activities as part of the “3Rs” approach (replacement, reduction,

refinement) to ethical use of live animals (European Union, 2010; Goodman et al., 2015). For these reasons, most bioaccumulation assessments are performed using predictive computational models, alone or in combination with *in vitro* methods (Nichols et al., 2009).

Quantitative Structure Activity Relationship (QSAR) models that predict chemical bioconcentration in fish have been widely used since the late 1970s (Pavan et al., 2008; Nichols et al., 2009). These models vary in complexity; however, all are based on the well-known pattern that exists between measured BCFs and chemical hydrophobicity, typically expressed as the log of a compound's n-octanol/water partition coefficient (log K_{OW}; Devillers et al., 1998; Nichols et al., 2009). More specifically, it was discovered that the log of the BCF for many neutral chemicals increased linearly with chemical log K_{OW} up to a log K_{OW} value of approximately 6. At higher log K_{OW} values, log BCFs tended to level off or even decrease (Meylan et al., 1999). The rationale given for this relationship is that log K_{OW} is a quantitative surrogate for chemical partitioning to tissue lipids, which is considered to be a key process underlying most observed bioaccumulation (Nichols et al., 2009). At very high log K_{OW} values, log BCFs decline because chemical bioavailability in water is reduced by non-specific binding to dissolved and particulate organic carbon. This has the effect of reducing the "free" or unbound concentration with which the animal comes to equilibrium.

Chemical accumulation in fish may also be predicted using mechanistic mass-balance models. Generally, these models represent the fish as a single well-stirred compartment, and the processes that control chemical uptake and elimination are described by first-order rate constants (Gobas and Morrison, 2000; Arnot and Gobas, 2003, 2004). Many of the inputs to these models, including rate constants for chemical

flux across the gills, may be estimated from K_{OW}-based relationships. Other terms, such as water temperature and the fish's lipid content, are tailored to a particular species and exposure scenario. These models can be expanded to describe chemical uptake and elimination in the gastrointestinal tract. As such, they may be used to predict chemical BCFs or BAFs. When incorporated into an appropriate food web model, such descriptions may also be used to predict chemical BMFs.

Multi-compartment mass-balance models for fish have also been provided, including physiologically based toxicokinetic (PBTK) descriptions that predict chemical uptake and accumulation from physiological, biochemical, and physio-chemical information (Nichols et al., 1990). Within such models, K_{OW} may play an important role as a predictor of chemical flux at various exchange surfaces (e.g., gills, gut and skin), as well as partitioning to blood and tissues. PBTK models are now gaining increased acceptance as tools for chemical bioaccumulation assessment (Stadnicka et al., 2014; Brinkman et al., 2016). Because they describe chemical accumulation in specific tissues and organs, PBTK models are well suited for relating *in vitro* effects information to exposures that would be required to elicit these effects *in vivo* (an application commonly referred to as "reverse toxicokinetics").

Existing QSAR and mass-balance models have been shown to accurately predict observed levels of bioconcentration and bioaccumulation in fish for a broad range of substances (Nichols et al., 2009). Nevertheless, both model types are subject to errors. One potential source of error in modeled predictions is chemical biotransformation. QSAR models do not generally consider the possibility of biotransformation. When this activity occurs in fish, these models tend to overestimate true levels of accumulation (Oliver and Niimi, 1985; Clark et al., 1990; de Wolf et al., 1992). Mass-balance models

may incorporate one or more metabolism rate constants; however, they tend to be quite sensitive to the specified value(s) of this(ese) term(s), which may vary widely between chemicals, organisms, and exposure conditions. A second potential source of error in current predictive models relates to chemical transport across biological membranes. In general, these models assume (explicitly or implicitly) that chemical flux across biological membranes occurs by simple passive diffusion. Restricted diffusion across membranes is likely for very high molecular-weight and/or extremely hydrophobic compounds (Nichols et al., 2009), as well as compounds that ionize at physiological pH values (Armitage et al., 2017). Finally, both model types generally ignore the possible role of membrane transport proteins which may promote chemical uptake or elimination, depending on their activities and the tissues in which they are expressed.

Applicability of current models for predicting PFAA bioaccumulation

The use of traditional K_{OW}-based models to predict bioaccumulation of PFAAs in fish is problematic for several reasons. First, because they are somewhat lipophobic, PFAAs do not preferentially partition to tissue lipids. Instead, they bind to specific proteins in blood plasma and selected tissues (e.g., liver and kidney). A second problem is that K_{OW} can't be directly measured for these compounds, as PFAAs are surfactants which tend to partition to the octanol-water interface, though QSAR methods may be used to predict K_{OW} values. Also, because the PFAAs are virtually 100% ionized at physiological pH values, simple diffusion across biological membranes is likely to be highly restricted.

To date, three models have been developed to describe the accumulation of PFAAs in fish. The first, given by Webster and Ellis (2010), described this accumulation using a modification of the traditional QSAR K_{OW}-based approach, where the BCF is

predicted from chemical K_{OW} and a fish's fractional lipid content. For this application, the standard BCF QSAR was modified to incorporate the Hendersen-Hasselbach relationship, which predicts ionized and neutral fractions of a chemical from pH and the chemical's p*K*a value. This approach yields a BCF which is corrected to account for partitioning of only the neutral fraction of a PFAA. When this model was applied to the chemicals tested by Martin et al. (2003a, 2003b), predicted BCFs were in reasonably good agreement with measured values. However, the p*K*a value used to parameterize the model (3.8) is probably much higher than true values (generally thought to be 1.0 or lower; Moroi et al., 2001; Goss et al., 2008; Cheng et al., 2009). As such, this model is highly suspect.

A second model, given by Armitage et al. (2013), is a modification of the one-compartment K_{OW}-based model given by Arnot and Gobas (2004), and was developed as a generalized description for ionizable substances. The model predicts the accumulation of ionized chemicals by fish from K_{OW}-based binding to tissue phospholipids, and employs a gill uptake description which accounts for restricted uptake of ionized forms from water. Renal clearance as a route of elimination was not considered. Model performance for PFAAs was highly dependent on the specification of p*K*a and K_{OW}. Regardless of which values were chosen, however, the model could not explain the large observed differences in measured BCFs for PFOS and PFOA. Finally, it may be questioned whether this type of model is appropriate for simulating the accumulation of PFAAs, given their known affinity for specific proteins and not membrane phospholipids.

A mechanistic model for PFAAs in fish was provided by Ng and Hungerbühler (2013). Unlike the models given by Webster and Ellis (2010) and Armitage et al. (2013),

this model contains a number of features which reflect current knowledge regarding factors that control uptake, distribution, and elimination of PFAAs in mammals. Within this model, the fish is described as a collection of compartments corresponding to tissues (cells) and tissue interstitial fluids which contain relevant binding proteins. Owing to the lack of mechanistic data for fish, the model was extensively parameterized using information from mammals. Thus, mammalian data was relied upon to describe PFAA binding to albumin and fatty acid binding proteins, while transfer rates across the gills were based on diffusion rates measured in mammalian *in vitro* cell cultures. Similarly, modeled rates of renal secretion and reabsorption were based on the measured activities of rat membrane transporters (Oats, Oatps, and Urat) which had been transfected into various mammalian cell lines. The model was largely successful in predicting the tissue distribution of PFAAs studied by Martin et al. (2003b), at least in terms of relative concentrations, though it tended to under-predict measured kidney and plasma concentrations. Of particular interest to the studies described in this dissertation, the authors noted that their model was hampered by a lack of information regarding renal elimination of PFAAs in fish.

Study Goals and Objectives

Concern regarding potential adverse effects of PFAAs on human health and the environment derives from the historical scope of PFAA production and use, and the demonstration that PFOS, PFOA, and other long-chain PFAAs can persist in the environment and accumulate in biota. Though initially regarded as biologically inert, accumulating evidence suggests that some PFAAs are toxic, particularly in repeated exposures. In mammals, toxicity associated with an individual PFAA may vary substantially between species and between the sexes within a species. These

differences appear to be correlated with the ability of different organisms to eliminate these compounds, as indicated by differences in $T_{½}$. Additional studies have shown that difference in $T_{½}$ are largely due to differences in CL_R. Differences in CL_R are due, in turn, to differences in the expression and activities of renal membrane transporters which secrete PFAAs into urine and promote their reuptake into plasma.

Numerous field surveys have shown that PFAAs in the environment accumulate in fish and the animals that consume fish, including aquatic-dependent birds and mammals. Additional, limited studies indicate that PFAAs may exert adverse effects on fish via toxicity pathways similar to those observed in mammals. The extent to which individual PFAAs accumulate in fish varies widely; in general, the greatest extent of accumulation is observed for longer chain PFAAs, and (for a given perfluorocarbon chain length) for sulfonates. Shorter chain PFAAs exhibit little or no tendency to bioaccumulate. As in mammals, difference in accumulation are associated with differences in $T_{½}$. However, the mechanistic basis for these observed differences in $T_{½}$ has been poorly understood.

The primary goal of studies described in this dissertation was to obtain a detailed mechanistic understanding of PFAA uptake and elimination in fish, while the larger aim was to support ongoing development of mechanistic models used to predict PFAA accumulation in fish. The working hypothesis, based on previous research with mammals, was that renal elimination contributes substantially to PFAA elimination in fish, and that differences in renal elimination are a primary determinant in differences in bioaccumulation of individual PFAAs. These investigations were performed using the two most well-studied PFAAs, PFOS and PFOA. Previous work has shown that PFOS exhibits a marked tendency to accumulate in fish while PFOA does not.

Detailed kinetic studies with PFOS and PFOA were performed with large, chambered trout using methods developed by McKim and Goeden (1982). The experiments were conducted at the US EPA research facility in Duluth, MN (US EPA/NHEERL/Mid-Continent Ecology Division) using exposure facilities which are unique with respect to the surgical preparation of study animals, control of chemical dosing, and collection of supporting physiological information. Individual animals were instrumented to permit repeated sampling of plasma, urine, and expired water. Dosing was performed by injecting animals with a bolus dose of chemical (to characterize elimination) or by introducing the chemical to respired water (to characterize branchial uptake). The resulting datasets were analyzed using a two-compartment clearance-volume kinetic model. Importantly, this modeling approach makes no assumptions regarding factors which influence a compound's apparent volume of distribution. As such, it does not rely on potentially problematic specification of chemical K_{OW}. Moreover, k_U and CL_R values determined in this manner may be compared directly to existing physiological information for trout, including the volumetric flow rate of water across the gills, renal blood flow, GFR, and urine flow rate.

Chapter 2 of this dissertation, which was published in Aquatic Toxicology (Consoer et al., 2014), describes the kinetics of PFOA in trout. Chapter 3, which was published in Environmental Toxicology and Chemistry (Consoer et al., 2016) describes the kinetics of PFOS in trout. Data and fitted model simulations for individual study animals are provided at the end of each chapter. Additional supporting information in Chapter 2 describes preliminary studies which were performed to ensure that blood and urine could be sampled without loss of PFOA to blood cannulas and urine catheters. Chapter 4 contrasts and compares the major findings these two kinetic studies, and

relates this information to existing knowledge regarding the kinetics of PFAAs in mammals (Chapter 1). Concluding sections of Chapter 4 discuss the implications of these findings for bioaccumulation assessment of PFAAs in fish, and provide guidance on future research directions.

Chapter 2: Toxicokinetics of perfluorooctanoate (PFOA) in rainbow trout (*Oncorhynchus mykiss*)

Daniel M. Consoer, Alex D. Hoffman, Patrick N. Fitzsimmons, Patricia A. Kosian, John W. Nichols.

Published in *Aquatic Toxicology*, 12 May, 2014. Volume 156, p 65-73.

Abstract

Rainbow trout (*Oncorhynchus mykiss*) confined to respirometer-metabolism chambers were dosed with perfluorooctanoate (PFOA) by intra-arterial (i.a.) injection and sampled to obtain concentration time course data for plasma, urine, and expired water. The data were then analyzed by compartmental modeling to estimate rates of renal and branchial clearance. Averaged across all animals, the renal clearance rate (1.35 mL/h/kg) was more than ten times greater than the branchial clearance rate (0.12 mL/h/kg). The average whole-body elimination half-life was 12.6 d, which is somewhat longer than values obtained in previous studies with smaller trout. The tissue distribution of PFOA was assessed by collecting tissues at the end of chambered exposures and in a separate tissue time-course experiment. From the time-course study it appeared that an internal steady-state was established within 24 h of i.a. injection. Consistent with previous studies, the rank order of PFOA concentration in tissues at steady state was: plasma > liver > kidney > muscle. In a second set of chambered experiments, fish were exposed to PFOA in water to determine the rate of branchial uptake. Branchial uptake rates were too low to assess directly by measuring PFOA concentrations in inspired and expired water. Uptake rate constants (mean 0.19 L/d/kg; 0.1% uptake efficiency) were therefore estimated by compartmental modeling using plasma concentration time-course

data and model parameters derived from the elimination experiments. It is clear from this effort that elimination of PFOA by trout occurs primarily via the renal route. This finding is consistent with numerous studies of mammals and suggests that trout possess membrane transporters that facilitate the movement of PFOA from plasma to urine.

1. Introduction

Perfluoroalkyl acids (PFAAs) are used in stain-resistant coatings, and as surfactants, fire-fighting foams, and photographic developers (Lau et al., 2007; Renner, 2001). Concern that these chemicals may constitute an important class of environmental contaminants can be traced back to 2001, when Giesy and Kannan (2001) published their findings on the global distribution of perfluorooctane sulfonate (PFOS) in wildlife. Since then, PFAAs have been measured in humans, surface waters, and aquatic sediments, and have been found in environmental matrices far from any known sources (Prevedouros et al., 2006). Although initially thought to be biologically inert, several PFAAs have been shown to cause toxic effects in mammals, including developmental toxicity, immunotoxicity, and hepatotoxicity (Andersen et al., 2008; Lau et al., 2004, 2007). Toxicity studies with fish have largely, though not solely focused on PFOS as a model compound. These investigations have demonstrated reproductive effects (Han and Fang, 2010), developmental toxicity (Han and Fang, 2010; Huang et al., 2010; Shi et al., 2008), hepatotoxicity (Hoff et al., 2003; Wei et al., 2008a, 2008b) and behavioral effects (Huang et al., 2010). Individual PFAAs have been shown to accumulate in aquatic species, although the extent of this accumulation varies widely. Measured log bioaccumulation factors (BAFs) for six PFAAs in Great Lakes lake trout (*Salvelinus namaycush*) ranged from 2.7 for perfluorohexane sulfonate (PFHxS) to 4.1 for PFOS (Furdui et al., 2007). Liu et al. (2011) reported BAFs for green mussels (*Perna*

viridis) ranging from 15 for perfluoroctanoate (PFOA) to 859 for perfluorodecanoate (PFDA), along with evidence for dependence of these values on the exposure concentration. In general, the tendency of individual PFAAs to accumulate in fish is directly related to the length of a compound's fluorinated carbon chain as well as the identity of the terminal group (sulfonate or carboxylate), which confers to the molecule its amphipathic character. PFOA and other shorter chain carboxylates accumulate to a lesser extent than perfluorosulfonates of comparable chain length, to the point that they are not considered to be bioaccumulative based on standard regulatory criteria (e.g., BAF > 1000; Martin et al., 2003a, 2003b; Conder et al., 2008).

Models commonly used to predict the accumulation of lipophilic chemicals in fish are generally thought to be inadequate for PFAAs. Such models describe uptake and accumulation as a consequence of passive diffusion across the gills and gut, and subsequent partitioning to whole-body lipid, taking into consideration the possibility of biotransformation (Arnot and Gobas, 2004). It is well known, however, that PFAAs do not partition to tissue lipid but instead bind to protein. Moreover, the character of this binding suggests high affinity for specific protein sub-classes including serum albumin and fatty-acid binding proteins in the liver (Han et al., 2003; Ng and Hungerbühler, 2013). Thus, PFAAs exhibit little tendency to accumulate in the white muscle of fish, which constitutes by far the largest store of total whole-body protein, and instead accumulate in blood plasma, liver, and kidney (Martin et al., 2003a). Complicating matters further, PFAAs are ionogenic and exist as a mixture of charged and neutral species. Uptake, distribution, and elimination are likely to depend, therefore, on an individual compound's p*Ka* and the pH of the local environment.

In mammals, large differences in the biological half-life of specific PFAAs have been noted for different test organisms (Butenhoff et al., 2004a; Andersen et al., 2008; Han et al., 2012). These differences have been attributed to different rates of renal elimination (Andersen et al., 2006; Butenhoff et al., 2004b; Han et al., 2012) which are due, in turn, to differences in the activities of specific transporters including the organic anion transporters Oat1 and Oat3, the organic anion transporting polypeptide Oatp1a1, and uric acid transporter 1 (Urat1), all of which facilitate reuptake of PFAAs from renal filtrate in the proximal tubule (Kudo et al., 2002; Nakagawa et al., 2008; Weaver et al., 2010). The presence/absence and activities of these transporters are also thought to explain large differences in elimination that often exist between the sexes, as the expression of many renal transporters is known to be under the control of sex hormones (Cheng and Klaassen, 2009; Ljubojevic et al., 2007; Morris et al., 2003).

Recent efforts to model PFAA accumulation in fish have attempted to account for some or all of these characteristic behaviors. For example, a model given by Armitage et al. (2013) predicts the uptake and accumulation of PFAAs based on partitioning of neutral and ionized chemical species to both neutral lipids and phospholipids. This model does not, however, account for the possible role of renal clearance. A second model, given by Ng and Hungerbühler (2013) explicitly describes protein binding interactions in several tissue compartments and includes a renal clearance description which operates against the unbound chemical mass in kidney tissue. Model terms that describe renal elimination were based on data from studies with mammals, as data for fish were lacking.

Given the importance of PFAAs as a class of environmental contaminants as well as the need to advance current modeling efforts, it is critical to obtain mechanistic

information regarding the kinetics of these compounds in fish, and in particular data regarding renal clearance. Although direct evidence for renal elimination of PFAAs in fish is lacking, indirect evidence suggests that it may play a role in the elimination of some compounds. For example, Lee and Schultz (2010) suggested that differences in renal elimination may have accounted for observed sex-linked differences in elimination of PFOA by fathead minnows. The goal of the present study was to characterize the routes and rates of PFOA elimination in large rainbow trout which had been chronically catheterized to permit continuous collection of urine. Additional experiments were performed to evaluate the uptake of PFOA across trout gills and the kinetics of its distribution to tissues.

2. Materials and methods

2.1. Chemicals

PFOA (>98% pure) was purchased from Sigma Aldrich (St. Louis, MO). PFOA and mass-labeled [^{13}C] PFOA standards were purchased from Wellington Laboratories (Guelph, ON, Canada). HPLC grade acetonitrile, methanol, and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing approximately 100 g were obtained from the USGS Upper Midwest Environmental Sciences Center in La Crosse, WI, and grown up to the desired size for each experiment. The animals were held in sand-filtered Lake Superior water at 11 ± 1 ºC under a natural photoperiod and fed a commercial trout chow (Silver Cup, Nelson and Sons Inc., Murray, UT). Water chemistry

characteristics were: total hardness 45–46 mg/L as $CaCO_3$; alkalinity 41–44 mg/L as $CaCO_3$; pH 7.6–7.8; total ammonia < 1 mg/L; dissolved O_2 85–100% of saturation.

2.3. Surgical preparation of animals

Elimination and branchial uptake studies were performed using trout confined to respirometer-metabolism chambers (McKim and Goeden, 1982). All of these fish had gone through at least one spawning cycle prior to use, and varied with respect to their state of sexual maturity at the time of data collection. Fish weighing 700–1500 g were sedated with tricaine methanesulfonate (MS- 222; Finquel, Argent Laboratories, Redmond, WA), weighed, and placed on a surgical table designed to provide continuous anesthesia with temperature-controlled, oxygenated water. Each fish was immobilized by spinal transection and surgically fitted with a dorsal aortic cannula (Intramedic PE 50; Becton, Dickinson and Co., Parsippany, NJ) to permit periodic blood sampling. Fish used for elimination experiments also were fitted with a urinary catheter (Intramedic PE 90) for continuous collection of urine. A latex membrane sewn to the fish's mouth allowed separate collection of inspired and expired water. A second latex membrane just posterior to the pectoral fins prevented further dilution of expired water. Fish were allowed 24 h to recover from surgery before starting an experiment. Water temperature (T; ºC), ventilation volume (V_{VOL}; mL/min), and oxygen consumption (VO_2; mg/h/kg) were monitored continuously using an automated data collection system and customized software (Carlson et al., 1989). This information was then evaluated to assess the viability and representativeness of each preparation, and generate average values for the experimental time period.

2.4. Elimination experiments

Eight trout of mixed sex were given a bolus intra-arterial (i.a.) injection of PFOA dissolved in trout serum (1 mL/kg), with each fish receiving a nominal dose of 1.0 mg/kg, equivalent to approximately 2 µmol/kg. Blood, urine, and expired water were sampled periodically for 72 h to evaluate the kinetics of PFOA in plasma and characterize its elimination by renal and branchial routes. Blood samples (100 µL) were centrifuged at 6000 RCF for 6 min (10 ºC) to obtain plasma. Expired water (1 L) was withdrawn directly from the respirometer-metabolism chamber. Urine samples (50–100 µL) were collected into pre-weighed 1.5-mL microcentrifuge tubes via the renal catheter. These tubes were then reweighed to obtain the mass of urine collected. The volume of urine produced between sampling times was recorded to calculate the urine flow rate (UFR) for each time period as well as an average UFR for the entire experiment.

Fish were euthanized after the final sampling period with an overdose of MS-222, and samples of kidney, liver, muscle, and bile were collected and stored at −20 ºC for later analysis. Muscle was sampled from above the lateral line, anterior to the dorsal fin. The liver, kidney, and bile were collected in their entirety.

2.5. Branchial uptake experiments

By providing for separate collection of inspired and expired water, respirometer-metabolism chambers allow for direct measurement of chemical uptake across the gills (McKim and Goeden, 1982). In practice, however, this method depends on there being a discernible difference in chemical concentration between inspired and expired water, taking into account the variance associated with these measurements. Alternatively, branchial uptake can be estimated indirectly by modeling to measured chemical concentrations in plasma, provided that the model adequately accounts for the internal distribution of a compound as well as its elimination by non-branchial routes.

Eight trout of mixed sex were exposed to PFOA in water for 72 h. Fish were surgically prepared as described above with the exception that they were not fitted with a urinary catheter. A stock solution of PFOA was prepared by dissolving PFOA in de-ionized water (Milli-Q; EMD Millipore, Billerica, MA). The stock solution was metered into a mixing cell and diluted with Lake Superior water to achieve a nominal target concentration of 500 µg/L. This water was then supplied to each chambered animal in excess of its respiratory requirement (approx. 440 mL/min). Blood samples (100 µL) were obtained at 2, 4, 8, 24, 36, 48, 54, and 72 h, and processed as described above. Tissue samples (liver, kidney, muscle) were again collected at test termination and stored at −20 ºC for later analysis.

2.6. Tissue distribution study

Sixteen trout of mixed sex weighing 150–250 g were given a bolus i.a. injection of PFOA to study the tissue distribution time course. All of these animals were sexually immature juveniles that had not yet gone through a spawning cycle. Fish were given a nominal dose of 1 mg/kg dissolved in trout plasma (2 mg/mL, administered at 0.5 mL/kg injection volume). Animals were sedated with MS-222. The dose was then injected directly into the dorsal aorta. Dosed animals were transferred to 2 ft circular tanks (4 per tank) supplied with Lake Superior water (11 ± 1 ºC). One tank of animals was sacrificed at 1, 3, 7, and 14 days post dosing to obtain samples of plasma, liver, kidney, and muscle. Tissues from unexposed fish served as controls. All samples were stored at −20 ºC for later analysis.

2.7. Plasma binding

PFOA binding in trout plasma was evaluated using an ultra-filtration method (Centrifree YM-30 filter units; EMD Millipore, Billerica, MA). Preliminary studies showed that PFOA spiked into physiological saline exhibited a high level of non-specific binding to the filter cartridge. This non-specific binding was subsequently reduced to an acceptable level by spiking PFOA into plasma ultrafiltrate, as recommended by the manufacturer. PFOA was spiked into trout plasma or plasma ultrafiltrate at 1 and 10 g/mL (n = 5 each). Samples (200 µL) were loaded into pre-conditioned filter units and centrifuged at 2000 RCF for 30 min. Binding was then determined from the difference in measured concentrations of PFOA in spiked samples and sample filtrates.

2.8. Quality controls

2.8.1. PFOA recovery

Preliminary experiments were conducted to measure the recovery of PFOA from spiked samples of plasma, urine, and trout holding water. Samples of plasma and urine were spiked with 10, 100, and 1000 g/mL of non-labeled PFOA standard (n = 9 each), mixed by vortexing for 30 s, and allowed to equilibrate overnight. The samples were then extracted and analyzed as described under analytical methods. Recoveries from plasma (mean ± SD) averaged 112.3 ± 9.9, 114.4 ± 3.9, and 109.6 ± 2.2% for the 10, 100, and 1000 g/mL samples, respectively. An overall average recovery of 112.1% was subsequently used to correct measured values from toxicokinetic studies. Recoveries from urine averaged 99.4 ± 8.7, 101.7 ± 5.8, and 99.6 ± 4.5% for the10, 100, and 1000 g/mL samples, respectively, and were not used to correct measured values. Tank water samples were spiked with 0.01 and 0.1 g/mL of PFOA standard (n = 5 each). Recoveries averaged 112.3 ± 7.7 and 101.5 ± 7.6% for the 0.01 and 0.1 g/mL samples,

respectively. An overall average recovery of 106.9% was subsequently used to correct measured values.

2.8.2. PFOA adsorption

Due to their surfactant properties, PFAAs in aqueous solution tend to adsorb to solid phases such as glass and plastic. Preliminary work was therefore conducted to evaluate the potential for PFOA to adsorb to the dorsal aortic cannula, as this could interfere with both i.a. dose administration and subsequent efforts to measure PFOA in blood. A syringe pump (CMA/100; Harvard Apparatus, Holliston,MA) was used to infuse a cannula with trout plasma spiked with 25 ng/mL PFOA. The pumping rate was set to 1 mL/min, and the plasma was collected at 30-s intervals for 9 min. The cannula was then infused with physiological saline to determine how rapidly PFOA was flushed from the system. The concentration of PFOA in saline declined rapidly over time (>95% by 1 min; supplementary data, Appendix 1, Fig. S2-1). Because some mixing occurs within the cannula when solutions are changed, it is not clear whether the small amount of PFOA "retained" at early time points was due to desorption of bound chemical or mixing effects. In any case, the i.a. dose administered to each fish was chased with 0.5 mL of clean saline. The cannula was then filled with heparinized saline prior to the first blood sampling period to maintain its patency. Blood sampling procedures, which include discarding the first 250 µL of blood and filling the cannula with heparinized saline between sampling times, also were designed to minimize sample-to-sample contamination. Under these circumstances, binding and/or mixing effects are unlikely to have a discernible impact on measured PFOA concentrations.

Additional tests were conducted for the urine catheters using urine samples spiked with 10 ng/mL PFOA. Again, these tests suggested a small degree of binding

and/or mixing effects. However, PFOA concentrations rapidly approached zero when the catheters were flushed with clean saline (data not shown).

2.9. Analytical methods

2.9.1. Plasma samples

Plasma samples were extracted using an acetonitrile protein precipitation method (Flaherty et al., 2005; Reagen et al., 2008). Briefly, samples (10 µL) were diluted into 990 µL (bolus dose elimination experiments) or 490 µL (branchial uptake experiments) ice-cold 75% acetonitrile (ACN)/25% de-ionized (DI) water in 1.5- mL microcentrifuge tubes. The tubes were vortexed for 30 s, placed on ice for 15 min, and centrifuged at 6000 RCF for 6 min to pellet precipitated proteins. A subsample (98 µL) of the supernatant was transferred to a 2-mL liquid chromatography (LC) vial with insert and spiked with 2 L of [^{13}C] PFOA in 75% ACN/25% DI water as an internal standard (final concentration of 5 ng/mL). These subsamples were then vortexed briefly and analyzed by LC/MS/MS.

2.9.2. Water samples

PFOA concentrations in water samples collected during the elimination studies were too low for direct measurement. It was therefore necessary to extract and concentrate the samples using a solid phase extraction (SPE) method (Taniyasu et al., 2005, 2008). SPE cartridges (Oasis WAX 6 cm^3, 150 mg, 30 µm) were conditioned by passing 4.0 mL of 0.1% ammonium hydroxide in methanol at 2 drops per sec, followed by 4 mL of pure methanol and finally 4.0 mL of de-ionized water. A sample (1.0 L) was passed through a cartridge at the same rate, after which the cartridge was washed with 4.0 mL of 25 mM sodium acetate buffer (fraction 1, discarded). PFOA was eluted by

passing 4.0 mL of methanol through the cartridge (fraction 2), followed by 4.0 mL of 0.1% ammonium hydroxide in methanol (fraction 3). PFOA was found to elute entirely in fraction 3. The fraction 3 eluant was diluted 3:4 with 75% ACN/25% DI water, spiked with internal standard to a final concentration or 5 ng/mL, and analyzed by LC/MS/MS. Water samples from the branchial uptake experiments were diluted 1:4 with 75% ACN/25% DI water and mixed by vortexing for 30 s. Subsamples (98 µL) were then transferred to LC vials with inserts, spiked with 2 µL of internal standard to a final concentration of 5 ng/mL, and analyzed by LC/MS/MS.

2.9.3. Urine samples

Urine samples were diluted 1:9 with 75% ACN/25% DI water and vortexed for 30 s. Subsamples (98 µL) were then transferred to LC vials with inserts, spiked with 2 µL of internal standard to a final concentration of 5 ng/mL, and analyzed by LC/MS/MS.

2.9.4. Tissue samples

Samples of liver, kidney, and muscle (0.5 g) were placed in 15 mL polypropylene centrifuge tubes and spiked with internal standard to a final concentration of 5 ng/mL. Samples were homogenized (Polytron tissue homogenizer, Brinkman Instruments, Westbury, NY) in nine volumes of 75% ACN/25% DI water for 2 min at approximately 15,000 rpm, and centrifuged for 20 min at 10,000 RCF to form a solid pellet. A subsample (100 µL) of the supernatant was then transferred to an LC vial with insert for analysis by LC/MS/MS.

2.9.5. Bile samples

Initially, we attempted to analyze bile samples by diluting them in solvent and centrifuging to remove particulates. However, this caused significant changes in the

retention time of PFOA, presumably due to the effects of bile acids on the column. In addition, there was a loss in signal, thought to be due to accumulation of material on the lens and spray chamber of the MS detector. Because of this, we used an SPE cleanup step similar to that used to concentrate and extract water samples from the elimination studies. A subsample of the collected bile (40 µL) was diluted into 200 mL of de-ionized water. This solution was then spiked with internal standard to a final concentration of 5 ng/mL and mixed by inverting. Samples were extracted and concentrated using the SPE extraction procedure described above for water samples, and analyzed by LC/MS/MS.

2.9.6. Quantitation

Samples were analyzed on an Agilent HPLC/Triple Quadrupole 6410 tandem mass spectrometer (LC/MS/MS). Chromatographic separation of PFOA was carried out on an Agilent Zorbax ExtendC18 column (3.5 m, 2.1 mm × 100 mm). Mobile phase A was 2% ethanol, 1% methanol, 2% 250 mM ammonium acetate in methanol, 96% water, and 0.01% of 25% ammonium hydroxide, pH 8.2. Mobile phase B was 2% ethanol, 2% water, 1% 250 mM ammonium acetate in methanol, 95% methanol, and 0.01% of 25% ammonium hydroxide. The isocratic flow rate was 0.2 mL/min at 69% mobile phase B, and the injection volume was 10 L. PFOA eluted at 2.7 min and was detected using an electrospray ionization (ESI) source operating in the negative mode. Detection was based on the transition from 413 to 369 (m/z) for PFOA and 417 to 372 (m/z) for mass-labeled PFOA internal standard. Quantitation was carried out against a standard curve constructed from known quantities of standard and a known, constant quantity of mass-labeled internal standard in 75% ACN/25% DI water. Blank samples spiked with internal standard were run for solvents used in extractions, and each animal provided its own

pre-dosing samples for analysis of background concentrations of PFOA in plasma, urine, and expired water.

2.10. Modeling and data analysis

Plasma, urine, and expired water time-course data were analyzed by compartmental modeling using the SAAM-II kinetic analysis software package (The Epsilon Group, Charlottesville, VA; Barrett et al., 1998). An examination of plasma data from preliminary elimination studies showed that there was a distinct distribution phase followed by a longer, log-linear elimination phase. Based on these findings we elected to use a two-compartment model with elimination from the central compartment (Fig. 2-1A). The two elimination routes (urine and expired water) were modeled as additional compartments. This allowed the model to fit the two elimination routes simultaneously. The models were fitted using data-relative variance, assigning a fractional standard deviation (FSD) of 0.1 to the plasma data, and an FSD of 0.5 to urine and expired water data. Fitted values were obtained from the model for kinetic parameters for distribution ($k_{1,2}$, $k_{2,1}$; 1/h), renal elimination ($k_{3,1}$; 1/h), branchial elimination ($k_{4,1}$; 1/h), and the apparent volume of the central compartment (V_C; mL/kg). Renal (CL_R; mL/h/kg) and branchial clearance (CL_B; mL/h/kg) were calculated as the product terms $V_C \times k_{3,1}$ and $V_C \times k_{4,1}$, respectively. Total clearance (CL_T; mL/h/kg) was calculated as the sum of renal and branchial clearances. Steady-state volume of distribution (V_{SS}; mL/kg) was calculated as the sum of V_C and the volume of the peripheral compartment (V_P; mL/kg), calculated as $(V_C \times k_{1,2})/k_{2,1}$. Plasma data from the branchial uptake studies were modeled using a two-compartment model (Fig. 2-1B), with elimination from the central compartment represented by a single clearance constant ($k_{3,1}$; mL/h/kg). Kinetic parameters for transfer to and from the peripheral compartment ($k_{1,2}$ and $k_{2,1}$) were fixed

at mean values determined from previous elimination studies (model 1A). Similarly, the value of $k_{3,1}$ was calculated as the sum of previously determined mean values for $k_{3,1}$ and $k_{4,1}$. Using this approach, the only term fitted by the model is the clearance constant for chemical uptake by the central compartment (k_U; L/d/kg), which operates against the measured PFOA concentration in inspired water (CINSP).

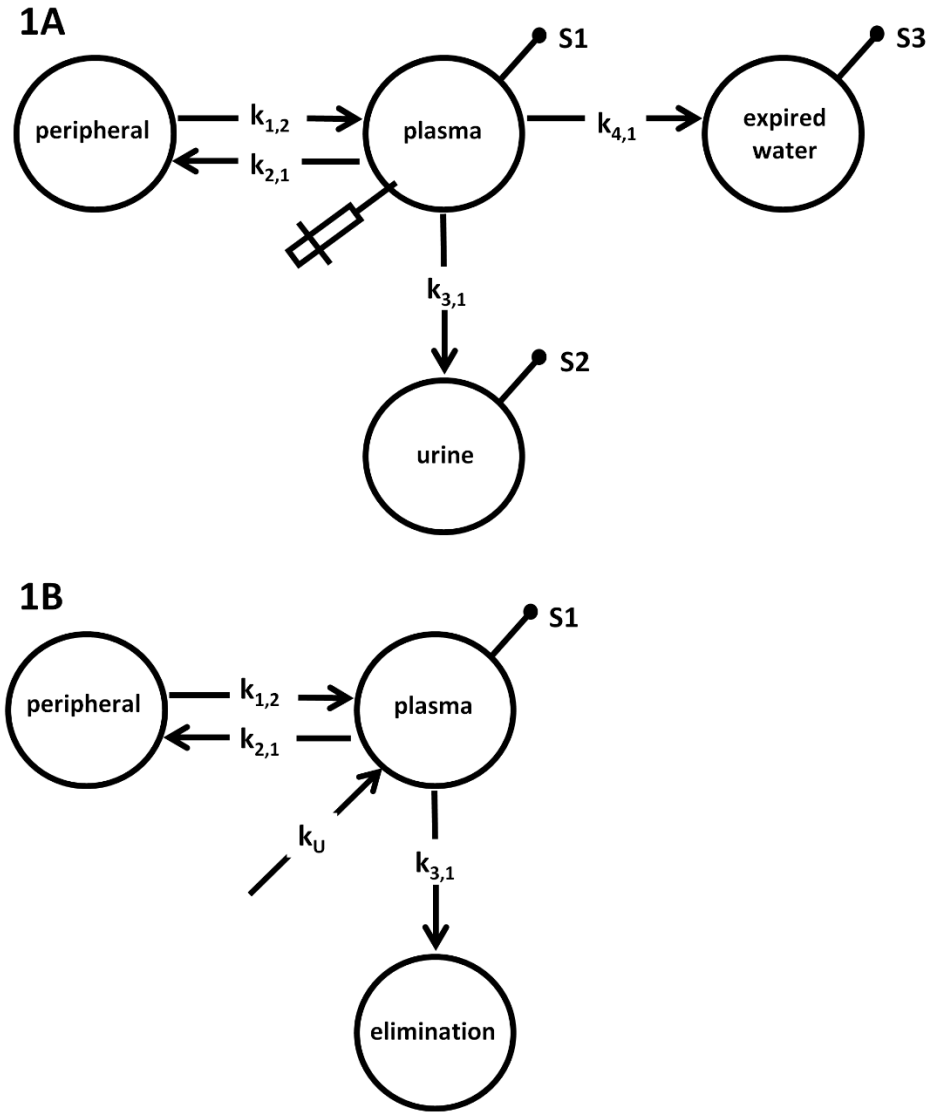

Fig. 2-1. Compartmental models used to describe PFOA kinetics in trout following bolus intra-arterial injection (1A) or continuous waterborne exposure (1B). Samples collected to evaluate the kinetics of PFOA over time are denoted with the symbol "S". The identities of individual rate constants ($k_{1,2}$, etc.) and the means by which they were determined are described in the text. Inter-compartmental rate constants are labeled using the naming convention employed by the SAAM-II modeling software. Under this convention, a rate constant designated $k_{a,b}$ refers to chemical flux to compartment "a" from compartment "b."

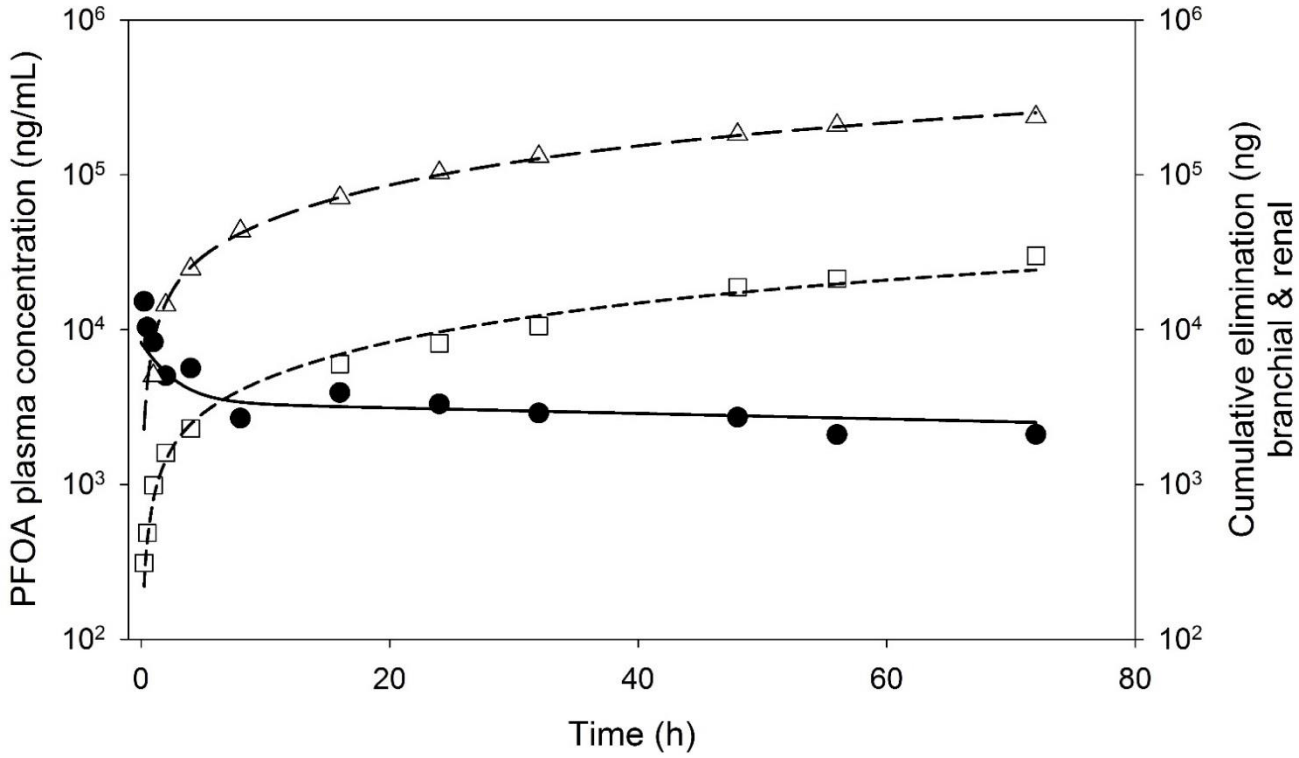

Fig. 2-2. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection. Data and model simulations are shown for Fish 1. Measured concentrations in plasma are shown as solid dots. Open triangles and open squares denote the cumulative mass of PFOA eliminated in urine and expired water, respectively. Lines show the optimized fit of model simulations to measured values: solid line – plasma, short dashes – expired water, long dashes – urine.

3. Results

3.1. Performance of chambered fish

Table 2-1 provides physiological parameters for all trout used in kinetic studies with PFOA. These values are similar to those reported in previous studies of chambered trout (Fitzsimmons et al., 2009; McKim and Goeden, 1982; McKim et al., 1994, 1999), suggesting the tested animals were of acceptable quality.

Table 2-1
Physiological performance of chambered fish[a]

	Study design	
Parameter	Bolus dose, elimination studies	Branchial uptake studies
Weight (g)	1146 (68)	1147 (345)
Gender	6 ♂, 2♀	5 ♂, 3 ♀
Ventilation volume (V_{VOL}; mL/min)	156 (36.2)	163 (33.8)
Oxygen consumption (VO_2; mg/h/kg)	41.8 (19.3)	46.3 (21.0)
Urine flow rate (UFR; mL/d/kg)	92.1 (28.1)	not measured

[a]Reported values are given as the mean (SD) for each study design

3.2. Bolus dose PFOA elimination experiments

Fig. 2-2 shows a log-scale plasma elimination curve for a representative animal given a bolus dose of PFOA. Plots for the other seven animals comprising this portion of the study are provided as supplementary data (Appendix 1, Figs. S2-2–S2-8). The log-transformed plasma concentration curve demonstrates a clear distribution phase for PFOA, followed by an extended log-linear elimination phase. The data for urine and branchial elimination represent the cumulative amount (ng) of PFOA eliminated over

time. For this particular animal, the cumulative renal elimination was approximately ten-fold higher than that for branchial elimination. Calculated kinetic parameters for fish used in the elimination studies are provided in Table 2-2. Means (±SD) determined for V_{SS} and $T_{1/2}$ were 523.7 (±138.2) mL/kg and 12.6 (±7.2) d, respectively. The variance in fitted V_{SS} values was small relative to the mean value (CV= 26%) while that observed for $T_{1/2}$ was somewhat larger (C_V= 51%). The ratio of renal clearance to branchial clearance varied from approximately 2.8–18.1 (mean of 11.2). Across all the animals, the average fraction of the overall elimination attributable to urine was 88.5%. These experiments were not designed to study sex-differences in PFOA elimination by rainbow trout, and it is not possible to draw a statistically meaningful conclusion based on them. An examination of derived kinetic parameters suggests, however, that there were no consistent gender-specific differences in distribution or clearance. Thus, while the lowest rates of total and renal clearance were exhibited by a female fish (no. 7), clearance rates determined for the other female (no. 2) were similar to those calculated for several males.

Table 2-2
Kinetic parameters and tissue/plasma concentration ratios from elimination studies with chambered trout

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Fish 7	Fish 8	Mean (SD)
Sex	♂	♀	♂	♂	♂	♂	♀	♂	
Weight	1032	1179	1134	1274	1172	1120	1132	1024	1146 (68)
Kinetic parameters									
V_{SS} (mL/kg)	296.4	432.9	510.2	732.3	526.5	680.6	456.5	554.4	523.7 (138.2)
Cl_R (mL/h/kg)	1.1	1.26	0.91	2.25	0.59	0.68	0.39	3.6	1.35 (1.07)
CL_B (mL/h/kg)	0.11	0.09	0.05	0.12	0.14	0.11	0.14	0.23	0.12 (0.05)
Cl_T (mL/h/kg)	1.21	1.35	0.97	2.37	0.73	0.8	0.53	3.83	1.47 (1.11)
$T_{1/2}$ (d)	6.9	7.9	13.4	7	17.7	22	21.9	3.7	12.6 (7.2)
Tissue/plasma, urine/plasma, and bile/plasma concentration ratios at takedown									
Liver	0.22	0.16	0.39	0.41	0.24	0.28	0.33	0.53	0.32 (0.12)
Kidney	0.24	0.24	0.26	0.46	0.25	0.24	0.37	0.46	0.31 (0.10)
Muscle	0.015	0.008	0.009	0.011	0.011	0.026	0.013	0.013	0.013 (0.006)
Urine	0.24	0.57	0.65	0.72	0.17	0.52	0.09	0.76	0.46 (0.24)
Bile	0.13	0.1	0.14	0.46	0.12	0.14	0.21	0.12	0.18 (0.12)

Although the methods employed in this study did not permit a kinetic evaluation of PFOA elimination to bile, bile produced by chambered fish tends to be retained within the gallbladder. It is possible, therefore, to evaluate this route of elimination by measuring the volume of stored bile and resulting concentration of PFOA. The bile/plasma concentration ratio for these animals averaged 0.18, which suggests that PFOA was not being actively secreted into bile. The mean PFOA concentration at the conclusion of the exposure was 264 ng/mL, while the average volume of collected bile was 2.8 mL. Multiplying gives an average total mass of 739 ng, which is substantially lower than the cumulative mass of chemical eliminated by renal and branchial routes (255.1 and 19.9 g, on average, respectively).

3.3. Branchial uptake experiments

Initial uptake studies were carried out at nominal aqueous concentrations of 5 and 50 g/L PFOA. In each case there were no measurable differences in concentration between inspired and expired water, and no increase in plasma PFOA concentration above that of background (data not shown). Subsequent exposures were conducted at a nominal concentration of 500 g/L. As before, there were no differences in PFOA concentration in water entering and exiting the gills. However, measured PFOA concentrations in plasma were well above those of background, increasing rapidly during the first 24 h and then approaching an apparent steady-state value.

Table 2-3
Kinetic parameters and tissue/plasma concentration ratios from branchial uptake studies with chambered trout

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Fish 7	Fish 8	Mean (SD)
Sex	♀	♂	♂	♂	♀	♂	♀	♂	
Weight	1496	1295	1563	1470	968	728	844	814	1147 (345)
PFOA water exposure (ng/mL)	296.4	296.4	296.4	296.4	288.4	288.4	288.4	288.4	
Kinetic parameters									
k_U (L/d/kg)	0.12	0.13	0.08	0.09	0.26	0.30	0.30	0.21	0.19 (0.09)
Uptake efficiency (%)	0.07	0.06	0.03	0.03	0.19	0.18	0.17	0.07	0.10 (0.07)
Tissue/plasma concentration ratios at takedown									
Liver	0.62	0.18	0.75	NS	0.16	0.34	0.30	0.20	0.36 (0.23)
Kidney	0.66	0.18	0.76	NS	0.59	0.35	0.61	0.24	0.48 (0.22)
Muscle	0.021	0.010	0.016	NS	0.076	0.012	0.014	0.012	0.023 (0.024)

Branchial uptake rate constants (k_U; L/d/kg) were obtained by fitting model simulations to measured plasma concentration time-course data (Table 2-3). Table 2-3 also provides chemical uptake efficiency values for each animal, calculated as k_U/V_{VOL}. A representative plot of modeled PFOA concentrations, along with measured values, is shown in Fig. 2-3. Plots for the other seven animals are provided as supplementary data (Appendix 1, Figs. S2-9–S2-15). Uptake rate constants for all animals were low, ranging from 0.08 to 0.30 L/d/kg. Branchial uptake efficiencies ranged from 0.03 to 0.19%, averaging 0.13%.

3.4. Tissue distribution experiment

Data from the tissue distribution study were developed as a set of tissue/plasma concentration ratios to investigate how rapidly the fish approached an internal steady-state (Table 2-4). Tissue/plasma ratios for all tissues did not change significantly after the first sampling time, suggesting that the majority of the distribution phase was complete by 24 h. This finding is consistent with the kinetics of PFOA in plasma observed in elimination studies. The tissue distribution data were not sufficient to calculate a full chemical mass-balance. Nevertheless, it was possible to estimate the fraction of the administered dose remaining in the sampled tissues at different time points, assuming that each tissue comprises a fixed fraction of total body weight. A fractional body weight of 2.45% was used for plasma, assuming a fractional blood volume of 3.5% and a hematocrit of 30% (Barron et al., 1987). Fractional body weights for other tissues were taken to be 67% for muscle, 1.27% for liver, and 0.76% for kidney (Nichols et al., 1990). These calculated values, which are given in Table 2-4, suggest that more than half of the administered dose was eliminated during the first 24 h post

dosing, before the chemical had a chance to achieve an internal steady-state distribution.

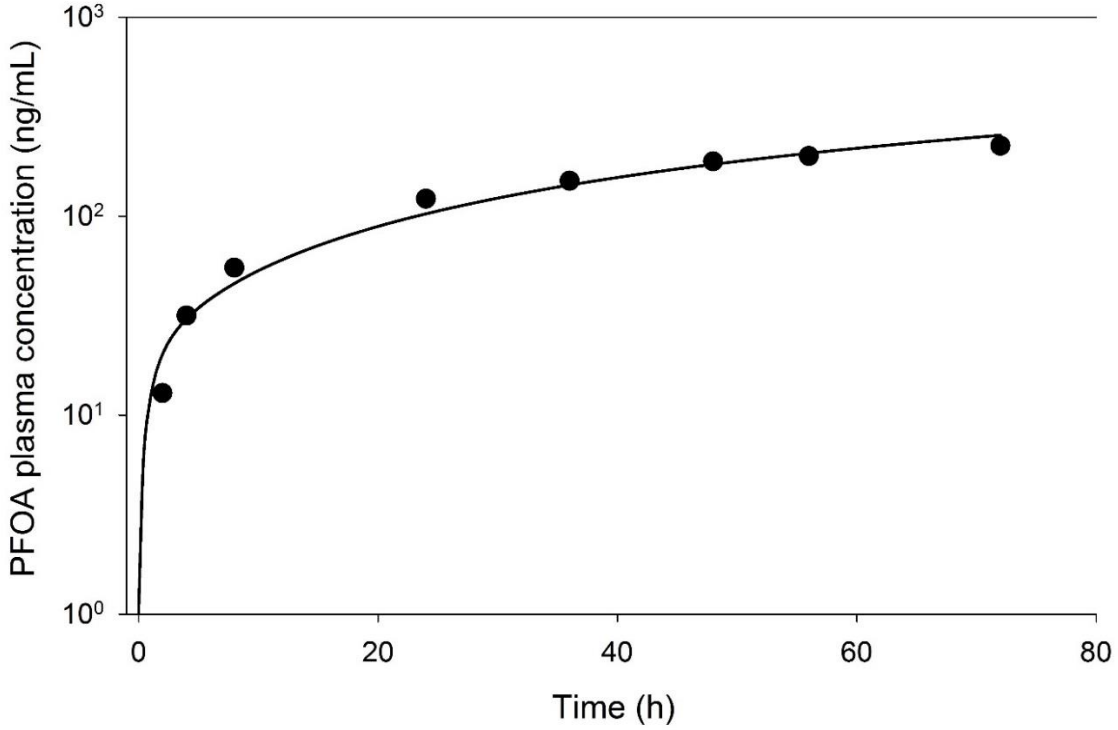

Fig. 2-3. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 1. Measured values are shown as individual points. The fitted model simulation is shown as a solid line.

Table 2-4
Kinetics of PFOA distribution to selected tissues and organs following bolus intra-arterial injection[a]

	Controls	Day 1	Day 3	Day 7	Day 14
Sex	1 ♂, 3♀	2 ♂, 2♀	2 ♂, 1♀	2 ♂, 2♀	1 ♂, 3♀
Weight (g)	214.5 (8.7)	271.8 (3.8)	278.7 (33.3)	295.8 (36.6)	242.0 (28.7)
$[PFOA]_{plasma}$ (ng/mL)	BQ[b]	4762 (913)	2511 (791)	1252 (361)	346 (179)
Tissue/plasma ratios					
Liver	BQ[b]	0.145 (0.036)	0.156 (0.023)	0.161 (0.019)	0.193 (0.058)
Kidney	BQ[b]	0.121 (0.041)	0.154 (0.041)	0.137 (0.014)	0.177 (0.068)
Muscle	BQ[b]	0.011 (0.004)	0.011 (0.001)	0.010 (0.001)	0.015 (0.007)
Bile	BQ[b]	0.308 (0.039)	0.552 (0.204)	0.384 (0.052)	0.557 (0.249)
Mass-balance estimates - % of initial dose remaining					
Plasma	BQ[b]	11.67 (2.24)	6.15 (1.94)	3.07 (0.88)	0.85 (0.44)
Muscle	BQ[b]	3.49 (0.77)	1.90 (0.45)	0.87 (0.27)	0.32 (0.12)
Liver	BQ[b]	0.87 (0.26)	0.48 (0.08)	0.25 (0.05)	0.08 (0.02)
Kidney	BQ[b]	0.42 (0.08)	0.29 (0.08)	0.13 (0.03)	0.05 (0.04)

[a.] Data presented as mean (SD), n=4.
[b.] BQ = Concentration too low to quantitatively measure

3.5. Plasma binding

The mean (±SD) recovery of PFOA from spiked samples of plasma ultrafiltrate was 71.9 ± 7.4%. In contrast, PFOA concentrations in filtrates of whole plasma were all below the limit of quantitation (5 ng/mL). It was not possible, therefore, to calculate plasma binding of PFOA with any certainty. However, it appears that this binding exceeded 99%.

4. Discussion

The PFAAs represent an important class of environmental contaminants. Although individual members of this chemical class have been banned or are voluntarily being phased out of production, new, generally shorter chain compounds have been developed as replacements (Lau, 2009; Lau et al., 2007). It is critical, therefore, to develop an improved understanding of their behavior in the environment, including their uptake and elimination by fish. The results of the present study indicate that renal clearance is the dominant route of PFOA elimination in rainbow trout. Based on modeled values, the ratio of CL_R to CL_B varied somewhat among animals (ranging from 2.8 to 18.1). Some of this variability may have been due to differences in the gender of tested animals as well as their state of sexual maturity. Nevertheless, CL_R as a fraction of CL_T exceeded 73% in all fish and averaged 88.5%. To our knowledge, this is the first direct demonstration that renal clearance contributes substantially to elimination of a PFAA by fish.

Until now, the only detailed studies of the kinetics of PFOA in rainbow trout were those conducted by Martin et al. (2003a, 2003b). Our model-fitted PFOA half-life from the elimination experiments averaged 12.6 d, which compares to 5.2 d reported by Martin et al. (2003a; carcass value). While this is slightly more than a twofold difference, it is important to note that Martin et al. (2003a) obtained fitted uptake and elimination values for animals weighing approximately 7 g. To the degree that processes (e.g., gill ventilation, urine production) responsible for PFOA elimination scale to a fractional exponent of body weight, the slower elimination rate observed in the present study may reflect the large size of fish (averaging over 1 kg) required for chambered experiments. De Silva et al. (2009) reported a PFOA half-life of 5.6 d in trout weighing 36 g. This

result agrees well with that obtained by Martin et al. (2003a) and is subject to the same allometric considerations when compared to the present effort. Lee and Schultz (2010) used an oral dosing approach to study the uptake and elimination of PFOA in fathead minnows of both genders. The whole-body half-life in male minnows was approximately 68.5 h, while that determined for females was 6.3 h. This difference was greatly reduced by either pretreatment of females with 17-trenbolone or males with estradiol. Although the present study was not designed to test sex-based differences in elimination, it is clear that there were no consistent differences on the same order as those reported in fathead minnows.

While it was not possible to characterize the kinetics of PFOA elimination in bile, the fact that bile accumulated in the gallbladder during the course of each experiment allowed for a quantitative recovery of compound eliminated by this route. The resulting mass balance estimate for PFOA elimination in bile was far smaller than values calculated for renal or branchial elimination. This observation is consistent with an earlier finding that PFOA concentrations in bile were similar to those in plasma after a 28-d aqueous exposure (Martin et al., 2003a). Because the oral bioavailability of PFOA is relatively high (60%; Martin et al., 2003b), it is also likely that PFOA eliminated to feces would be reabsorbed by the animal. Taken together, these findings suggest that bile is an unimportant route of elimination for this compound.

Chambered trout were also used to assess branchial uptake of PFOA. Slow uptake across the gills made it infeasible to directly evaluate this process using measured chemical concentrations in inspired and expired water. We therefore modeled uptake using measured plasma time-course data and an average concentration in water. The average model-fitted value for k_U was 0.19 L/d/kg. Using individual uptake terms

from the models and measured ventilation volumes for each fish, we calculated a mean uptake efficiency of 0.1%.

For comparison, Martin et al. (2003a) reported a fitted uptake clearance of 0.53 L/d/kg. In order to relate this value to the present study, however, it is necessary to account for the effect of fish size on ventilation volume. Arnot and Gobas (2004) used data provided by Thurston and Gehrke (1990) to develop an allometric relationship for oxygen consumption in fish (V_{OX}; mg O_2/d): $V_{OX} = 980W_B^{0.65}$, where W_B is the fish's weight in kg. To develop a similar expression for Q_V in trout we adopted the same allometric exponent and applied this to the measured Q_V for a 1 kg animal (10.6 L/h; Nichols et al., 1990): $Q_V = 10.6W_B^{0.65}$. Using this relationship, the predicted Q_V for a 7 g trout is approximately 60.2 L/h/kg. Dividing this value into the uptake clearance given by Martin et al. (2003a) yields an average uptake efficiency of 0.037%, which is within the range of values determined in the present study. These calculations provide only a rough basis for comparing the present study to that of Martin et al. (2003a), and other factors may have contributed to the modest difference in study results including differences in fish strain and stage of development. Nevertheless, it is clear from both efforts that the efficiency of PFOA uptake directly from water is very low.

Tissue data from all three sets of experiments showed that PFOA has a high affinity for plasma relative to that for other tissues. This finding is consistent with previous observations in both fish and mammals, and provides a likely explanation for the low estimated V_{SS} (0.52 L/kg) determined in bolus injection studies. A detailed comparison of our tissue data to that provided by Martin et al. (2003a) is complicated by the fact that this latter study reports measured concentrations graphically as log-scaled values (Fig. 2 of the cited paper). However, a general comparison is still instructive. As

in this earlier study, the concentration of PFOA in tissues followed the general pattern: plasma > liver > kidney > muscle. Tissue/plasma concentration ratios at equilibrium (based on means for chambered fish as well as means for days 3, 7, and 14 of the tissue distribution study) ranged from 0.16 to 0.36 for liver, 0.16–0.48 for kidney, and 0.010–0.023 for muscle. Using data reported by Martin et al. (2003a), similar ratios may be calculated for liver (approximately 0.5) and kidney (approximately 0.15). PFOA concentrations in muscle reported by Martin et al. (2003a) suggest a muscle/plasma ratio of approximately 0.1. This value is 5–10 times higher than that determined in the present study. Nevertheless, both studies indicate that PFOA has little affinity for muscle tissue relative to that for plasma.

As noted previously, existing models for accumulation of PFAAs in fish either do not explicitly account for renal elimination (Armitage et al., 2013) or have been parameterized using data from mammals (Ng and Hungerbühler, 2013). The present work confirms the importance of renal elimination for one member of this chemical class, and provides an initial dataset that can be used to model this process. Although additional work is needed to determine whether other PFAAs are eliminated by fish in the same manner, the results of this effort suggest that differences in renal handling of individual PFAAs may substantially determine their rate of elimination by fish, and by extension their relative propensity to accumulate.

Finally, we note with interest the possibility that membrane transporters play an important role in renal elimination of PFAAs by fish, as is understood to occur in mammals. Renal elimination is the primary determinant of differences in the overall rate of elimination among different species of mammals, and between sexes of the same species (Andersen et al., 2006; Butenhoff et al., 2004b; Han et al., 2012). Additional

work has shown that these differences are due to variable expression of, and complex interactions between, specific renal transporters (Kudo et al., 2002; Nakagawa et al., 2009; Weaver et al., 2010). The sex-based difference in PFOA elimination observed in at least one fish species (fathead minnows; Lee and Schultz, 2010), along with the observed predominance of urine as a route of PFOA elimination in trout, suggest that similar processes are operating in fish.

Gene expression studies indicate that fish possess a diverse array of membrane transport proteins including ATP binding cassette (ABC)transporters (Fischer et al., 2011; Loncar et al., 2010) and solute carriers (SLC; Verri et al., 2012), the latter of which include organic anion transporters (Oats) and organic anion transporting polypeptides (Oatps; Popovic et al., 2010). Most transporters probably evolved to transport endogenous substrates (e.g., bile salts, cyclic nucleotides, hormones) or naturally-derived toxins. However, functional studies suggest that many are capable of transporting xenobiotic substances (Aslamkhan et al., 2006; Bard, 2000; Doi et al., 2001; Fischer et al., 2011, 2013; Miller et al., 1998; Sturm et al., 2001; Sturm and Segner, 2005; Zaja et al., 2008, 2011). In this regard, ABC transporters have been given special attention because of their ability to transport compounds against their electrochemical gradient. By comparison, functional studies of Oats and Oatps from fish are fewer in number (Boaru et al., 2006; Meier-Abt et al., 2007; Wolff et al., 1997).

With respect to renal transporters, a substantial literature indicates that fish kidneys secrete both “classical” substrates for Oats (e.g., p-aminohippurate) as well as some anionic environmental contaminants (e.g., 2,4-dichlorophenoxyacetic acid; Pritchard and James, 1979). The role of the kidney in secreting products of Phase I and II biotransformation also is well-established (McKim et al., 1999; Pritchard and Bend,

1991, 1984). To date, however, there have been few studies showing that membrane transporters, renal or otherwise, limit the accumulation of an important environmental contaminant in fish, absent biotransformation.

Although the present study does not prove that transporters are responsible for renal clearance of PFOA by trout, a close examination of the data provides indirect evidence supporting this conclusion. Previously, McKim et al. (1999) determined the glomerular filtration rate (GFR) in chambered trout to be 6.1 mL/h/kg. The average urine flow rate (UFR) for the same animals was 4.3 mL/h/kg. In the present study, the measured UFR, averaged across all animals (mean ± SD), was 3.8 ± 1.1 mL/h/kg. We may assume, therefore, that the GFR determined by McKim et al. (1999) reasonably approximates that of fish exposed to PFOA. Binding studies conducted as part of the current study indicated that PFOA is nearly 100% bound in trout plasma. Based on this information, the renal clearance that would have been predicted to occur due to glomerular filtration is negligible (0.1 mL/h/kg). Instead, the average CLR was 1.35 mL/h/kg, which is 22% of the estimated GFR.

Averaged across all fish in Table 2-2, the total PFOA concentration in urine at test termination was 46% of that in plasma. We did not measure PFOA binding in urine so it is unclear whether free concentrations in urine exceeded those in plasma. However, freshwater fish are known to produce highly dilute urine (Hickman and Trump, 1969), so it is possible that much of the PFOA in urine was freely dissolved. Characterizing the free concentrations of PFOA in urine and plasma is important because this may indicate whether the mechanisms responsible for renal clearance require energy to overcome an electrochemical gradient. Additional experiments utilizing chemical inhibitors of specific Oats may confirm whether renal transporters

contribute to the elimination of PFOA by trout, while studies conducted at different dosing levels would indicate whether these systems saturate at toxicologically relevant concentrations. The chambered trout model provides an excellent system for exploring these questions.

Chapter 3: Toxicokinetics of perfluorooctane sulfonate in rainbow trout (*Oncorhynchus mykiss*)

Daniel M. Consoer, Alex D. Hoffman, Patrick N. Fitzsimmons, Patricia A. Kosian, and John W. Nichols.

Published in *Environmental Toxicology and Chemistry*, March, 2016. Volume 35(3), p 717-27.

Abstract

Rainbow trout (*Oncorhynchus mykiss*) confined to respirometer-metabolism chambers were dosed with perfluorooctane sulfonate (PFOS) by intra-arterial injection and sampled to obtain concentration time-course data for plasma and either urine or expired water. The data were then analyzed using a 2-compartment clearance-volume model. Renal and branchial clearance rates (mL/d/kg) determined for all experiments averaged 19% and 81% of total clearance, respectively. Expressed as mean values for all experiments, the steady-state volume of distribution was 277 mL/kg and the terminal half-life was 86.8 d. Additional animals were exposed to PFOS in water, resulting in an average calculated branchial uptake efficiency of 0.36%. The renal clearance rate determined in the present study is approximately 75 times lower than that determined in earlier studies with perfluorooctanoate (PFOA). Previously, it was suggested that PFOA is a substrate for membrane transporters in the trout kidney. The present study suggests that glomerular filtration may be sufficient to explain the observed renal clearance rate for PFOS, although a role for membrane transporters cannot be ruled out. These findings demonstrate that models developed to predict the bioaccumulation of

perfluoroalkyl acids by fish must account for differences in renal clearance of individual compounds.

Introduction

The perfluoroalkyl acids (PFAAs), including the perfluorosulfonates (PFSAs) and perfluorocarboxylates (PFCAs), are widely used as lubricants, surfactants, stain-resistant coatings, fire-fighting foams, and photographic developers. Initial concerns regarding the PFAAs as a class of environmental contaminants date to a 2001 article by Geisy and Kannan (2001) on the global distribution of perfluorooctane sulfonate (PFOS) in wildlife. This finding prompted numerous studies which have demonstrated the accumulation of PFOS and other PFAAs in aquatic and terrestrial animals, the environment, and humans. Subsequent work has shown that bioaccumulation and the potential for biomagnification of PFAAs in fish depends on the properties of individual compounds (Kannan et al., 2005; Furdui et al., 2007; Conder et al., 2008; Ishibashi et al., 2008; Ahrens et al., 2014). Generally, bioaccumulation is greater for the PFSAs than the PFCAs and increases with the length of the perfluorocarbon chain. The production and use of PFOS and its precursors have been restricted under the terms of the Stockholm Convention on Persistent Organic Pollutants (i.e., as an Annex B substance), and other PFAAs are being phased out voluntarily (Buck et al., 2011). Nevertheless, modified and shorter-chain members of this chemical class continue to be produced in large quantities. It is of interest, therefore, to understand how PFAAs behave in the environment and to develop predictive models that describe this behavior.

Mechanistic models used to predict bioaccumulation of neutral organic chemicals in fish generally assume that uptake and elimination occur by passive diffusion and that chemicals partition nonspecifically to tissue lipids in accordance with their relative hydrophobicity, typically represented by the octanol– water partition coefficient (K_{OW}). Because of their surfactant properties, it is difficult to accurately measure K_{OW} values for PFAAs. Moreover, these compounds are >99.9% ionized at physiological pH values. Research with mammals also suggests that PFAAs do not partition to tissue lipids but instead bind to specific proteins including serum albumin in the blood (Han et al., 2003; Chen and Guo, 2009; Bischel et al., 2011) and fatty-acid binding proteins in liver (Luebker et al., 2002). The determinants of PFAA distribution in fish are less well known; however, observed patterns of distribution are qualitatively consistent with those seen in mammals (Hoff et al., 2003; Martin et al., 2003a; Shi et al., 2012; Goeritz et al., 2013).

Recognizing the limitations of existing approaches, Ng and Hungerbühler (2013) developed a model for PFAA accumulation in fish that accounts for protein binding interactions. This model describes the animal as a set of 4 tissues, each of which is comprised of tissue and interstitial fluid compartments. Characteristic binding relationships were used to determine bound and free fractions in each compartment, as well as in blood. This model also accounts for possible elimination of PFAAs to urine. Lacking measured information for fish, however, model parameters that describe renal elimination were obtained from studies with rats. Modeled simulations accurately described observed patterns of accumulation in blood and tissues. Generally, however, the model tended to underestimate reported whole-body bioconcentration factors for PFAAs in fish.

In a previous effort, we used large trout confined to respirometer-metabolism chambers to study the kinetics of PFOA uptake and elimination (Consoer et al., 2014). When PFOA was given to fish as a bolus intra-arterial dose, approximately 90% was eliminated to urine. The average renal clearance was determined to be approximately one-fourth the reported glomerular filtration rate (GFR) for similarly prepared animals. However, additional experiments showed that PFOA in trout plasma is nearly 100% bound. Taken together, these observations suggest that renal clearance of PFOA in trout is mediated, at least in part, by membrane transporters and that these transporters are capable of operating against an electrochemical gradient. The purpose of the present study was to use chambered trout to investigate the uptake and elimination of PFOS. Because PFOS has been tested extensively to determine its toxicity to fish, information relating to its uptake and accumulation has value in its own right. Our larger goal, however, was to support the development of mechanistic models for bioaccumulation of PFAAs in fish. The results of the present study clearly demonstrate the critical importance of renal elimination as a determinant of this behavior and suggest that differences in active secretion and/or reabsorption within the kidney are responsible for observed differences in bioaccumulation of individual compounds.

Materials and methods

Chemicals

Perfluorooctane sulfonate (>98% pure) was purchased from Sigma-Aldrich. Both PFOS and mass-labeled [^{13}C] PFOS standards were purchased from Wellington Laboratories. Amersham Radiochemicals supplied the [^{14}C] polyethylene glycol (PEG; 50 mCi/mL in ethanol), and the Ultima Gold scintillation cocktail was obtained from

Packard Instruments. High-performance liquid chromatography (HPLC)–grade acetonitrile, methanol, and glacial acetic acid were purchased from Fisher Scientific.

Test organism source, care, and handling

Rainbow trout (*Oncorhynchus mykiss*) weighing approximately 100 g were obtained from the US Geological Survey's Upper Midwest Environmental Sciences Center in La Crosse, Wisconsin, and grown to the desired size for each experiment. The fish were fed a commercial trout chow (Silver Cup; Nelson and Sons) and maintained on a 16:8-h light:dark cycle at 11 ± 1ºC. Water used for fish holding was obtained directly from Lake Superior (single pass, sand-filtered, and ultraviolet treated) and had the following characteristics: alkalinity 41 mg/L to 44 mg/L as CaCO3, pH 7.6 to 7.8, total ammonia <1 mg/L, dissolved O_2 85% to 100% of saturation. All animals were fasted for 24 h prior to use.

Surgical preparation of animals

Elimination and branchial uptake studies were performed using trout confined to respirometer-metabolism chambers (McKim and Goeden, 1982). Fish weighing 700 g to 1500 g were surgically prepared as described (Consoer et al., 2014). Briefly, fish were placed under continuous anesthesia with tricaine methane sulfonate (MS-222; Finquel; Argent Laboratories) and fitted with a dorsal aortic cannula (Intramedic PE 50; Becton, Dickinson). Animals used for the renal elimination studies were also fitted with a urinary catheter (Intramedic PE 90). A latex membrane sewn to the fish's mouth allowed separate collection of inspired and expired water. A second membrane just posterior to the pectoral fins prevented further dilution of expired water. Fish were allowed 24 h to recover after surgery before starting an experiment. Water temperature (Celsius),

ventilation volume (V_{VOL}; L/d/kg), and O_2 consumption (VO_2; mg/d/kg) were monitored continuously using an automated data collection system and customized software Carlson et al., 1989). This information was then evaluated to assess the viability and representativeness of each preparation and to generate average values for the experimental time period.

Elimination studies

Six fish of mixed sex (3 male, 3 female) were given a bolus intra-arterial injection of PFOS to study the kinetics of renal elimination. An additional 6 animals (1 male, 5 female) were then dosed in the same manner to study the kinetics of branchial elimination. Each fish was given a nominal dose of 1.0 mg/kg, dissolved in trout plasma (1 mL/kg injection volume). Blood (100 µL) was sampled periodically for 72 h to evaluate the kinetics of PFOS in plasma. Urine (50–100 µL) or expired water (1 L) was then sampled in conjunction with blood to characterize elimination by each route. All samples were collected and processed as described (Consoer et al., 2014).

For the renal elimination studies, the volume of urine produced between sampling times was recorded to calculate the urine flow rate for each time period as well as an average urine flow rate for the entire experiment. Two of these animals were injected with [^{14}C] PEG to measure GFR. The methods used to perform these measurements are described in detail elsewhere (McKim et al., 1999). Briefly, fish were given a 300-µL injection of [^{14}C] PEG (1.5 µCi in 90% physiological saline/10% ethanol) just after dosing with PFOS. Blood (200 µL) and urine (200 µL) samples were then collected for determination of GFR at 8 h, 16 h, 32 h, and 72 h after [^{14}C] PEG injection using the relationship

$GFR_{[14C]\ PEG}$ (mL/min/kg) = (urine DPM)(urine flow rate) / plasma DPM (1)

where urine and plasma DPM refer to disintegrations/min/mL of sample and urine flow rate is in mL/min/kg. Sample counting was performed for 20 min using a scintillation counter (Beckman Coulter).

Fish were euthanized after the final sampling period with an overdose of MS-222. Samples of kidney, liver, muscle, and bile (branchial elimination studies only) were collected and stored at –20 ºC for later analysis. Muscle was sampled from above the lateral line, anterior to the dorsal fin. The liver, kidney, and bile were collected in their entirety.

Branchial uptake studies

Six trout of mixed sex (1 male, 5 female) were exposed to PFOS in water for 72 h. A stock solution was prepared by dissolving PFOS in deionized water (Milli-Q; EMD Millipore). This solution was metered into a mixing cell and diluted with Lake Superior water. The dilute solution was then supplied to each chambered animal in excess of its respiratory requirement (440 mL/min). A preliminary uptake study was carried out at a nominal aqueous concentration of 0.5 ng/mL. Under these conditions, there were no measurable differences in concentration between inspired and expired water, and the plasma PFOS concentration did not increase above that of background (data not shown). All subsequent exposures were conducted at a nominal concentration of 5 ng/mL. Blood (100 µL), inspired water (1 L), and expired water (1 L) were sampled at 2 h, 4 h, 8 h, 24 h, 36 h, 48 h, 54 h, and 72 h. These samples were then processed in the same manner as samples obtained from PFOS elimination studies. Samples of liver,

kidney, muscle, and bile were again collected attest termination and stored at –20 ºC for later analysis.

Tissue distribution studies

Twenty trout of mixed sex, weighing 150 g to 250 g each, were used to study the distribution time course of PFOS in liver, kidney, muscle, and plasma. Fish were divided into 5 groups of 4 animals each (4 dosed and 1 control group). The control group was processed immediately, and the resulting samples were stored at –20 ºC for later analysis. The remaining animals were given a bolus intra-arterial injection of PFOS dissolved in trout plasma at a nominal dose of 1 mg/kg and placed in 2-foot circular tanks supplied with Lake Superior water (4 fish/tank). One tank of animals was euthanized at 1 d, 3 d, 7 d, and 14 d post dosing. All tissue samples were stored at –20 ºC for later analysis.

Plasma binding

Binding of PFOS in trout plasma was evaluated using an ultrafiltration method (Centrifree UM-30 filter units; EMD Millipore). Nonspecific binding to the filter cartridge was reduced to an acceptable level by spiking PFOS into plasma ultrafiltrate, as recommended by the manufacturer. Samples of trout plasma or plasma ultrafiltrate (200 µL) spiked with 10 µg/mL, 100 µg/mL, or 1000 µg/mL PFOS (n = 3 each) were centrifuged at 2000 g for 30 min. Binding was then determined from the difference in measured concentrations of PFOS in spiked samples and sample filtrates.

Quality controls

Preliminary experiments were conducted to measure the recovery of PFOS from samples of plasma, urine, and Lake Superior water. Plasma samples from unexposed

fish were spiked with 10 µg/mL, 50 µg/mL, and 100 µg/mL PFOS (n = 3 each), mixed by vortexing for 30 s and allowed to equilibrate overnight. These samples were then extracted and analyzed as described below. Recovery from plasma (mean ± standard deviation [SD]) averaged 93.0 ± 1.3%, 107.5 ± 12.3%, and 109.5 ± 6.5% for the 10 µg/mL, 50 µg/mL, and 100 µg/mL samples, respectively. An overall recovery of 103.0% was subsequently used to correct measured values from the kinetic studies. Blank urine samples from unexposed animals were spiked with 5 µg/mL, 10 µg/mL, and 50 µg/mL PFOS (n = 3 each). Recoveries averaged 92.9 ± 11.9%, 80.4 ± 3.3%, and 79.0 ± 2.5%, respectively. Overall recovery from urine averaged 84.1%, and results from toxicokinetic studies were corrected to this value. Tank water samples were spiked with PFOS at concentrations of 0.05 µg/mL, 0.5 µg/mL, and 5 µg/mL (n= 3 each). Recoveries averaged 103.4 ± 16.7%, 96.9 ± 8.0%, and 107.7 ± 5.1%, respectively. The overall recovery from water averaged 102.6% and was used to correct measured concentrations in both elimination (i.e., expired water samples) and waterborne (inspired water and expired water) dosing studies.

Using methods developed for PFOA (Consoer et al., 2014) additional experiments were conducted to evaluate the potential for PFOS to adsorb to the blood cannulae and urine catheters used to dose animals and obtain samples of plasma and urine. Briefly, spiked samples of plasma and urine were pumped through the appropriate tubing material (Intramedic PE 50 or PE 90, cut to length) at 1 mL/min for 9 min. Each tube was then flushed with physiological saline to determine how rapidly PFOS was lost from the system. As with PFOA, these experiments suggested a small degree of adsorption and/or mixing effects. However, measured concentrations of PFOS rapidly approached 0 when the cannula or catheter was flushed with clean saline.

Based on these findings, it is highly unlikely that adsorption of PFOS to blood cannulae or urine catheters had any impact on the present study results.

Sample preparation and analysis

Samples of plasma, water, urine, and tissues were processed using methods developed for analysis of PFOA (Consoer et al., 2014). Briefly, plasma samples were extracted using an acetonitrile protein precipitation method (Flaherty et al., 2005; Reagen et al., 2008). A subsample (98 µL) of the extract in 75% acetonitrile/25% deionized water was then transferred to a 2-mL liquid chromatography (LC) vial with insert and spiked with 2 µL of [^{13}C] PFOS in 75% acetonitrile/25% deionized water as an internal standard (final concentration 5 ng/mL).

Water samples from branchial elimination studies were extracted and concentrated using a solid-phase extraction (SPE) method (Taniyasu et al., 2005; Taniyasu et al., 2008). Each sample was passed through a conditioned SPE cartridge (Oasis WAX 6 cm^3, 150 mg, 30mM; Waters) at 2 drops/s. The cartridge was then washed with 4.0 mL of 25 mM sodium acetate buffer (fraction 1, discarded). The PFOS was eluted by passing 4.0 mL of methanol through the cartridge (fraction 2), followed by 4.0 mL of 0.1% ammonium hydroxide in methanol (fraction 3). As with PFOA, PFOS was found to elute entirely in fraction 3. The fraction 3 eluant was then diluted 3:4 with 75% acetonitrile/25% deionized water. Water samples from the branchial uptake experiments were diluted 1:4 with 75% acetonitrile/25% deionized water. Urine samples were diluted 1:9 with 75% acetonitrile/25% deionized water. Subsamples (98 µL) derived from the processing of water and urine samples were transferred to LC vials with inserts and spiked with 2 µL of internal standard to a final concentration of 5 ng/mL.

Samples of liver, kidney, and muscle (~0.5 g) were spiked with internal standard to a final concentration of 5 ng/mL. The samples were homogenized (Polytron tissue homogenizer; Brinkman Instruments) in 9 volumes of 75% acetonitrile/25% deionized water for 2 min at approximately 15 000 rpm and centrifuged for 20 min at 10 000xg to form a solid pellet. A subsample (100 µL) of the supernatant was then transferred to an LC vial with insert. Bile samples (40 µL) were diluted into 200 mL of deionized water. This solution was spiked with internal standard to a final concentration of 5 ng/mL and processed using the same SPE procedure described for water samples.

Quantitation

Samples were analyzed on a HPLC–Triple Quadrupole 6410 tandem mass spectrometer (Agilent Technologies). Chromatographic separation was carried out on an Agilent Zorbax Extend-C18 column (3.5 µ, 2.1 x 100 mm). Mobile phase A was 2% ethanol, 1% methanol, 2% 250 mM ammonium acetate in methanol, 96% water, and 0.01% of 25% ammonium hydroxide, pH 8.2. Mobile phase B was 2% ethanol, 2% water, 1% 250 mM ammonium acetate in methanol, 95% methanol, and 0.01% of 25% ammonium hydroxide. The isocratic flow rate was 0.2 mL/min at 69% mobile phase B, and the injection volume was 10 µL. The PFOS eluted at 4.1 min and was detected using tandem mass spectrometry as a transition from 499 m/z to 80 m/z for PFOS and 503 m/z to 84 m/z for mass labeled PFOS internal standard. Transitions from 499 m/z to 99 m/z and from 503 m/z to 103 m/z were used as qualifier transitions. Bile samples were quantitated on the basis of the 499 m/z to 99 m/z transition because of high background rates of taurdeoxycholate, a bile acid that shares the 499 m/z to 80 m/z transition. Quantitation was carried out against a standard curve constructed from known quantities of standard and a known, constant quantity of mass-labeled internal

standard in 75% acetonitrile/25% deionized water. Blank samples spiked with internal standard were run for solvents used in extractions, and each animal provided its own predosing samples for analysis of background concentrations of PFOS in plasma, urine, and expired water.

Modeling and data analysis

Data obtained from the elimination experiments were analyzed by compartmental modeling using the SAAM II kinetic analysis software package (Epsilon Group; Barrett et al., 1998). In all cases, the kinetics of PFOS elimination from plasma exhibited a distinct distribution phase followed by a longer, log-linear elimination phase. We therefore modeled these data sets using a 2-compartment model with elimination from the central compartment (Figure 3-1A). The models were fitted by nonlinear regression, applying a data-relative variance model. Variance was modeled by assigning a fractional SD of 0.1 (equivalent to a SD of 10% of the measured value) to the plasma data and a fractional SD of 0.5 to the urine or expired water data. Fitted values were obtained from the model for kinetic parameters that describe PFOS distribution ($k_{1,2}$, $k_{2,1}$; 1/d), renal or branchial elimination ($k_{3,1}$; 1/d), and the apparent volume of the central compartment (V_C; mL/kg). Initially, the renal elimination data sets were modeled by fitting a second "other" elimination term ($k_{4,1}$; 1/d), as described below. Renal clearance (CL_R; mL/d/kg) and branchial clearance (CL_B; mL/d/kg) were calculated as the product of V_C and $k_{3,1}$ for each set of experiments. The steady state volume of distribution (V_{SS}; mL/kg) was calculated as the sum of V_C and the volume of the peripheral compartment (mL/kg), calculated as $(V_C \times k_{1,2})/k_{2,1}$. Assuming that very little PFOS would be eliminated in bile, this potential elimination route was ignored in the model. Measured PFOS

concentrations in bile determined in the branchial elimination studies provided support for this assumption (see below, *PFOS elimination to bile*).

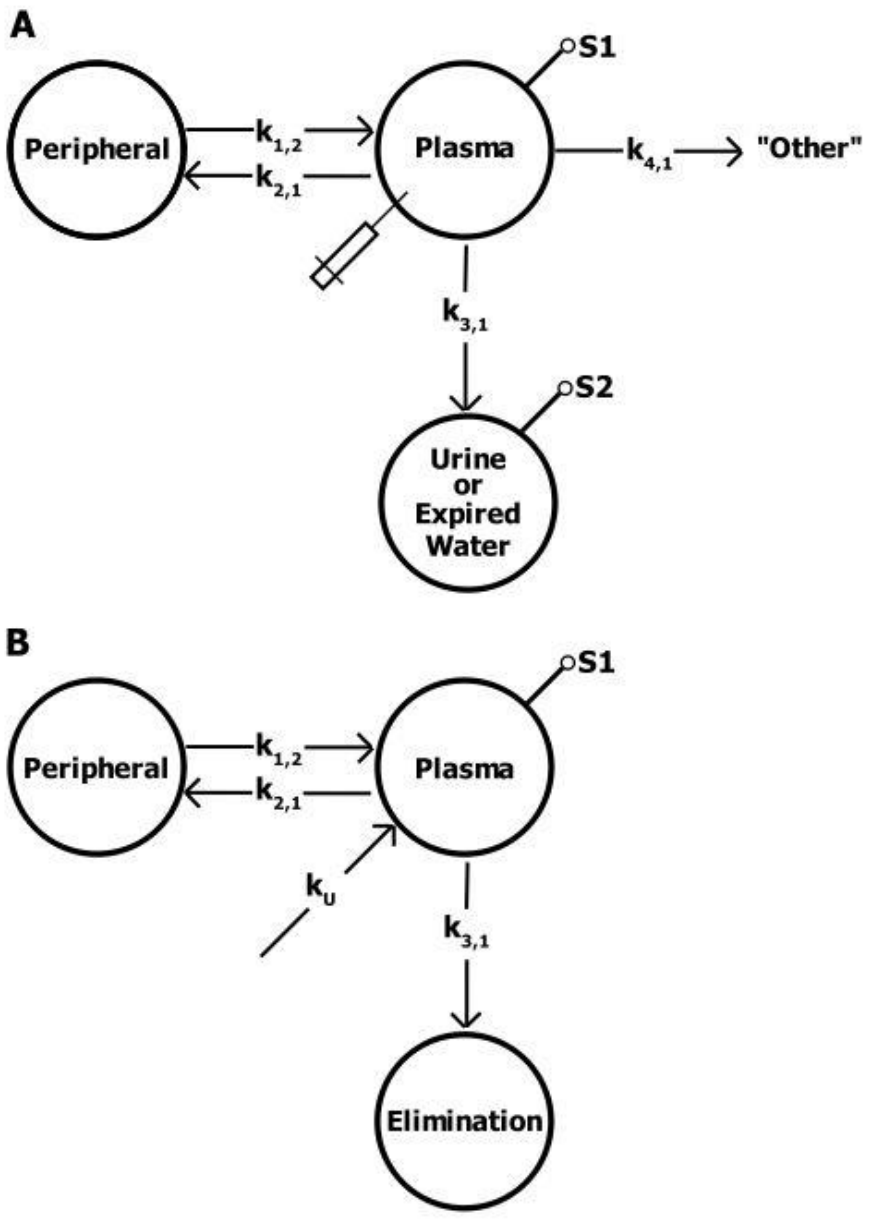

Figure 3-1. Compartmental models used to describe renal and branchial elimination of perfluorooctane sulfonate (PFOS) following bolus intra-arterial injection (**A**), as well as PFOS uptake (**B**) in continuous waterborne exposures. Samples collected to evaluate the kinetics of PFOS over time are denoted with the symbol "S". The identities of individual rate constants ($k_{1,2}$, etc.) and the means by which they were determined are described in the text. Inter-compartmental rate constants are labeled using the naming convention employed by the SAAM-II modeling software. Using this convention, a rate constant designated $k_{a,b}$ refers to chemical flux to compartment "a" from compartment "b".

Previously, we found that trout eliminate PFOA primarily via the urine (Consoer et al., 2014). Initially, therefore, we investigated PFOS elimination by the same route. Renal elimination was characterized by modeling to measured concentrations of PFOS in urine. An "other" elimination term ($k_{4,1}$), operating against the central compartment, was then included as a means of accounting for nonrenal elimination. These fitted models provided an excellent description of measured data but suggested that renal clearance was substantially lower than total clearance. Additional studies were therefore conducted to characterize branchial elimination of PFOS. In this second effort, the "other" elimination rate constant, $k_{4,1}$, was set equal to the mean of fitted $k_{3,1}$ values for renal elimination, obtained from the initial renal models. To obtain a final set of consistent models, the renal elimination experiments were modeled a second time, replacing the "other" elimination rate constant $k_{4,1}$ with the mean of fitted $k_{3,1}$ values for branchial elimination, obtained from the branchial elimination models. For both the renal and branchial elimination models, total clearance (CL_T; mL/h/kg) was calculated as V_C x ($k_{3,1}$ + $k_{4,1}$). The terminal elimination half-life ($T_{1/2}$; d), which is the time required for the plasma concentration to decrease by one-half during the log-linear elimination phase, was then calculated for each fish as 0.693/β, where (Toutain and Bousquet-Melou, 2004)

$$\beta = \frac{(k_{2,1}+k_{1,2}+k_{3,1}+k_{4,1})-r}{2} \quad (2)$$

and

$$r = \sqrt[2]{\left(k_{2,1} + k_{1,2} + k_{3,1} + k_{4,1}\right)^2 - (4\ x\ \left(k_{3,1} + k_{3,1}\right)\ x\ k_{1,2})} \quad (3)$$

Plasma data from the branchial uptake studies were modeled using a 2-compartment description (Figure 3-1B), with elimination from the central compartment represented by a single rate constant ($k_{3,1}$; mL/h/kg). Kinetic parameters for transfer to and from the peripheral compartment ($k_{1,2}$ and $k_{2,1}$) were fixed at mean values determined from the renal and branchial elimination studies (i.e., values averaged across the entire set of 12 fitted models). The value of $k_{3,1}$ was calculated as the sum of previously determined mean values for $k_{3,1}$ and $k_{4,1}$, again averaged across all 12 fish used in the elimination studies. Using this approach, the only term fitted by the branchial uptake model is the clearance constant for chemical uptake by the central compartment (k_U; L/d/kg), which operates against the measured PFOS concentration in inspired water (ng/mL).

The quality of model fit was characterized by calculating Akaike information criterion (AIC) and Bayesian information criterion (BIC) values. The SAAM-II files containing compartmental model schematics, example data, and model parameters are available on request from the corresponding author.

Results

Performance of chambered fish

Table 3-1 provides physiological parameters for all chambered trout used in kinetic studies with PFOS. These values are similar to those reported in previous studies (Consoer et al., 2014; McKim et al., 1982; McKim et al., 1999) suggesting that the tested animals were of acceptable quality. Two fish from the renal elimination studies were used to measure the GFR. The means (±SD) determined for these animals were 5.40 (±1.33) mL/h/kg and 5.14 (±0.56) mL/h/kg. Both values are close to

that given previously for chambered trout (6.1 mL/h/kg; McKim et al., 1999). Based on this agreement and the fact that GFR determinations require a substantial amount of blood, we decided to forego these measurements on other fish used to characterize urinary elimination of PFOS.

Models developed from renal and branchial elimination experiments

Representative plasma and urine data from a renal elimination experiment are shown in Figure 3-2. The log-transformed plasma concentration-time course demonstrates a clear distribution phase lasting approximately 24 h, followed by a log-linear elimination phase. From the slope of this log-linear phase, it is apparent that the terminal elimination half-life is much longer than the total study period. The renal elimination data represent the cumulative amount (nanograms) of PFOS eliminated over time. These data show that fish eliminated measurable quantities of PFOS to urine throughout the experiment. Initially, data from the renal elimination experiments were simulated using a model that includes a fitted "other" elimination term, $k_{4,1}$ (Supplemental Data, Appendix 2, Figures S3-1–S3-6). Based on this evaluation, CL_R was determined on average to account for only 1% of CL_T, whereas the balance of clearance (99%) was attributed to branchial elimination (Supplemental Data, Appendix 2, Table S3-1). Importantly, the term ($k_{3,1}$) which describes renal elimination of PFOS was informed by measured concentrations of PFOS in urine during the entire experimental time course. For this reason, our confidence in fitted values of $k_{3,1}$ was relatively high. In contrast, $k_{4,1}$ was largely determined by the kinetics of PFOS in plasma during the terminal elimination phase, which was typically represented by 4 data points. Because of this lack of data at later time points as well as the observed low rate of PFOS elimination from plasma, our confidence in fitted values of $k_{4,1}$ was relatively low.

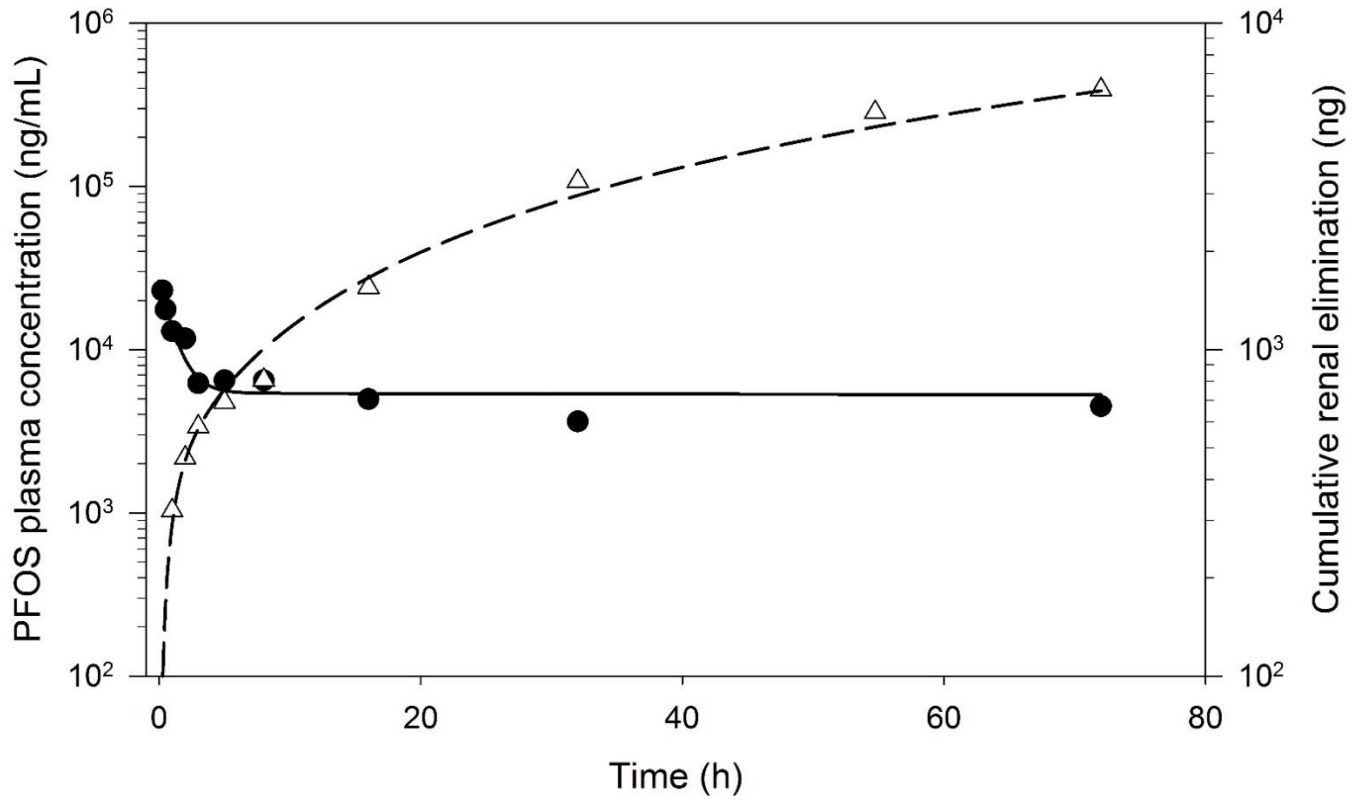

Figure 3-2. Kinetics of perfluorooctane sulfonate (PFOS) elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 1. These simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as an estimate of the elimination rate constant $k_{4,1}$. Measured concentrations in plasma are shown as solid dots. Open triangles denote the cumulative mass of PFOS eliminated in urine. Lines show the optimized fit of model simulations to measured values: solid line – plasma; dashed line – urine.

Table 3-1. Physiological performance of chambered fish[a]

Parameter	Study design		
	Bolus dose, renal elimination studies	Bolus dose, branchial elimination studies	Branchial uptake studies
Weight (g)	1037 (285)	928 (131)	1147 (345)
Ventilation volume (V_{VOL}; L/d/kg)	304.1 (159.1)	318.9 (86.0)	295.6 (165.7)
Oxygen consumption ($VO_{2;}$ (mg/d/kg)	1941 (815)	1125 (289)	1718 (732)
Urine flow rate (UFR; L/d/kg)	0.073 (0.019)	not measured	not measured

[a]Reported values are given as the mean (SD) for each study design.

Table 3-2. Kinetic parameters and tissue–plasma concentration ratios for fish used in branchial elimination studies

	Fish 7	Fish 8	Fish 9	Fish 10	Fish 11	Fish 12	Mean (SD)
Gender	♀	♀	♂	♀	♂	♀	
Weight (g)	1130	870	774	838	929	1025	928 (131)
Kinetic parameters							
V_C (mL/kg)	55.0	79.3	51.7	87.0	102.8	81.6	76.23 (19.56)
$k_{1,2}$ (1/d)	1.69	0.64	3.96	1.22	1.63	2.29	1.90 (1.15)
$k_{2,1}$ (1/d)	4.64	1.30	15.56	5.34	3.33	5.71	5.98 (4.96)
$k_{3,1}$ (1/d)	0.023	0.028	0.032	0.043	0.015	0.021	0.027 (0.010)
$k_{4,1}$ (1/d)	0.0069[a]	0.0069[a]	0.0069[a]	0.0069[a]	0.0069[a]	0.0069[a]	0.0069[a]
V_{SS} (mL/kg)	206	241	255	469	313	285	295 (93)
CL_B (mL/d/kg)	1.24	2.21	1.64	3.73	1.56	1.70	2.01 (0.90)
CL_R (mL/d/kg)	0.38[b]	0.55[b]	0.36[b]	0.60[b]	0.71[b]	0.57[b]	0.53 (0.14)[b]
CL_T (mL/d/kg)	1.62	2.76	2.00	4.33	2.27	2.26	2.54 (0.95)
$T_{1/2}$ (d)	88.0	60.6	88.2	75.0	95.3	87.1	82.3 (12.5)
AIC[c]	8.44	8.88	7.38	8.17	8.69	8.52	8.42 (0.38)
BIC[d]	8.59	9.03	7.96	8.31	8.84	8.67	8.57 (0.38)
Tissue–plasma ratios at takedown							
Liver	0.38	0.59	0.84	0.39	0.32	0.38	0.48 (0.20)
Kidney	0.23	0.38	0.31	0.30	0.17	0.16	0.26 (0.09)
Muscle	0.039	0.024	0.031	0.020	0.012	0.014	0.023 (0.010)
Bile	0.022	0.033	0.017	0.004	0.001	0.020	0.016 (0.012)

[a]Renal elimination rate constant; set equal to average value determined in the "renal plus other" modeling exercise (Table S3-1). See text for details.

[b]Calculated as the product of V_C and a fixed value of $k_{4,1}$. The reported variance in the mean therefore reflects only the variance in V_C.

[c]Akaike information criterion (AIC)
[d]Bayesian information criterion (BIC)

Table 3-3. Kinetic parameters and tissue–plasma concentration ratios for fish used in renal elimination studies

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Mean (SD)
Gender	♀	♀	♂	♂	♂	♀	
Weight (g)	1346	814	1400	1033	934	694	1037 (285)
Kinetic parameters							
V_C (mL/kg)	29.4	49.8	122.0	77.4	45.5	41.6	60.9 (33.9)
$k_{1,2}$ (1/d)	4.53	3.21	14.66	1.38	3.44	8.23	5.91 (4.85)
$k_{2,1}$ (1/d)	17.60	13.36	33.52	5.23	14.19	21.37	17.54 (9.49)
$k_{3,1}$ (1/d)	0.0093	0.0064	0.0029	0.0043	0.0092	0.0082	0.0067 (0.0027)
$k_{4,1}$ (1/d)	0.027[a]	0.027[a]	0.027[a]	0.027[a]	0.027[a]	0.027[a]	0.027[a]
V_{SS} (mL/kg)	143	257	401	372	233	150	259 (109)
CL_B (mL/d/kg)	0.79[b]	1.38[b]	3.28[b]	2.08[b]	1.26[b]	1.15[b]	1.66 (0.90)[b]
CL_R (mL/d/kg)	0.27	0.32	0.35	0.33	0.42	0.34	0.34 (0.05)
CL_T (mL/d/kg)	1.06	1.70	3.63	2.41	1.68	1.49	1.99 (0.91)
$T_{1/2}$ (d)	93.8	105.0	76.7	106.9	96.3	69.5	91.3 (15.2)
AIC[c]	7.86	7.31	7.08	7.75	8.47	7.11	7.60 (0.54)
BIC[d]	8.01	7.46	7.23	7.90	8.62	7.25	7.75 (0.54)
Tissue–plasma concentration ratios at takedown							
Liver	0.42	NS[e]	0.83	1.71	0.70	0.70	0.87 (0.49)
Kidney	0.22	NS[e]	0.49	0.75	0.27	0.29	0.41 (0.2)
Muscle	0.027	NS[e]	0.042	0.075	NS[e]	NS[e]	0.048 (0.025)

[a]Branchial elimination rate constant; set equal to the average value determined by modeling data from branchial elimination experiments (Table 3-2). See text for details.

[b]Calculated as the product of V_C and a fixed value of $k_{4,1}$. The reported variance in the mean therefore reflects only the variance in V_C.
[c]Akaike information criterion (AIC)
[d] Bayesian information criterion (BIC)
[e]NS = Sample lost or not collected

Data and model simulations from a representative branchial elimination experiment are shown in Figure 3-3. Additional plots are provided elsewhere (Supplemental Data, Appendix 2, Figures S3-7–S3-11), and calculated parameters for all 6 fish are given in Table 3-2. To simulate these data sets, we used the average CL_R determined from the "renal plus other" modeling exercise as a fixed input. The model was then used to fit the branchial elimination rate constant by simulating measured concentrations of PFOS in expired water. The average CL_B determined in this manner was 79% of CL_T. Thus, 79% of total PFOS clearance was attributed to elimination in expired water, whereas the balance (21%) was assumed to be the result of urinary elimination. The mean V_{SS} determined in this modeling effort was 295 mL/kg, whereas the average $T_{1/2}$ was 82.3 d. Finally, using the average branchial elimination rate constant from the branchial elimination studies as a fixed input, we remodeled data from the original set of renal elimination experiments. Model simulations generated in this manner are shown in Figure 3-2. Additional plots for the other renal elimination experiments are given elsewhere (Supplemental Data, Appendix 2, Figures S3-12–S3-16). For each animal, the quality of model fit was nearly identical to that obtained in the initial modeling effort (Table 3-3). Moreover, fitted values of $k_{3,1}$ were very close to those determined in the original modeling exercise. In contrast, fitted values for V_C and V_{SS} were higher than those determined initially, whereas calculated CL_T values were substantially lower. It is clear, therefore, that although models developed in both efforts performed similarly (as judged by AIC and BIC values), optimized model fits were obtained differently.

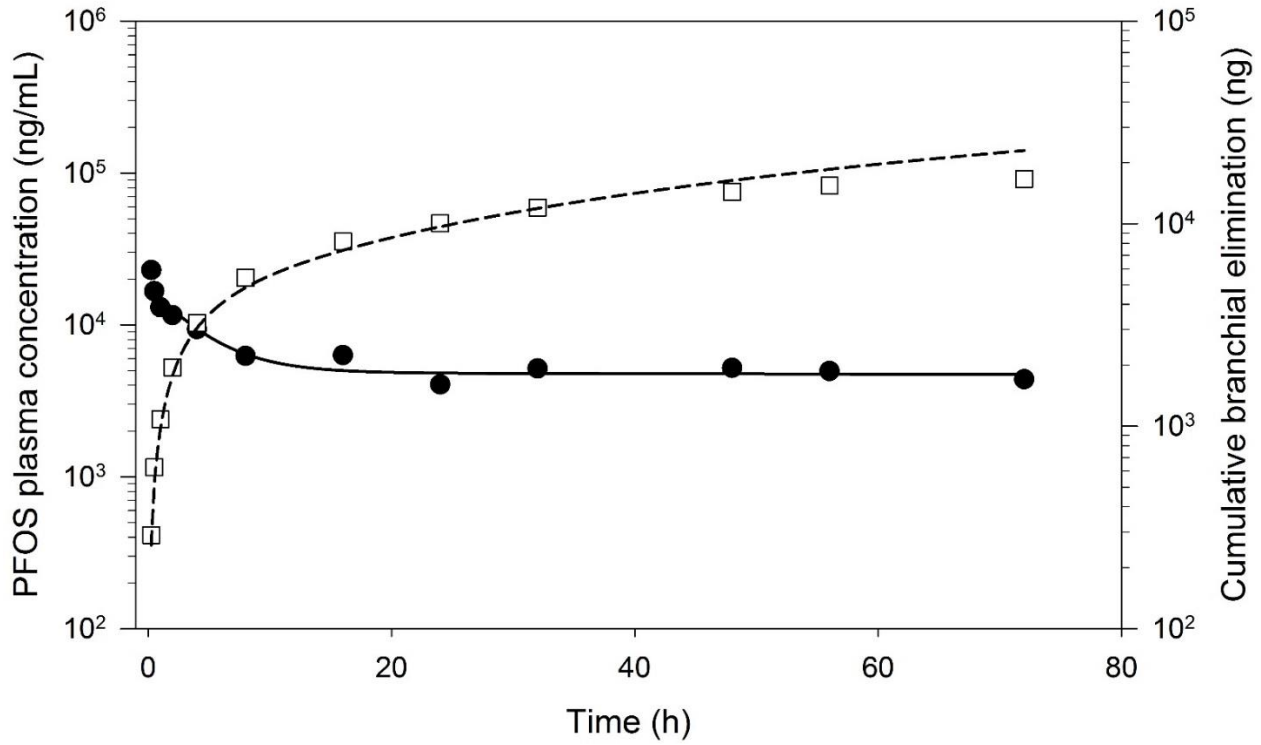

Figure 3-3. Kinetics of perfluorooctane sulfonate (PFOS) elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 7. Measured concentrations in plasma are shown as solid dots. Open squares denote the cumulative mass of PFOS eliminated in expired water. Lines show the optimized fit of model simulations to measured values: solid line = plasma; dashed line = water.

Because the second modeling effort was informed by measured PFOS concentrations in urine and expired water (albeit from separate experiments), our confidence in the second set of fitted parameters is much greater than that in the first set of fitted terms. Further confidence in these models was gained by noting consistencies that exist between calculated parameters and parameters developed from the branchial elimination studies. Thus, CL_R was determined, on average, to account for 17% of CL_T, whereas CL_B accounted for the remainder (83%). The average CL_T (1.99 mL/d/kg) was close to that (2.54 mL/d/kg) determined in branchial elimination studies, as were the average V_{SS} (259 mL/kg vs 295 mL/kg) and $T_{1/2}$ (91.3 d vs 82.3 d).

Figure 3-2 shows that fish 1 eliminated approximately 6 µg of PFOS to urine during the 72-h test period. During the same time period, fish 7 (Figure 3-3) eliminated approximately 20 µg of PFOS to expired water. These values correspond to 0.6% and 2% of the administered dose, respectively. Qualitatively similar results were obtained for the other test animals. These findings indicate that >95% of the injected dose was retained by the fish at test termination, which is consistent with the long estimated $T_{1/2}$ values.

These experiments were not designed to study sex differences in PFOS elimination by trout, and it is not possible to draw a statistically meaningful conclusion based on them. It should be noted, however, that the average CL_R values (mean ± SD) determined for each sex in the renal elimination studies were nearly identical (0.37 ± 0.05 mL/d/kg and 0.31 ± 0.04 mL/d/kg for males and females, respectively; n = 3). An examination of calculated CL_T values from both the renal and branchial elimination studies also suggests that there were no trends with respect to gender.

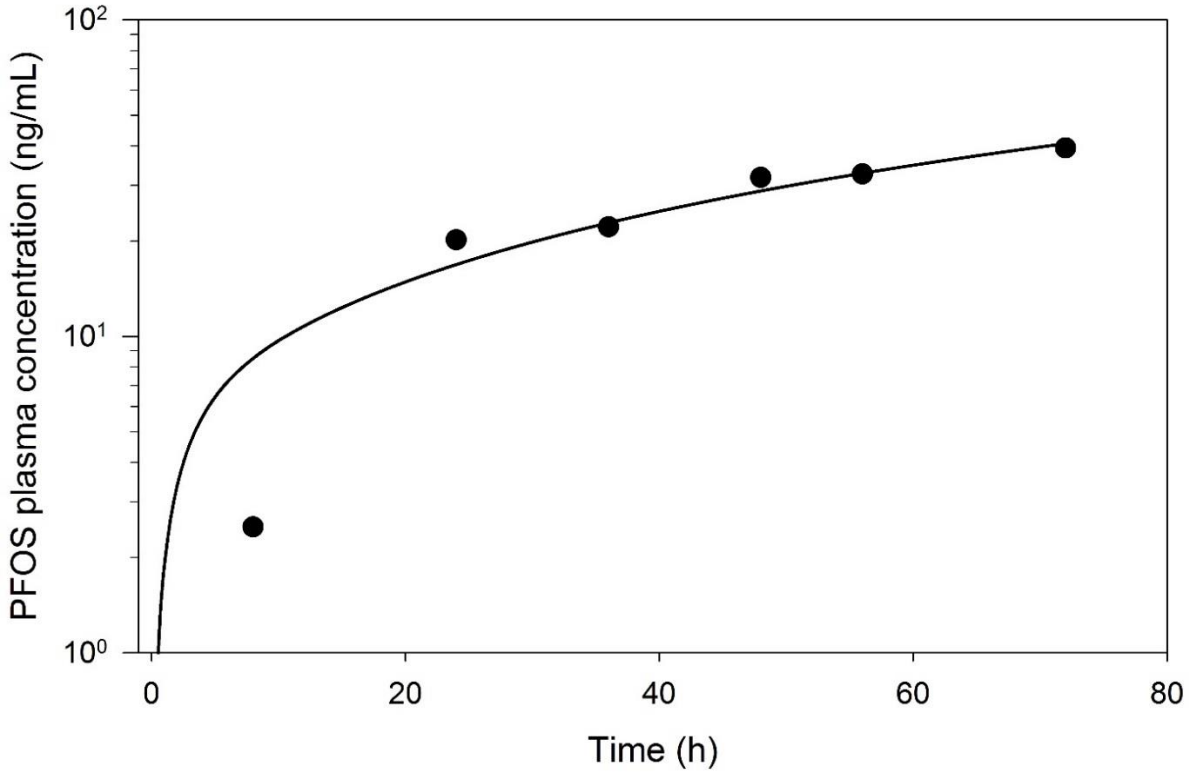

Figure 3-4. Kinetics of perfluorooctane sulfonate (PFOS) in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 13. Measured values are shown as individual points. The fitted model simulation is shown as a solid line.

Table 3-4. Kinetic parameters and tissue–plasma concentration ratios for fish used in branchial uptake studies

	Fish 13	Fish 14	Fish 15	Fish 16	Fish 17	Fish 18	Mean (SD)
Gender	♀	♀	♀	♀	♀	♂	
Weight (g)	1035	1102	998	1432	1237	1565	1147 (345)
PFOS water exposure (ng/mL)[a]	3.25	3.25	3.25	1.84	1.84	3.31	
Kinetic parameters							
k_U (L/d/kg)	0.87	0.94	0.39	0.58	1.27	1.07	0.85 (0.29)
Uptake efficiency (%)	0.28	0.56	0.06	0.30	0.48	0.48	0.36 (0.18)
Tissue–plasma ratios							
Liver	1.39	0.62	2.10	0.64	1.90	1.06	1.28 (0.63)
Kidney	0.69	0.27	0.56	0.06	0.12	0.07	0.29 (0.27)
Muscle	0.042	0.017	0.064	BQ[b]	BQ[b]	BQ[b]	0.041 (0.024)
Bile	NS[c]	0.033	0.031	0.044	0.131	0.070	0.062 (0.042)

[a]PFOS = perfluorooctane sulfonate
[b]BQ = Concentration too low to quantify
[c]NS = Sample lost or not collected

PFOS elimination to bile

Although the methods employed in the present study did not permit a time-dependent assessment of PFOS elimination to bile, bile produced by chambered trout tends to be retained within the gallbladder. It is possible, therefore, to evaluate this route of elimination by measuring the volume of stored bile and resulting concentration of PFOS. Bile was collected for all animals in the branchial elimination experiments. The bile to plasma concentration ratios for these animals averaged 0.016, which suggests that PFOS was not actively secreted into bile. The mean PFOS concentration in bile at the conclusion of the exposure was 56.5 ng/mL, and the average volume of collected bile was 3.1 mL. Multiplying these values gives an average total mass of 175 ng, which is substantially lower than the average cumulative mass of chemical eliminated by the renal and branchial routes (4.1 µg and 22.1 µg, respectively). It remains possible that biliary elimination of PFOS is more important in feeding animals because of higher bile flow rates. However, the oral bioavailability of PFOS in trout is close to 100% (Martin et al., 2003a). It is likely, therefore, that any PFOS eliminated to bile would be reabsorbed in the gastrointestinal tract. Taken together, these observations suggest that bile represents a minor route for PFOS elimination in trout. Branchial uptake experiments Branchial uptake rate constants (k_U; L/d/kg) were obtained by fitting a 2-compartment model (Figure 3-1B) to measured plasma concentration time-course data from continuous waterborne exposures. A representative plot is shown in Figure 3-4. Plots for the other 5 animals are provided elsewhere (Supplemental Data, Appendix 2, Figures S3-17–S3-21). Fitted rate constants were then used along with the measured V_{VOL} for each animal to calculate a set of chemical uptake efficiencies (as k_U/V_{VOL}; Table 3-4).

Branchial uptake rate constants averaged 0.85 L/d/kg. Calculated branchial uptake efficiencies were very low, averaging 0.36%.

Tissue distribution experiment

Data from the tissue distribution experiment were developed as a set of tissue to plasma concentration ratios to investigate how rapidly the fish approached an internal steady state (Table 3-5). The lowest liver to plasma, muscle to plasma, and bile to plasma ratios were calculated for fish sampled on day 1. However, these values did not differ statistically from ratios calculated at later time points (one-way analysis of variance, $\alpha = 0.05$). The rank order of measured PFOS concentrations in tissues on day 3, day 7, and day 14 was plasma > liver > kidney >> muscle ≈ bile.

The tissue distribution data were not sufficient to calculate a full chemical mass balance. Nevertheless, it was possible to estimate the fraction of the administered dose in sampled tissues at different time points, assuming that each tissue comprises a fixed fraction of total body weight (Table 3-5). A fractional body weight of 2.45% was used for plasma, assuming a fractional blood volume of 3.5% and hematocrit of 30% (Barron et al., 1987). Fractional body weights for the other tissues were taken to be 67% for muscle, 1.27% for liver, and 0.76% for kidney (Nichols et al., 1990). At first glance, these calculated values suggest that more than half of the administered dose was eliminated during the first 24 h post dosing. However, this assessment does not account for PFOS contained in nonsampled tissues, which may have been substantial. Based on the chambered studies, it is clear that PFOS injected into the bloodstream distributes to other tissues in <24 h, substantially reducing the measured concentration in plasma. From 24 h on, calculated mass-balance values from the tissue distribution experiment

changed little, if at all. Thus, PFOS contained in these tissues after the distribution phase tended to remain within the animal.

Plasma binding

The recovery of PFOS from spiked samples of plasma ultrafiltrate averaged 66.5 ± 15.2%. In contrast, PFOS concentrations in filtrates of whole plasma were below the limit of quantitation (5 ng/mL). It was not possible, therefore, to estimate plasma binding with any certainty. However, it appears that this binding exceeded 99%.

Table 3-5. Perfluorooctane sulfonate (PFOS) tissue–plasma concentration ratios at 1, 3, 7 and 14 days post dosing[a]

	Controls	Day 1	Day 3	Day 7	Day 14
Gender	1 ♂, 3♀	2 ♂, 2♀	2 ♂, 1♀	4♀	3 ♂, 1♀
Weight	333 (52)	308 (36)	268 (47)	245 (33)	288 (35)
PFOS plasma conc.	BQ[b]	8046 (413)	5983 (820)	6779 (794)	6737 (902)
Tissue–plasma ratios					
Liver	BQ[b]	0.29 (0.06)	0.51 (0.21)	0.65 (0.33)	0.58 (0.34)
Kidney	BQ[b]	0.25 (0.02)	0.25 (0.02)	0.22 (0.07)	0.21 (0.04)
Muscle	BQ[b]	0.010 (0.001)	0.020 (0.004)	0.014 (0.005)	0.016 (0.002)
Bile	BQ[b]	0.002 (0.001)	0.022 (0.013)	0.019 (0.002)	0.035 (0.026)
Mass-balance estimates - % of initial dose remaining					
Plasma	BQ[b]	19.7 (1.0)	14.7 (2.0)	16.6 (1.9)	16.5 (2.2)
Muscle	BQ[b]	5.3 (0.7)	7.8 (0.8)	6.1 (1.7)	7.0 (0.3)
Liver	BQ[b]	3.0 (0.7)	3.8 (1.5)	5.4 (2.6)	4.8 (2.3)
Kidney	BQ[b]	1.5 (0.0)	1.1 (0.1)	1.2 (0.3)	1.0 (0.1)

[a]Data presented as the mean (SD), *n* = 4
[b]BQ = Concentration too low to quantify

Table 3-6. Comparison of kinetic parameters for perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) determined in chambered studies with rainbow trout[a]

	PFOS	PFOA[b]
Branchial uptake efficiency (%)[c]	0.36 (0.18)	0.10 (0.07)
CL_B (mL/d/kg)	1.83 (0.88)[d]	2.88 (1.20)
CL_R (mL/d/kg)	0.43 (0.14)[d]	32.40 (25.68)
CL_T (mL/d/kg)	2.26 (0.94)[d]	35.28 (26.64)
V_{SS} (mL/kg)	277 (98)[d]	524 (138)
$T_{1/2}$ (d)	86.9 (14.1)[d]	12.6 (7.2)

[a]Reported values are given as the mean (SD)

[b]Kinetic parameters for PFOA were obtained from Consoer et al. (2014)

[c]Determined in branchial uptake studies

[d]Calculated from values determined in both the renal and branchial elimination studies.

Discussion

Although commercial production of PFOS has stopped, this compound continues to receive attention because of its persistence in the environment, large one-time production volumes, accumulation in biota, and demonstrated toxicity (Lau et al., 2007). Previous studies with fish have shown reproductive impairment in swordtails (*Xiphophorus hellerii*; Han, 2010) and developmental toxicity in both swordtail (Han and Fang, 2010) and zebrafish (*Danio rerio*; Shi et al., 2008; Huang et al., 2010) embryos. Additional work has provided evidence for hepatotoxicity in common carp (*Cyprinus carpio*; Hoff et al., 2003) and effects on zebrafish embryo behavior (Huang et al., 2010). To date, the only detailed study of PFOS kinetics in fish is that provided by Martin et al. (Martin et al., 2003a) which was performed as part of a larger effort to characterize the bioconcentration and tissue distribution of several PFAAs in rainbow trout. The branchial uptake rate (k_U) determined in this earlier effort was 53 L/d/kg, and the reported $T_{1/2}$ was 15 d. By comparison, the k_U determined in the present study is approximately 50 times lower (0.85 L/d/kg) and calculated $T_{1/2}$ values are 5 to 6 times longer (82.3 d and 91.3 d for the branchial and renal elimination experiments, respectively).

When making these comparisons, it is important to consider that trout used in chambered experiments averaged more than 1 kg, whereas the fish used by Martin et al. (2003a) weighed approximately 7 g. For example, the average measured ventilation rate (V_{VOL}) of chambered fish used for branchial uptake studies was 295.6 L/d/kg. The average V_{VOL} of a 7-g trout is unknown. However, using the allometric relationship developed previously by Consoer et al. (2014) ($V_{VOL} = 10.6\ W_B^{0.65}$, where V_{VOL} is in

L/h/kg and W_B is the fish's weight in kilograms), we may estimate its value to be approximately 60.2 L/h/kg, or 1444.8 L/d/kg. Based on this information, it is clear that some of the observed difference in fitted k_U values from the present study and that performed by Martin et al. (2003a) can be attributed to likely differences in V_{VOL}.

Similar considerations apply to the interpretation of differences in $T_{1/2}$. Thus, higher ventilation rates (weight-normalized) in smaller fish can be expected to result in higher rates of PFOS elimination across the gills. We are not aware of any allometric data on urine production in fish. However, because the need to eliminate water can be expected to increase with increased rates of gill ventilation, it is reasonable to assume that urine production scales to a fractional exponent of body weight.

Measured concentrations of PFOS from the tissue distribution experiment suggested that the injected chemical approaches an internal steady state in 1 d to 3 d. This finding is consistent with the short distributional phase evident in measured plasma time-course data from bolus injection experiments. The rank order of PFOS concentration in tissues at 3 d, 7 d, and 14 d post dosing was plasma > liver > kidney >> bile ~ muscle. Comparisons with the study performed by Martin et al. are complicated by the fact that these authors reported measured concentrations graphically as log-scaled values (Martin et al., 2003a; see Figure 2). However, an examination of these data suggests that the only difference between this and the present study relates to measured concentrations in liver and kidney. Thus, Martin et al. (2003a) found that PFOS concentrations in these tissues were approximately equal, whereas in the present study measured concentrations in liver were 2 to 3 times greater than those in kidney.

A major objective of the present study was to obtain data needed to compare the kinetics of PFOS and PFOA in rainbow trout. Although structurally similar, these

compounds differ with respect to their acid terminal groups and the total number of fluorinated carbons (PFOS has 8, whereas PFOA has 7). Perfluorooctane sulfonate has been shown to accumulate in trout, whereas PFOA does not (Martin et al., 2003a). These differences cannot be explained by differences in ionization state since dissociation constant values for both compounds are far lower than environmental pH values.

In Table 3-6, modeled kinetic parameters from the present study are compared with those obtained in an earlier study with PFOA (Consoer et al., 2014). Branchial uptake efficiencies for both PFOS and PFOA are <1%. This finding is consistent with a substantial body of work showing that ionized compounds are poorly absorbed across fish gills (Saarikoski et al., 1986; Erickson et al., 2006; Nichols et al., 2015). Nevertheless, the mean branchial uptake efficiency for PFOS is 3.6 times higher than that for PFOA. Taken alone, this difference would be expected to result in modestly greater accumulation of the sulfonate.

The mean CL_B for PFOA is approximately 50% greater than that for PFOS. However, to estimate rate constants for branchial elimination of each compound (k_B; 1/d), these clearance rates must be divided by modeled estimates of V_{SS} (i.e., $k_B = CL_B/V_{SS}$). Although fitted V_{SS} values for both compounds are substantially less than 1.0, the value determined for PFOA is somewhat larger than that for PFOS. Taking this difference into account, the calculated k_B for PFOS (0.0072/d) is slightly larger than that for PFOA (0.0055/d) and favors greater accumulation of the carboxylate.

The most striking difference in kinetic parameters provided in Table 3-6 relates to modeled CL_R values. In earlier studies with PFOA, CL_R averaged 89% of CL_T, with CL_B making up the remainder (Consoer et al., 2014). In percentage terms, the results of the

present study indicate that for PFOS the relative importance of these processes is reversed. Thus, the CL_R for PFOS determined by averaging data from all 12 elimination experiments was 19% of CL_T. Expressed in absolute terms, differences in CL_R for PFOS and PFOA (0.43 mL/d/kg vs 32.4 mL/d/kg) are even more apparent and suggest that the processes responsible for renal elimination of PFOA are approximately 75 times more efficient that those for PFOS. Based on this information, it is clear that differences in $T_{1/2}$ for PFOS and PFOA can be largely attributed to differences in CL_R.

The mechanistic basis for this difference in renal handling of PFOS and PFOA remains to be determined. In mammals, however, it is well known that species and gender differences in the plasma half-life of different PFAAs are strongly associated with differences in urinary elimination (Butenhoff et al., 2004; Andersen et al., 2006; Han et al., 2012). For the PFCAs, these differences are particularly large, and a substantial amount of work has been performed to understand the basis for these observations. This research suggests that renal clearance within a particular species (or gender) reflects the net result of secretion to urine and reuptake from urine to plasma (Han et al., 2012). Both processes are mediated by membrane transporters localized to renal proximal tubule cells. These proteins operate at both the basolateral and apical membranes and include several organic ion transporters (OATs/Oats; capital letters are used to denote human transporters) and organic anion transport polypeptides (OATPs/Oatps).

Gene expression studies indicate that fish possess a diverse array of membrane transport proteins (Loncar et al., 2010; Popovic et al., 2010; Fischer et al., 2011; Verri et al., 2012; Luckenbach et al., 2014) many of which have been shown to transport xenobiotic compounds (Miller et al., 1998; Bard, 2000; Doi et al., 2001; Sturm et al.,

2001; Sturm and Segner, 2005; Zaja et al., 2008; Zaja et al., 2011; Fischer et al., 2013; Uchea et al., 2015). Functional characterization studies have been performed on several adenosine 5'-triphosphate binding cassette (ABC) transporters from fish. By comparison, functional studies of solute carrier proteins (including Oats and Oatps) are more limited. Organic ion transporters cloned from several fish species have been expressed in *Xenopus laevis* oocytes to study their substrate specificity (Wolff et al., 1997; Aslamkhan et al., 2006; Meier et al., 2007). All were shown to promote uptake of well-known substrates for mammalian OATs/Oats including p-aminohippuric acid, adenofir, 2,4-dichlorophenoxyacetic acid (2,4-D), estrone sulfate, phalloidin, and microcystin. More recently, Popovic et al. (2014) evaluated interactions between zebrafish Oatp 1d1 and a number of environmental contaminants, including both PFOA and PFOS. These studies showed that PFOA is a strong uncompetitive inhibitor of Oatp 1d1 and PFOS is a high affinity substrate. Winter flounder efficiently secreted chlorophenol red (an organic acid) and p-aminohippuric acid into urine (Mackenzie et al., 1977). Subsequent work showed that flounder eliminate 2,4-D by the same pathway (Pritchard and James, 1979). Finally, several studies with aglomerular fish have shown that endogenous organic acids are eliminated to urine by mechanisms that do not involve glomerular filtration (Bayenbach, 2004).

The results of the present study do not prove that differences in renal tubular transport of PFOS and PFOA are responsible for observed differences in urinary elimination of these compounds; nevertheless, they are highly suggestive. Using the approach outlined by Consoer et al. (2014) we may compare the measured CL_R for PFOS to that expected from measured rates of glomerular filtration and an estimated level of plasma binding. For example, assuming a GFR of 5.5 mL/h/kg (based on the

present study as well as McKim et al. (1999) and plasma binding of 99% or 99.9%, the renal clearance predicted from simple glomerular filtration would be 1.3 mL/d/kg or 0.13 mL/d/kg, respectively. Given the uncertainty that exists in plasma binding estimates for PFOS, either prediction is indistinguishable from the CL_R (0.43 mL/d/kg) determined in the present study for trout. In contrast, the measured CL_R for PFOA is much higher than that predicted from measured glomerular filtration and any plausible level of plasma binding.

Research is needed to better understand the processes responsible for renal elimination of PFOS, PFOA, and other PFAAs by fish. Of special interest is the possibility that PFCAs are actively secreted in the fish kidney, whereas PFSAs are not as this would provide a simple explanation for observed differences in the bioaccumulation of these chemical subclasses. Alternatively, it is possible that both PFCAs and PFSAs are secreted to urine; however, different transporters may promote the reabsorption of PFOS and other PFSAs, prolonging their elimination half-lives. Thus, the underlying basis for differences in elimination of PFOA and PFOS by trout may be analogous to that responsible for observed species and gender differences in elimination of PFOA by mammals. Finally, differences in renal handling of PFOS and PFOA by trout may be related to the total number of fluorinated carbons. One way to address this possibility would be to measure the renal clearance of perfluorononanoic acid, which has the same number of fluorinated carbons as PFOS.

Additional work is required to evaluate possible species-based and gender-based differences in renal handling of PFAAs, as well as the concentration dependence of these processes. Lee and Schultz (2010) reported that female fathead minnows eliminate PFOA more rapidly than males. This difference was partially reversed by

pretreatment of females with a synthetic androgen, suggesting that the mechanisms responsible for elimination of PFOA are controlled in part by sex hormones. Similar findings reported in studies with rodents have been attributed to hormonal control of membrane transporter expression (Kudo et al., 2002). In principal, renal clearance mediated by a specific transport protein would have the potential to saturate at high substrate concentrations. This could, in turn, result in concentration-dependent changes in $T_{1/2}$. To address this possibility, De Silva et al. (2009) exposed trout to a high (81.1 ng/g) and a low (6.9 ng/g) dietary dose of technical PFOA (78% n-isomer). Half-life values determined from the depuration phase of these exposures did not vary with dose. The authors noted, however, that both dose levels may have saturated the pathways responsible for elimination.

The present study, together with that of Consoer et al. (2014), clearly demonstrates the critical importance of renal clearance as a determinant of PFAA accumulation in fish. Mechanistic models that explicitly consider this process, such as that given by Ng and Hungerbühler (2013) are therefore appropriate. Both studies also provide detailed information on rates of chemical uptake and elimination across the gills, as well as elimination to bile, which may be used to further refine such models. As such, these efforts substantially advance current understanding of this important class of environmental contaminants.

Chapter 4: Conclusion

Overview of kinetic studies with PFOA (Chapter 2)

The goal of studies described by Consoer et al. (2014) was to better understand the processes which control uptake and elimination of PFOA in rainbow trout. Initially, large trout confined to respirometer-metabolism chambers were given a bolus dose of PFOA to characterize the kinetics of elimination. In these experiments, branchial and renal elimination were studied concurrently, allowing a direct comparison for each animal in the study, as well as in aggregate. The resulting data sets were analyzed using a two-compartment clearance-volume model with elimination from the central compartment, and parameter estimation was achieved by simultaneously fitting measured PFOA concentrations in plasma, urine, and expired water. The results showed that trout eliminate approximately 90% of PFOA in urine (mean, all experiments), with a smaller, but still measurable fraction eliminated in expired water. The mean terminal elimination half-life ($T_{1/2}$) for PFOA was 12.6 d, while the mean steady-state volume of distribution (V_{SS}) was 524 mL/kg.

Branchial uptake of PFOA was studied in a separate set of experiments in which trout were exposed to a constant concentration of PFOA in water. An attempt was made to directly measure PFOA uptake based on differences in concentration in inspired and expired water. However, these concentration differences were too small to calculate a meaningful uptake rate. Instead, branchial uptake was evaluated using a model-based analysis of plasma concentration time-course data. For this analysis, model parameters that describe chemical elimination in urine and expired water were set equal to average values determined in bolus dosing studies. Using this approach, the branchial uptake efficiency for PFOA was determined to be approximately 0.1%. This low value is

consistent with earlier work on branchial uptake of ionized substances by fish (Armitage et al., 2017).

The rank order of measured PFOA concentrations in tissues at the end of the uptake and elimination experiments was: plasma > liver ≈ kidney > muscle. This pattern is similar to that reported in previous studies with trout (Martin et al., 2003). Measurable quantities of PFOA were detected in bile; however, a simple mass-balance analysis indicated this was a minor route elimination. A separate tissue distribution experiment was performed to evaluate changes in PFOA concentration over time. The tissue concentration time-course data suggested that PFOA distributes rapidly within the animal, reaching an internal equilibrium in less than 24 h. This finding is consistent with plasma time-course data from the bolus dosing studies, which demonstrated a relatively short distributional phase (< 12 h) followed by an extended log-linear elimination phase.

Overview of kinetic studies with PFOS (Chapter 3)

In a follow-up study (Consoer et al., 2016), trout confined to respirometer-metabolism chambers were used to investigate uptake and elimination of PFOS. The fish were again administered the chemical as a bolus dose, and the resulting data sets were evaluated using a two-compartment clearance-volume model. Unlike earlier studies with PFOA, however, renal and branchial elimination were evaluated using two different sets of animals. Initially, these data sets were used to independently fit rate constants for urinary and branchial elimination. The results from both studies were then combined to develop a single coherent model description.

Based on these investigations, it was determined that trout eliminate approximately 81% of PFOS across the gills (mean, all experiments), with renal

elimination accounting for most of the remainder. A simple mass-balance calculation again showed that PFOS elimination in bile did not contribute substantially to overall clearance. The mean $T_{1/2}$ for PFOS was 86.9 d, while the mean fitted V_{SS} was 277 ml/kg.

Additional studies were conducted to investigate the uptake of PFOS from respired water. As with PFOA, uptake from water was characterized by modeling to measured chemical concentrations in plasma. The mean branchial uptake efficiency determined in this manner was 0.36%.

Measured concentrations of PFOS in tissues at the conclusion of the elimination studies exhibited the rank order: plasma > liver > kidney > muscle. These findings were again consistent with distribution patterns reported by Martin et al. (2003). The results of a separate time-course study indicated that the distribution of PFOS to tissues was rapid, although the liver/plasma concentration ratio appeared to increase somewhat between 24 and 72 h. A mass-balance analysis of measured PFOS concentrations in tissues suggested that there was little loss of chemical from fish over the 14-day time-course experiment. This finding is consistent with the long estimated $T_{1/2}$ for this compound.

Comparison of the PFOA and PFOS studies

A detailed comparison of modeled kinetic parameters for PFOA and PFOS was provided in Chapter 3 of this dissertation. A brief summary of this discussion is provided here. Qualitatively, PFOS was found to be eliminated primarily across the gills, while PFOA was eliminated primarily in urine. The mean total clearance rate (CL_T) for PFOA was 35.3 mL/d/kg, while that determined for PFOS was 2.0 mL/d/kg. This large

difference in CL_T was associated with a similarly large difference in $T_{1/2}$ (mean of 12.6 d for PFOA vs. 86.9 d for PFOS). The mean V_{SS} for PFOS (277 mL/kg) was approximately half of that determined for PFOA (524 mL/kg). The basis for this difference in V_{SS} is unknown. However, the fact that fitted V_{SS} values for both chemicals were substantially less than 1.0 is consistent with the general finding that chemical concentrations in plasma were higher than those in tissues.

Modeled branchial clearance rates (CL_B) for PFOA and PFOS differed by less than a factor of 2 (mean of 2.88 ml/d/kg for PFOA vs. 1.88 ml/d/kg for PFOS). In contrast, the renal clearance rate (CL_R) for PFOA (mean of 32.40 ml/d/kg) was approximately 75 time faster than that for PFOS (mean of 0.43 ml/d/kg). From these findings it may be concluded that differences in CL_T between the two compounds are driven largely by differences in CL_R. Modeled branchial uptake efficiencies for PFOA (mean of 0.1%) were somewhat lower than those determined for PFOS (mean of 0.36%). Taken alone, these differences in branchial uptake would be expected to result in modestly greater accumulation of PFOS, compared to the carboxylate. It is clear, however, differences in renal clearance provide the most likely explanation for observed differences in accumulation of PFOS and PFOA in fish.

Implications for bioaccumulation assessment of PFAAs in fish

The larger aim of this research was to better understand the kinetics of PFAAs in fish in order to inform bioaccumulation assessments, and to provide an improved basis for developing predictive computational models. A variety of models have been used to predict chemical bioaccumulation in fish. Most of these models rely heavily on the specification of physicochemical characteristics such as K_{OW} and pKa, and reflect, explicitly or implicitly, two simplifying assumptions: 1) chemical accumulation largely

reflects the tendency of neutral chemicals to partition out of water and into tissue lipids, and 2) membrane transport is dominated by passive diffusion of the neutral chemical species. As noted previously, the reliance of these models on factors such as K_{OW} limits their ability to describe the accumulation of compounds that do not fit the assumptions on which they are based. Further, differences in accumulation of PFAAs in mammals have been shown to be dependent on differences in renal elimination. These differences in renal elimination are believed to be due, in turn, to differences in chemical affinity for various renal transport proteins, as well as differences in expression of renal transporters in different species and sexes. Because renal elimination is a key determinant of PFAA accumulation, and as renal elimination is based on factors not easily predicted from simple physiochemical properties, the use of most current models to predict PFAA accumulation in fish is problematic.

To date, three models have been developed to describe the accumulation of PFAAs in fish (Webster and Ellis, 2010; Armitage et al., 2013; Ng and Hungerbühler, 2013). However, the model given by Ng and Hungerbühler (2013) is the only description which explicitly considers factors that are likely to control the rate and extent of accumulation, including interactions with specific binding proteins (serum albumin, fatty acid binding proteins) and the activities of renal membrane transporters. Because of the dearth of data for many of these interactions in fish, most of the parameters in the model were specified using data from studies in mammals. While there was some information available on interactions of PFAAs with fish plasma proteins, there were no data on interactions with fatty acid binding proteins, or direct measurements of gill uptake, gill elimination, and renal elimination in fish.

In a recent publication, Armitage et al. (2017) used information provided by Consoer et al. (2014, 2016) to re-evaluate the model given by Ng and Hungerbühler (2013). Branchial transport rates predicted by the model were substantially higher than those measured by Consoer et al. (2014, 2016). The model was therefore modified to decrease predicted rates of gill flux, resulting in a better match to measured values for both PFOA and PFOS. $T_{1/2}$ values predicted by the adjusted model were somewhat lower than reported values (2.67 days predicted vs. 12.6 days measured for PFOA; 68.7 days predicted vs. 86.9 days measured for PFOS). However, the model was parameterized using a range of measured albumin interaction values in fish, and $T_{1/2}$ predictions generated across this range of binding values overlapped measured $T_{1/2}$ values for both PFOA and PFOS. Of particular note, renal clearance rates for PFOA and PFOS given by Consoer et al. (2014, 2016) were fairly well predicted by both the original and updated Ng and Hungerbühler (2013) models. Based on these findings, Armitage et al. (2017) concluded that the binding of PFAAs to specific proteins and their elimination by membrane transporters in the kidney may be "read across" from mammals to fish; that is, both processes are critical determinants of PFAA accumulation in mammals and fish. As such, the studies performed by Consoer et al. (2014, 2016) provide a concrete example of the need for detailed, mechanistic data on uptake and elimination of PFAAs in fish.

Study retrospective

If I had an opportunity to perform this research over again, the only thing I would do differently is conduct the PFOS and PFOA elimination studies the same way; that is, by using animals prepared for simultaneous collection of urine and expired water. There are advantages to using separate animals for renal and branchial elimination studies.

These include ease of animal preparation, which results in a greater percentage of successful surgeries, and a simpler data acquisition regimen (fewer samples per test and fewer sample types, processed using fewer sample-specific methods). In hindsight, however, it turned out to be far more advantageous to collect renal and branchial elimination data from each animal. By doing so, it was possible to perform direct comparisons for each animal, rather than relying on aggregate measured values for two groups of animals. This approach introduced more complexity to the modeling effort, as the models were developed by simultaneously fitting data for three concentrations of PFOA at each time point: plasma, urine, and expired water. In the end, however, this approach resulted in a more a robust analysis which was easier to describe.

Future directions

Additional mechanistic data on PFAA kinetics in fish is needed to understand processes that govern the bioaccumulation of these chemicals, and to aid in development of predictive computational models. One of the most obvious needs is to conduct kinetic studies with a wider range of chemical structures. These studies are necessary to determine how differences in gill uptake, gill elimination, and renal elimination noted for PFOS and PFOA, two PFAAs with comparable perfluocarbon chain lengths but different acidic moieties, fit into more generalized patterns with respect to PFAAs as a diverse chemical class.

Another research need is to better characterize plasma binding of PFAAs in fish. The binding experiments described in this dissertation showed only that plasma binding of PFOA and PFOS was very high (> 99%). This still leaves some room for differences in bound/free fractions in plasma to contribute to observed differences in renal clearance. As noted by Consoer et al. (2014), however, the renal clearance of PFOA

was far greater than that predicted from simple filtration of unbound chemical. Thus, while an improved understanding of PFAA binding in fish plasma is still needed, the data reported in this dissertation indicate that differences in glomerular filtration cannot explain observed differences in renal clearance of PFOA and PFOS in trout.

Finally, and perhaps most importantly, there is a need for additional research on processes that control renal clearance of PFAAs by fish. The studies described in this dissertation suggest that renal clearance is a major determinant of PFAA kinetics in fish, and that differences in renal clearance substantially determine the extent to which individual PFAAs accumulate over time. The experiments performed with chambered trout were not designed to directly evaluate the role of membrane transporters in renal elimination of PFAAs. However, the results of these studies, when coupled our current understanding of PFAA kinetics in mammals, suggest that these transporters play a critical role in fish, as they do in mammals.

A variety of *in vivo*, *ex vivo*, and *in vitro* methods have been used to study processes related to renal transport of PFAAs in mammals and (to a lesser degree) fish. For example, toxicokinetic studies using known inhibitors of renal transporters have been performed to study the role of specific classes of renal transporters. Additional studies using intact, castrated, and ovariectomized rats have been conducted to investigate the effects of sex hormones on transporter expression (Vanden Heuvel et al., 1992; Kudo et al., 2002). Previously, Lee and Schultz (2010) used hormone treatments to study differences in PFOA elimination by male and female fathead minnows. Additional studies employing sex hormones and/or specific transport inhibitors like probenecid could be conducted to investigate the role of specific transporter classes in fish, as well as sexually determined expression of this activity. Other *in vivo* studies with

mammals have been performed using animals deficient in the expression of specific transporters (Katakura et al., 2007). Similar genetic knockout models could be developed using, for example, zebrafish (Amacher, 2008) to study the role of specific transporters in fish.

In vitro methods used to study PFAA transport in mammals also could be adapted to study fish membrane transporters. For example, Nakagawa et al. (2008) used a culture of HEK293 (human embryonic kidney) cells transiently transfected to express a number of human and rat OAT/Oat proteins to probe the ability of PFOA to inhibit uptake of known substrates across a range of PFOA concentrations. Using similar methods, Steiner et al (2014) screened a rainbow trout cDNA library for a hypothesized trout ortholog for human OATP1. A novel Oatp1d1 protein was identified, cloned, and transfected into human HEK293 cells. Additional work was then performed to demonstrate the ability of this protein to transport a number of endogenous and exogenous substrates. Using these and other *in vitro* methods it may be possible to identify membrane transporters responsible for renal clearance of different PFAAs in fish, and determine how the expression of these transporters varies among species for which genetic sequence information is available.

Bibliography

[3M] Company. 1999. Fluorochemical use, distribution and release overview. USEPA Administrative Record AR226-0550. Available from: www.regulations.gov as document EPA-HQ-OPPT-2002-0051-0003.

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

Amacher, S.L., 2008. Emerging gene knockout technology in zebrafish: zinc-finger nucleases. Brief. Funct. Genomic. Proteomic. 7.6, 460–464.

Andersen, M.E., Butenhoff, J.L., Chang, S.-C., Farrar, D.G., Kennedy, G.L., Lau, C., Olsen, G.W., Seed, J., Wallace, K.B., 2008. Perfluoroalkyl acids and related chemistries –toxicokinetics and modes of action. Toxicol. Sci. 102, 3–14.

Andersen, M.E., Clewell, H.J., Tan Y-M., Butenhoff, J.L., Olsen, G.W., 2006. Pharmacokinetic modeling of saturable, renal resorption of perfluoroalkylacids in monkeys - probing the determinants of long plasma half-lives. Toxicology 227, 156–164.

Armitage, J.M., Arnot, J.A., Wania, F., Mackay, D., 2013. Development and evaluation of a mechanistic bioconcentration model for ionogenic organic chemicals in fish. Environ. Toxicol. Chem. 32, 115–128.

Armitage, J.M., Erickson, R.J., Luckenbach, T., Ng, C.A., Prosser, R.S., Arnot, J.A., Schirmer, K., Nichols, J.W., 2017. Assessing the bioaccumulation potential of ionizable organic compounds: current knowledge and research priorities. Environ. Toxicol. Chem. 36, 882-897.

Arnot, J.A., Gobas, F.A.P.C., 2003. A generic QSAR for assessing the bioaccumulation potential of organic chemicals in aquatic food webs. QSAR Comb. Sci. 22, 337-345.

Arnot, J.A., Gobas, F.A.P.C., 2004. A food web bioaccumulation model for organic chemicals in aquatic ecosystems. Environ. Toxicol. Chem. 23, 2343–2355.

Aslamkhan A.G., Thompson, D.M., Perry, J.L., Bleasby K., Wolff, N.A., Barros, S., Miller, D.S., Pritchard, J.B., Amy, G., Jennifer, L., David, S., 2006. The flounder organic anion transporter fOat has sequence, function, and substrate specificity similarity to both mammalian Oat1 and Oat3. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291, R1773–R1780.

Bard, S., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357–389.

Barrett, P.H., Bell, B.M., Cobelli, C., Golde, H., Schumitzky, A., Vicini, P., Foster, D.M., 1998. SAAM II: simulation, analysis, and modeling software for tracer and pharmacokinetic studies. Metabolism 47, 484–492.

Barron, M.G., Tarr, B.D., Hayton, W.L., 1987. Temperature-dependence of cardiac output and regional blood flow in rainbow trout, *Salmo gairdneri Richardson*. J. Fish Biol. 31, 735–744.

Bayenbach, K., 2004. Kidneys sans glomeruli. Am. J. Physiol., Renal Physiol. 286, F811-F827.

Bijland, S., Rensen, P.C.N., Pieterman, E.J., Maas, A.C.E., van der Hoorn, J.W., Van Erk, M.J., Havekes, L.M., van Dijk, K.W., Chang, S-C., Ehresman, D.J., Butenhoff, J.L., Princen, H.M.G., 2011. Perfluoroalkyl sulfonates cause alkyl chain length-dependent hepatic steatosis and hypolipidemia mainly by impairing lipoprotein production in APOE*3-Leiden CETP mice. Toxicol. Sci. 123, 290–303.

Bischel, H.N., MacManus-Spencer, L.A., Zhang, C., Luthy, R.G., 2011. Strong associations of short-chain perfluoroalkyl acids with serum albumin and investigation of binding mechanisms. Environ. Toxicol. Chem. 30, 2423-2430.

Bjork, J.A., Butenhoff, J.L., Wallace, K.B., 2011. Multiplicity of nuclear receptor activation by PFOA and PFOS in primary human and rodent hepatocytes. Toxicology 288, 8–17.

Bjork, J.A., Wallace, K.B., 2009. Structure-activity relationships and human relevance for perfluoroalkyl acid-induced transcriptional activation of peroxisome proliferation in liver cell cultures. Toxicol. Sci. 111, 89–99.

Boaru, D.A., Dragos, N., Schirmer, K., 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures and cell lines: a comparative study. Toxicology 218, 134–148.

Brinkman, M., Schlechtreim, C., Reininghaus, M., Eichbaum, K., Buchinger, S., Reifferscheid, G., Hollert, H., Preuss, T.G., 2016. Cross-species extrapolation of uptake and disposition of neutral organic chemicals in fish using a multispecies physiologically-based toxicokinetic model framework. Environ. Sci. Technol. 50, 1914-1923.

Buck, R.C., Franklin, J., Berger, U., Conder, J.M., Cousins, I.T., de Voogt, P., Jensen, A.A., Kannan, K., Mabury, S.A., van Leeuwen, S.P.J., 2011. Perfluoroalkyl and Polyfluoroalkyl Substances in the Environment: Terminology, Classification, and Origins. Integr. Environ. Assess. Manag. 7, 513-534.

Butenhoff, J.L., Gaylor, D.W., Moore, J.A., Olsen, G.W., Rodricks, J., Mandel, J.H., Zobel, L.R., 2004a. Characterization of risk for general population exposure to perfluorooctanoate. Regul. Toxicol. Pharmacol. 39, 363–380.

Butenhoff, J.L., Kennedy, G.L., Hinderliter, P.M., Lieder, P.H., Jung, R., Hansen, K.J., Gorman, G.S., Noker, P.E., Thomford, P.J., 2004b. Pharmacokinetics of perfluorooctanoate in *Cynomolgus* monkeys. Toxicol. Sci. 82, 394–406.

Carlson, R.W., Lien, G.W., Holmen, B.A., 1989. An Automated Monitoring System for Fish Physiology and Toxicology. EPA/600/3-89/011. U.S. Environmental Protection Agency, Duluth, MN.

[CEPA] Canadian Environmental Protection Act, 1999. Statutes of Canada 1999, Chapter 33. Act assented to September 14, 1999. Available at http://laws-lois.justice.gc.ca/eng/acts/c-15.31/ Accessed 28 March 2017.

Chang, S-C., Gorman, G.S., Gibson, S.J., Hart, J.A., Ehresman, D.J., Butenhoff, J.L., 2012. Comparative pharmacokinetics of perfluorooctanesulfonate (PFOS) in rats, mice and monkey. Reprod. Toxicol. 33, 428–440.

Chen, J., Tanguay, R.L., Tal, T.L., Gai, Z., Ma, X., Bai, C., Tilton, S.C., Jin, D., Yang, D., Huang, C., Dong, Q., 2014. Early life perfluorooctanesulphonic acid (PFOS) exposure impairs zebrafish organogenesis. Aquat. Toxicol. 156, 124-132.

Chen, Y., Guo, L., 2009. Fluorescence study on site-specific binding of perfluoroalkyl acids to human serum albumin. Arch. Toxicol. 83, 255-261.

Cheng, J., Psillakis, E., Hoffmann, M., Colussi, A., 2009. Acid dissociation versus molecular association of perfluoroalkyl oxoacids: Environmental implications. J. Phys. Chem. A 113, 8152−8156.

Cheng, X., Klaassen, C.D., 2009. Tissue distribution, ontogeny, and hormonal regulation of xenobiotic transporters in mouse kidneys. Drug Metab. Dispos. 37, 2178–2185.

Conder, J.M., Hoke, R.A., De Wolf, W., Russell, M.H., Buck, R.C., 2008. Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds. Environ. Sci. Technol. 42, 995–1003.

Consoer, D.M., Hoffman, A.D., Fitzsimmons, P.N., Kosian, P.A., Nichols, J.W., 2014. Toxicokinetics of perfluorooctanoate (PFOA) in rainbow trout. Aquat. Toxicol. 156, 65-73.

Consoer D.M., Hoffman A.D., Fitzsimmons P.N., Kosian P.A., Nichols J.W., 2016. Toxicokinetics of perfluorooctane sulfonate in rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 35, 717-727.

Corton, J.C., Cunningham, M.L., Hummer, B.T., Lau, C., Meek, B., Peters, J.M., Popp, J.A., Rhomberg, L., Seed, J., Klaunig, J.E., 2014. Mode of action framework analysis for receptor-mediated toxicity: the peroxisome proliferator-activated receptor alpha (PPARα) as a case study. Crit. Rev. Toxicol. 44, 1–49.

Council of the European Union, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. 673 p.

De Silva, A.O., Gledhill, M., Sekela, M., Syrgiannis, J., Evans, M.S., Armellin, A., 2010. Perfluorinated chemicals in freshwater fish in Canada. Organohalogen Compd. 72, 1277–80.

De Silva, A.O., Tseng, P.J., Mabury, S.A., 2009. Toxicokinetics of perfluorocarboxylate isomers in rainbow trout. Environ. Toxicol. Chem. 28, 330–337.

Devillers, J., Domine, D., Bintein, S., Karcher, W., 1998. Comparison of fish bioconcentration models. In: Comparative QSAR. Devillers J., (Ed) Washington DC: Taylor and Francis. p 1–50.

DeWitt, J.C., Copeland, C.B., Strynar, M.J., Luebke, R.W., 2008. Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ. Health Perspect. 116, 644–650.

DeWitt J.C. (Ed.), 2015. Toxicological effects of perfluoroalkyl and polyfluoroalkyl substances. Molecular and Integrative Toxicology, Springer, New York.

de Wolf, W., de Bruijn, J.H.M., Seinen, W., and Hermens, J., 1992. Influence of biotransformation on the relationship between bioconcentration factors and octanol-water partition coefficients. Environ. Sci. Technol. 26, 1197-1201.

Dinglasan, M.J., Ye, Y., Edwards, E.A., and Mabury, S.A., 2004. Fluorotelomer alcohol biodegradation yields poly- and perfluorinated acids. Environ. Sci. Technol. 38, 2857–2864.

Doi, A.M., Holmes, E., Kleinow, K.M., 2001. P-glycoprotein in the catfish intestine: inducibility by xenobiotics and functional properties. Aquat. Toxicol. 55, 157–170.

DuPont, 2017. DuPont position statement on PFOA. Available at http://www.dupont.com/corporate-functions/our-company/insights/articles/position-statements/articles/pfoa.html Accessed 28 March 2017.

Elcombe, C.R., Elcombe, B.M., Foster, J.R., Farrar, D.G., Jung, R., Chang, S.C., Kennedy, G.L., Butenhoff, J.L., 2010. Hepatocellular hypertrophy and cell proliferation in Sprague-Dawley rats following dietary exposure to ammonium perfl uorooctanoate occurs through increased activation of the xenosensor nuclear receptors PPARa and CAR/PXR. Arch. Toxicol. 84, 787–798.

Erickson, R.J., McKim, J.M., Lien, G.J., Hoffman, A.D., Batterman, S.L., 2006. Uptake and elimination of ionizable organic chemicals at fish gills: II. Observed and predicted effects of pH, alkalinity, and chemical properties. Environ. Toxicol. Chem. 25, 1522-1532.

European Union, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Available at http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32010L0063 Accessed 9 May 2017.

Fasano, W.J., Kennedy, G.L., Szostek, B., Farrar, D.G., Ward, R.J., Haroun, L., Hinderliter, P.M., 2005. Penetration of ammonium perfluorooctanoate through rat and human skin *in vitro*. Drug Chem. Toxicol. 28, 79-90.

Fischer, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A.-M., Wellner,P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as a multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biol. 11 (69), http://dx.doi.org/10.1187/1741-7007-11-69.

Fischer, S., Loncar, J., Zaja, R., Schnell, S., Schirmer, K., Smital, T., Luckenbach, T., 2011. Constitutive mRNA expression and protein activity levels of nine ABC efflux transporters in seven permanent cell lines derived from different tissues of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 101, 438–446.

Fitzsimmons, P.N., Hoffman,A.D., Lien, G.J., Hammermeister, D.E., Nichols,J.W., 2009. Kinetics and effects of dichloroacetic acid in rainbow trout. Aquat. Toxicol. 94, 186–194.

Flaherty, J.M., Connolly, P.D., Decker, E.R., Kennedy, S.M., Ellefson, M.E., Reagen, W.K., Szostek, B., 2005. Quantitative determination of perfluorooctanoic acid in serum and plasma by liquid chromatography tandem mass spectrometry. J. Chromatogr. B 819, 329–338.

Franko, J., Meade, B.J., Frasch, H.F., Barbero, A.M., Anderson, S.E., 2012. Dermal penetration potential of perfluorooctanoic acid (PFOA) in human and mouse skin. J. Toxicol. Environ. Health A 75, 50–62.

Furdui, V.I., Stock, N.L., Ellis, D.A., Butt, C.M., Whittle, D.M., Crozier, P.W., Reiner, E.J., Muir, D.C., Mabury, S.A., 2007. Spatial distribution of perfluoroalkyl contaminants in lake trout from the Great Lakes. Environ. Sci. Technol. 41, 1554–1559.

Gannon, S.A., Johnson, T., Nabb, D.L., Serex, T.L., Buck, R.C., Loveless, S.E., 2011. Absorption, distribution, metabolism and excretion of [1-^{14}C]-perfluorohexanoate ([^{14}C-PFHx) in rats and mice. Toxicology. 283, 55-62.

Giesy, J.P., Kannan, K., 2001. Global distribution of perfluorooctane sulfonate in wildlife. Environ. Sci. Technol. 35, 1339–1342.

Gobas, F.A., de Wolf, W., Burkhard, L.P. Verbruggen, E., Plotzke, K., 2009. Revisiting bioaccumulation criteria for POPs and PBT assessments. Integr. Environ. Assess. Manag. 5, 624-37.

Gobas, F.A.P.C., Morrison, H.A., 2000. Bioconcentration and biomagnification in the aquatic environment. In: Boethling, R.S., Mackay D., (Eds). Handbook of property estimation methods for chemicals. Environmental and health sciences. Boca Raton (FL): CRC. p 189–231.

Goeritz, I., Falk, S., Stahl, T., Schäfers, C., Schlechtreim, C., 2013. Biomagnification and tissue distribution of perfluoroalkyl substances (PFASs) in market-sized rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 32, 2078-2088.

Goodman, J., Chandna, A., Roe, K., 2015. Trends in animal use at US research facilities. J. Med. Ethics. 41, 563-566.

Goss, K.U., 2008. The pKa values of PFOA and other highly fluorinated carboxylic acids. Environ. Sci. Technol. 42, 456−458.

Han, J., Fang, Z., 2010. Estrogenic effects, reproductive impairment and developmental toxicity in ovoviparous swordtail fish (*Xiphophorus helleri*) exposed to perfluorooctane sulfonate (PFOS). Aquat. Toxicol. 99, 281-290.

Han, X., Nabb, D.L., Russell, M.H., Kennedy, G.L., Rickard, R.W., 2012. Renal elimination of perfluorocarboxylates (PFCAs). Chem. Res. Toxicol. 25, 35–46.

Han, X., Snow, T.A., Kemper, R.A., Jepson, G.W., 2003. Binding of perfluorooctanoic acid to rat and human plasma proteins. Chem. Res. Toxicol. 16, 775–781.

Han, X., Yang, C.H., Snajdr, S.I., Nabb, D.L., Mingoia, R.T., 2008. Uptake of perfluorooctanoate in freshly isolated hepatocytes from male and female rats. Toxicol. Lett. 181, 81-86.

Han, Z.-X., Lv, C.-X., Zheng, Z., 2009. Effects of perfluorooctane sulfonate on *Carassius auratus* and kinetics models. Int. J. Green Nanotechnol. Biomed. 1, B60–B68.

Harada, K., Inoue, K., Morikawa, A., Yoshinaga, T., Saito, N., Koizumi, A., 2005. Renal clearance of perfluorooctanoate in humans and species –specific excretion. Environ. Res. 99, 253–261.

Hebert, P., MacManus-Spencer, L.A., 2010. Development of a fluorescence model for binding of medium- to long-chain perfluoroalkyl acids to human serum albumin through a mechanistic evaluation of spectroscopic evidence. Anal. Chem. 82, 6463-6471.

Hickman, Jr., C.P., Trump, B.F., 1969. The kidney. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 1. Academic Press, New York, pp. 91–239.

Hinderliter, P.M., Mylchreest, E., Gannon, S.A., Butenhoff, J.L., Kennedy, G.L., Jr., 2005. Perfluorooctanoate placental and lactational transport pharmacokinetics in rats. Toxicology 211, 139–148.

Hoff, P.T., Van de Vijver, K., Van Dongen, W., Esmans, E.L., Blust, R., De Coen, W.M., 2003. Perfluorooctane sulfonic acid in bib (*Trisopterus luscus*) and plaice (*Pleuronectes platessa*) from the Western Scheldt and the Belgian North Sea: Distribution and biochemical effects. Environ. Toxicol. Chem. 22, 608-614.

Hoff P.T., Van Dongen, W., Esmans, E.L., Blust, R., De Coen, W.M., 2003. Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp (*Cyprinus carpio*). Aquat. Toxicol. 62, 349-359.

Houde, M., Martin, J.W., Letcher, R.J., Solomon, K.R., Muir, D.C., 2006. Biological monitoring of poly-fluoroalkyl substances: a review. Environ. Sci. Technol. 40, 3463–3473.

Houde, M., Czub, G., Small, J.M., Backus, S., Wang, X., Alaee, M, Muir, DC., 2008. Fractionation and bioaccumulation of perfluorooctane sulfonate (PFOS) isomers in a Lake Ontario food web. Environ. Sci. Technol. 42, 9397–9403.

Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M.T., Tanguay, R.L., Dong, Q., 2010. Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). Aquat. Toxicol. 98, 139–147.

Hundley, S.G., Sarrif, A.M., Kennedy, G.L., 2006. Absorption, distribution, and excretion of ammonium perfluorooctanoate (APFO) after oral administration to various species. Drug Chem. Toxicol. 29, 137-145.

Ishibashi, H., Iwata, H., Kim, E-Y., Tao, L., Kannan, K., Amano, M., Miyazaki, N., Tanabe, S., Batoev, V.B., Petrov, E.A., 2008. Contamination and effects of perfluorochemicals in Baikal seal (*Pusa sibirica*). 1. Residue level, tissue distribution, and temporal trend. Environ. Sci. Technol. 42, 2295-2301.

Jandacek, R.J., Rider, T., Keller, E.R., Tso, P., 2010. The effect of olestra on the absorption, excretion and storage of 2,2′,5,5′ tetrachlorobiphenyl; 3,3′,4,4′ tetrachlorobiphenyl; and perfluorooctanoic acid. Environ. Int. 36, 880–883.

Johnson, J.D., Gobson, S.J., Ober, R.E., 1979. Absorption of FC-95-14C in rat after a single oral dose. Riker Laboratories, St Paul. US EPA Administrative Record AR-226-0007.

Johnson, J.D., Gibson, S.J., Ober, R.E., 1984. Cholestyramine-enhanced fecal elimination of carbon-14 in rats after administration of ammonium [14C]perfluorooctanoate or potassium [14C]perfluorooctanesulfonate. Fundam. Appl. Toxicol. 4, 972-976.

Kannan, K., Franson, J.C., Bowerman, W.W., Hansen, K.J., Jones, J.D., and Giesy, J.P., 2001. Perfluorooctane sulfonate in fish-eating water birds including bald eagles and albatrosses. Environ. Sci. Technol. 35, 3065–3070.

Kannan, K., Tao, L., Sinclair, E., Pastva, S.D., Jude, D.J., Giesy, J.P., 2005. Perfluorinated compounds in aquatic organisms at various trophic levels in a great lakes food chain. Arch. Environ. Contam. Toxicol. 48, 559-566.

Katakura, M., Kudo, N., Tsuda, T., Hibino, Y., Mitsumoto, A., Kawashima, Y., 2007. Rat organic anion transporter 3 and organic anion transporting polypeptide 1 mediate perfluorooctanoic acid transport. J. Health Sci. 53, 77–83.

Kawashima, Y., Uy-Yu N., Kozuka, H., 1989. Sex-related difference in the inductions by perfluorooctanoic acid of peroxisomal beta-oxidation, microsomal 1-acylglycerophospho-choline acyltransferase and cytosolic long-chain acyl-CoA hydrolase in rat liver. Biochem. J. 261, 595–600.

Kemper, R.A., 2003. Perfluorooctanoic acid: toxicokinetics in the rat. DuPont Haskell Laboratories, report ID: Dupont-7473, USEPA Administrative Record AR-226.1449.

Kemper, R.A., Nabb, D.L., 2005. *In vitro* studies in microsomes from rat and human liver, kidney and intestine suggest that perfluorooctanoic acid is not a substrate for microsomal UDP-glucuronosyltransferases. Drug. Chem. Toxicol. 28, 281-287.

Kennedy, G.L., 1985. Dermal toxicity of ammonium perfluorooctanoate. Toxicol. Appl. Pharmacol. 81, 348-355.

Key, B. D., Howell, R. D., and Criddle, C. S., 1997. Fluorinated organic in the biosphere. Environ. Sci. Technol. 31, 2445–2454.

Key, B. D., Howell, R. D., and Criddle, C.S., 1998. Defluorination of organofluorine sulfur compounds by *Pseudomonas* sp. strain D2. Environ. Sci. Technol. 32, 2283–2287.

Kissa, E., 2001. Fluorinated Surfactants and Repellants, 2nd ed. Marcel Decker, New York.

Kjeldsen, L.S., Bonefeld-Jorgensen, E.C., 2013. Perfluorinated compounds affect the function of sex hormone receptors. Environ. Sci. Pollut. Res. Int. 20, 8031–8044.

Klaunig, J. E., Babich, M. A., Baetcke, K. P., Cook, J. C., Corton, J. C., David, R. M., DeLuca, J. G., Lai, D. Y., McKee, R. H., Peters, J. M., Roberts, R.A., Fenner-Crisp, P.A., 2003. PPARa agonist-induced rodent tumors: Modes of action and human relevance. Crit. Rev. Toxicol. 33, 655–780.

Kudo, N., 2015. Metabolism and Pharmacokinetics. In: Dewitt. J.C., (Ed.) Toxicological Effects of Perfluoroalkyl and Polyfluoroalkyl Substances. Springer, New York. p 151-176.

Kudo, N., Bandai, N., Suzuki, E., Katakura, M., Kawashima, Y., 2000. Induction by perfluorinated fatty acids with different carbon chain length of peroxisomal beta-oxidation in the liver of rats. Chem. Biol. Interact. 124, 119–132.

Kudo, N., Iwase, Y., Okayachi, H., Yamakawa, Y., Kawashima, Y., 2005. Induction of hepatic peroxisome proliferation by 8-2 telomer alcohol feeding in mice: formation of perfluorooctanoic acid in the liver. Toxicol. Sci. 86, 231–238.

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

Kudo, N., Kawashima, Y., 2003. Toxicity and toxicokinetics of perfluorooctanoic acid in humans and animals. J. Toxicol. Sci. 28, 49–57.

Kudo, N., Iwase, Y., Okayachi, H., Yamakawa, Y., Kawashima, Y., 2005. Induction of hepatic peroxisome proliferation by 8-2 telomer alcohol feeding in mice: formation of perfluorooctanoic acid in the liver. Toxicol. Sci. 86, 231–238.

Kudo, N., Sakai, A., Mitsumoto, A., Hibino, Y., Tsuda, T., Kawashima, Y., 2007. Tissue distribution and hepatic subcellular distribution of perfluorooctanoic acid at low dose are different from those at high dose in rats. Biol. Pharm. Bull. 30, 1535–1540.

Kudo, N., Suzuki, E., Katakura, M., Ohmori, K., Noshiro, R., Kawashima, Y., 2001. Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. Chem. Biol. Interact. 134, 203–216.

Langley, A.E., and Pilcher, G.D., 1985. Thyroid, bradycardic and hypothermic effects of perfluoro-*n*-decanoic acid in rats. J. Toxiciol. Environ. Health 15, 485–491.

Lau, C., 2009. Perfluoroalkyl acids: recent activities and research progress. Reprod. Toxicol. 27, 209–211.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., Seed, J., 2007. Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol. Sci. 99, 366–394.

Lau, C., Butenhoff, J.L., Rogers, J.M., 2004. The developmental toxicity of perfluoroalkyl acids and their derivatives. Toxicol. Appl. Pharmacol. 198, 231–241.

Lee, J.J., Schultz, I.R., 2010. Sex differences in the uptake and disposition of perfluorooctanoic acid in fathead minnows after oral dosing. Environ. Sci. Technol. 44, 491–496.

Lien, G.J., McKim, J.M., 1993. Predicting branchial and cutaneous uptake of 2,2',5,5'-tetrachlorobiphenyl in fathead minnows (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*): rate-limiting factors. Aquat. Toxicol. 27, 15-32.

Liu, C., Du, Y., Zhou, B., 2007. Evaluation of estrogenic activities and mechanism of action of perfl uorinated chemicals determined by vitellogenin induction in primary cultured tilapia hepatocytes. Aquat. Toxicol. 8, 267–277.

Liu, C., Gin, K.Y.H., Chang, V.W.C., Goh, B.P.L., Reinhard, M., 2011. Novel perspectives on the bioaccumulation of PFCs – the concentration dependency. Environ. Sci. Technol. 45, 9758–9764.

Ljubojevic, M., Balen, D., Breljak, D., Kusan, M., Anzai, N., Bahn, A., Burckhardt, G., Sabolic, I., 2007. Renal expression of organic anion transporter OAT2 in rats and mice is regulated by sex hormones. Am. J. Physiol. Renal Physiol. 292, F361–F372.

Loncar, J., Popovic, M., Zaja, R., Smital, T., 2010. Gene expression analysis of the ABC efflux transporters in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 151C, 209–215.

Lote, C.J., 2001. Principles of renal physiology, 5th Ed. Springer, New York.

Lou, I., Wambaugh, J.F., Lau, C., Hanson, R.G., Linstrom, A.B., Strynar, M.J., Zehr, R.D., Setzer, R.W., Barton, H.A., 2009. Modeling single and repeated dose pharmacokinetics of PFOA in mice. Toxicol. Sci. 107, 331-341.

Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. Comp. Biochem. Physiol. 165C, 28-52.

Luebker, D.J., Hansen, K.J., Bass, N.M., Butenhoff, J.L., Seacat, A.M., 2002. Interactions of fluorochemicals with rat liver fatty acid-binding protein. Toxicology 176, 175-185.

Mackenzie, D.D.S., Maack, T., Kinter, W.B., 1977. Renal excretion of chlorphenol red and related organic acids in the intact flounder (*Pseudopleuronectes americanus*). J. Exp. Zool. 199, 449-458.

Macon, M.B., Villanueva, L.R., Tatum-Gibbs, K., Zehr, R.D., Strynar, M.J., Stanko, J.P., White S.S., Helfant L., Fenton S.E., 2011. Prenatal perfluorooctanoic acid exposure in CD-1 mice: low dose developmental effects and internal dosimetry. Toxicol. Sci. 122, 134–145.

Maras, M., Vanparys, C., Muylle, F., Robbens, J., Berger, U., Barber, J.L., Blust, R., and De Coen, W., 2006. Estrogen-like properties of fluorotelomer alcohols as revealed by MCF-7 breast cancer cell proliferation. Environ. Health Perspect. 114, 100–105.

Martin, J.W., Mabury, S.A., Solomon, K.R., Muir, D.C.G., 2003a. Bioconcentration and tissue distribution of perfluorinated acids in rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 22, 196–204.

Martin, J.W., Mabury, S.A., Solomon, K.R., Muir, D.C.G., 2003b. Dietary accumulation of perfluorinated acids injuvenile rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 22, 189–195.

Martin, J.W., Smithwick, M.M., Braune, B.M., Hoekstra, P.F., Muir, D.C.G., Mabury, S.A., 2004. Identification of long-chain perfluorinated acids in biota from the Canadian Arctic. Environ. Sci. Technol. 38, 373–380.

McKim, J.M., Goeden, H.M., 1982. A direct measure of the uptake efficiency of a xenobiotic chemical across the gills of brook trout (*Salvelinus fontinalis*) under normoxic and hypoxic conditions. Comp. Biochem. Physiol. 72C, 65–74.

McKim, J.M., Kolanczyk, R.C., Lien, G.J., Hoffman, A.D., 1999. Dynamics of renal excretion of phenol and major metabolites in the rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 45, 265–277.

McKim, J.M., Nichols, J.W., Lien, G.J., Bertelsen, S.L., 1994. Respiratory-cardiovascular physiology and chloroethane gill flux in the channel catfish, *Ictalurus punctatus*. J. Fish Biol. 44, 527–547.

Meier-Abt, F., Hammann-Hänni, A., Stieger, B., Ballatori, N., Boyer, J.L., 2007. The organic anion transport polypeptide 1d1 (Oatp1d1) mediates hepatocellular uptake of phalloidin and microcystin into skate liver. Toxicol. Appl. Pharmacol. 218, 274–279.

Meylan, W.M., Howard, P.H., Boethling, R.S., Aronson,D., Printup, H., Gouchie, S., 1999. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. Environ. Toxicol. Chem. 18, 664–672.

Miller, D.S., Sussman, C.R., Renfro, J.L., David, S., Larry, J., 1998. Protein kinase C regulation of p-glycoprotein-mediated xenobiotic secretion in renal proximal tubule. Am. J. Physiol. Renal Physiol. 275, F785–F795.

Moody, C.A., Martin, J.W., Kwan, W.C., Muir, D.C.G., Mabury, S.A., 2001. Monitoring perfluorinated surfactants in biota and surface water samples following an accidental release of fire-fighting foam into Etobicoke Creek. Environ. Sci. Technol. 36, 545–551.

Moroi, Y., Yano, H., Shibata, O., Yonemitsu, T., 2001. Determination of acidity constants of perfluoroalkanoic acids. Bull. Chem. Soc. Jpn. 74, 667−672.

Morris, M.E., Lee, H.-J., Predko, L.M., 2003. Gender differences in the membrane transport of endogenous and exogenous compounds. Pharmacol. Rev. 55, 229–240.

Mulle,r C.E., de Silva, A.O., Small, J., Williamson, M., Wang, X., Morris, A., Katz, S., Gamberg, M., Muir D.C., 2011. Biomagnification of perfluorinated compounds in a remote terrestrial food chain: Lichen-Caribou-wolf. Environ. Sci. Technol. 45, 8665–8673.

Murakami, M., Adachi, N., Saha, M., Morita, C., Takada, H., 2011. Levels, temporal trends, and tissue distribution of perfluorinated surfactants in freshwater fish from Asian countries. Arch. Environ. Contam. Toxicol. 61, 631–641.

Nakagawa, H., Hirata, T., Terada, T., Jutabha, P., Miura, D., Harada, K.H., Inoue, K., Anzai, N., Endou, H., Inui, K.-I., Kanai, Y., Koizumi, A., 2008. Roles of organic anion transporters in the renal excretion of perfluorooctanoic acid. Basic Clin. Pharmacol. Toxicol. 103, 1–8.

Nakagawa, H., Terada, T., Harada, K.H., Hitomi, T., Inoue, K., Inui, K.-I., Koizumi, A., 2009. Human organic anion transporter hOAT4 is a transporter of perfluorooctanoic acid. Basic Clin. Pharmacol. Toxicol. 105, 136–138.

Nichols, J.W., Bonnell, M., Dimitrov, S., Escher, B.I., Han, X., Kramer, N.I., 2009. Bioaccumulation assessment using predictive approaches. Integr Environ. Assess. Manag. 5, 577-597.

Nichols, J.W., Du, B., Berninger, J.P., Connors, K.A., Chambliss, K., Erickson, R.J., Hoffman, A.D., Brooks, B.W., 2015. Observed and modeled effects of pH on bioconcentration of diphenhydramine, a weakly basic pharmaceutical, in fathead minnows. Environ Toxicol Chem. 6, 1425-1435.

Nichols, J.W., McKim, J.M., Andersen, M.E., Gargas, M.L., Clewell III, H.J., Erickson, R.J., 1990. A physiologically based toxicokinetic model for the uptake and disposition of waterborne organic chemicals in fish. Toxicol. Appl. Pharmacol. 106, 433–447.

Ng, C.A., Hungerbühler, K., 2013. Bioconcentration of perfluorinated alkyl acids: how important is specific binding? Environ. Sci. Technol. 47, 7214–7223.

Ohmori, K., Kudo, N., Katayama, K., Kawashima, Y., 2003. Comparison of the toxicokinetics between perfluorocarboxylic acids with different carbon chain length. Toxicology 184:135–140.

Oliver, B.G., and Niimi, A.J., 1985. Bioconcentration factors of some halogenated organics for rainbow trout: Limitations in their use for prediction of environmental residues. Environ. Sci. Technol. 19, 842-849.

Olsen, G.W., Burris J.M., Ehresman, D.J., Froelich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfljuorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health Perspect. 115, 1298-1305.

Ophaug, R.H., Singe,r L., 1980. Metabolic handling of perfluorooctanoic acid in rats. Proc. Soc. Exp. Biol. Med. 163:19-23.

Ostertag, S.K., Tague, B.A., Humphries M.M., Tittlemier S.A., Chan H.M., 2009. Estimated dietary exposure to fluorinated compounds from traditional foods among Inuit in Nunavut, Canada. Chemosphere 75, 1165–1172.

Paul, A.G., Jones, K.C., Sweetman, A.J., 2009. A first global production, emission, and environmental inventory for perfluorooctane sulfonate. Environ. Sci. Technol. 43, 386-392.

Pavan, M., Netzeva, T.I., Worth A.P., 2008. Review of literature-based quantitative structure-activity relationship models for bioconcentration. QSAR. Comb. Sci. 27, 21–31.

Popovic, M., Zaja, R., Smital, T., 2010. Organic anion transporting polypeptides (OATP) in zebrafish (*Danio rerio*): phylogenetic analysis and tissue distribution. Comp. Biochem. Physiol. 155A, 327–335.

Popovic, M., Zaja, R., Fent, K., Smital, T., 2014. Interaction of environmental contaminants with zebrafish organic anion transporting polypeptide, Oatp1d1 (*Slco1d1*). Toxicol. Appl. Pharmacol. 280, 149-158.

Prevedouros, K., Cousins, I.T., Buck, R.C., Korzeniowski, S.H., 2006. Sources, fate and transport of perfluorocarboxylates. Environ. Sci. Technol. 40, 32–44.

Pritchard, J.B., Bend, J.R., 1984. Mechanisms controlling the renal excretion of xenobiotics in fish – effects of chemical structure. Drug Metab. Rev. 15, 655–671.

Pritchard, J.B., James, M.O., 1979. Determinants of the renal handling of 2 4-dichlorophenoxyacetic acid by winter flounder. J. Pharmacol. Exp. Ther. 208, 280–286.

Pritchard, J.B., Bend, J.R., 1991. Relative roles of metabolism and renal excretory mechanisms in xenobiotic elimination by fish. Environ. Health Perspect. 90, 85–92.

Reagen, W.K., Ellefson, M.E., Kannan, K., Giesy, J.P., 2008. Comparison of extraction and quantification methods of perfluorinated compounds in human plasma, serum, and whole blood. Anal. Chim. Acta 628, 214–221.

Reiner, J.L., O'Connell, S.G., Moors, A.J., Kucklick, J.R., Becker, P.R., Keller, J.M., 2011. Spatial and temporal trends of perfl uorinated compounds in Beluga Whales (*Delphinapterus leucas*) from Alaska. Environ. Sci. Technol. 45, 8129–8136.

Renner, R., 2001. Growing concern over perfluorinated chemicals. Environ. Sci. Technol. 35, 154A-160A.

Rosen, M.B., Lee, J.S., Ren, H., Vallanat, B., Liu, J., Waalkes, M.P., Abbott, B.D., Lau, C., Corton, J.C., 2008. Toxicogenomic dissection of the perfluorooctanoic acid

transcript profile in mouse liver: evidence for the involvement of nuclear receptors PPAR alpha and CAR. Toxicol. Sci. 103, 46–56.

Saarikoski, J., Lindstrom, M., Tyynila, M., Viluksela, M., 1986. Factors affecting the absorption of phenolics and carboxylic acids in the guppy (*Poecilia reticulata*). Ecotoxicol. Environ. Safety 11, 158-173.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Olsen, G.W., Case, M.T., and Butenhoff, J.L., 2002. Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. Toxicol. Sci. 68, 249–264.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Clemen, L.A., Eldridge, S.R., Elcombe, C.R., and Butenhoff, J.L., 2003. Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. Toxicology 183, 117–131.

Shi, X., Du, Y., Lam, P.K.S., Wu, R.S.S., Zhou, B., 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicol. Appl. Pharmacol. 230, 23–32.

Shi, Y., Wang, J., Pan, Y., Cai, Y., 2012. Tissue distribution of perfluorinated compounds in farmed freshwater fish and human exposure by consumption. Environ. Toxicol. Chem. 31, 717-723.

Southworth, G.R., Keffer, C.C., and Beauchamp, J.J., 1980. Potential and realized bioconcentration. A comparison of observed and predicted bioconcentration of azaarenes in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 14, 1529-1531.

Stadnicka, J., Schirmer, K., Ashauer, R., 2012. Predicting concentrations of organic chemicals in fish using toxicokinetic models. Environ. Sci. Technol. 46, 3273-3280.

Sturm, A., Segner, H., 2005. P-glycoproteins and xenobiotic efflux transport in fish. In: Mommsen, T.P., Moon, T.W. (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 6. Elsevier B.V., Amsterdam, pp. 495–533.

Sturm, A., Ziemann, C., Hirsch-Ernst, K.I., Segner, H., 2001. Expression and functional activity of P-glycoprotein in cultured hepatocytes from *Oncorhynchus mykiss*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 281, R1119–R1126.

Takacs, M.L., Abbott, B.D., 2007. Activation of mouse and human peroxisome proliferator-activated receptors (alpha, beta/delta, gamma) by perfl uorooctanoic acid and perfl uorooctane sulfonate. Toxicol. Sci. 95, 108–117.

Taniyasu, S., Kannan, K., So, M.K., Gulkowska, A., Sinclair, E., Okazawa, T., Yamashita, N., 2005. Analysis of fluorotelomer alcohols, fluorotelomer acids, and short- and long-chain perfluorinated acids in water and biota. J. Chromatogr. A 1093, 89–97.

Taniyasu, S., Kannan, K., Yeung, L.W.Y., Kwok, K.Y., Lam, P.K.S., Yamashita, N., 2008. Analysis of trifluoroacetic acid and other short-chain perfluorinated acids (C2- C4) in precipitation by liquid chromatography–tandem mass spectrometry: comparison to patterns of long-chain perfluorinated acids (C5–C18). Anal. Chim. Acta 619, 221–230.

Thurston, R.V., Gehrke, P.C., 1990. Respiratory oxygen requirements of fishes: description of OXYREF, a data file based on test results reported in published literature. In: Russo, R.C., Thurston, R.V. (Eds.), Fish Physiology, Toxicology, and Water-Quality Management. EPA/600/R-93/157. U.S. Environmental Protection Agency, Washington, DC, pp. 95–108.

Toutain, P.L., Bousquet-Mélou, A., 2004. Plasma terminal half-life. J. Vet. Pharmacol. Therap. 27, 427-439.

Uchea, C., Owen S.F., Chipman J.K., 2015. Functional xenobiotic metabolism and efflux transporters in trout hepatocyte spheroid culture. Toxicol. Res. 4, 494-507.

[UNEP] United Nations Environment Program, 2001. Final act of the Conference of Pleniopotentiaries on The Stockholm Convention on Persistent Organic Pollutants. Geneva (CG): UNEP. 44 p. Available at http://www.pops.int/documents/meetings/dipcon/25june2001/conf4_finalact/en/FINALACT-English.PDF Accessed 5 March 2017.

[UNEP] United Nations Environment Program, 2009. Stockholm convention on persistent organic pollutants – amendment to annex A. Available at https://treaties.un.org/doc/Publication/CN/2013/CN.934.2013-Eng.pdf. Accessed 28 March 2017.

[US EPA] United States Environmental Protection Agency, 1976. Toxic substance control act (1976). Washington, DC. US EPA.

[US EPA] United States Environmental Protection Agency, 2009. Long-Chain Perfluorinated Chemicals (PFCs) Action Plan. Available at https://www.epa.gov/sites/production/files/2016-01/documents/pfcs_action_plan1230_09.pdf Accessed 17 April 2017.

[US EPA] United States Environmental Protection Agency, 2016. Frank R. Lautenberg Chemical Safety for the 21st Century Act. Washington, DC. US EPA.

[US EPA] United States Environmental Protection Agency, 2016. Fact sheet PFOA & PFOS drinking water health advisories. Available at https://www.epa.gov/sites/production/files/2016-06/documents/drinkingwaterhealthadvisories_pfoa_pfos_updated_5.31.16.pdf Accessed 28 March 2017.

[US EPA] United States Environmental Protection Agency, 2017. US EPA's Non-CBI summary tables for 2015 company progress reports (final progress reports).

Available at https://www.epa.gov/sites/production/files/2017-02/documents/2016_pfoa_stewardship_summary_table_0.pdf Accessed 28 March 2017.

Vanden Heuvel, J.P., Davis, J.W. 2nd, Sommers, R., Peterson, R.E., 1992. Renal excretion of perfluorooctanoic acid in male rats: inhibitory effect of testosterone. J. Biochem. Toxicol. 7, 31-36.

Vanden Heuvel, J.P., Kuslikis, B.I., Van Rafelghem, M.J., Peterson, R.E., 1991. Tissue distribution, metabolism, and elimination of perfluorooctanoic acid in male and female rats. J. Biochem. Toxicol. 6, 83–92.

Verri, T., Terova, G., Romano, A., Barca, A., Pisani, P., Storelli, C., Saroglia, M., 2012. The Solute Carrier (SLC) family series in teleost fish. In: Saroglia, M., Liu, Z. (Eds.), Functional Genomics in Aquaculture. John Wiley & Sons, Oxford, pp. 219–320.

Weaver, Y.M., Ehresman, D.J., Butenhoff, J.L., Hagenbuch, B., 2010. Roles of rat renal organic anion transporters in transporting perfluorinated carboxylates with different chain lengths. Toxicol. Sci. 113, 305–314.

Webster, E., Ellis, D., 2011. Equilibrium modeling: a pathway to understanding observed perfluorocarboxylic and perfluorosulfonic acid behavior. Environ. Toxicol. Chem. 30, 2229-2236.

Wei, Y., Chan, L.L., 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.

Weisbrod, A.V., Woodburn, K.B., Koelmans, A.A., Parkerton, T F., McElroy, A.E. and Borga, K., 2009. Evaluation of Bioaccumulation Using *In vivo* Laboratory and Field Studies. Integr. Environ. Assess. Manag. 5, 598–623.

White, S.S., Calafat, A.M., Kuklenyik, Z., Villanueva, L., Zehr, R.D., Helfant, L., Strynar, M.J., Lindstrom, A.B., Thibodeaux, J.R., Wood, C., Fenton, S.E., 2007. Gestational PFOA exposure of mice is associated with altered mammary gland development in dams and female offspring. Toxicol. Sci. 96, 133–144.

White, S.S., Kato, K., Jia, L.T., Basden, B.J., Calafat, A.M., Hines, E.P., Stanko, J.P., Wolf, C.J., Abbott, B.D., Fenton, S.E., 2009. Effects of perfl uorooctanoic acid on mouse mammary gland development and differentiation resulting from cross-foster and restricted gestational exposures. Reprod. Toxicol. 27, 289–298.

White, S.S., Stanko, J.P., Kato, K., Calafat, A.M., Hines, E.P., Fenton, S.E., 2011. Gestational and chronic low-dose PFOA exposures and mammary gland growth

and differentiation in three generations of CD-1 mice. Environ. Health Perspect. 119, 1070–1076.

Wolff, N.A., Werner, A., Burkhardt, S., Burckhardt, G., 1997. Expression cloning and characterization of a renal organic anion transporter from winter flounder. FEBS Lett. 417, 287–291.

Yang, Q., Abedi-Valugerdi, M., Xie, Y., Zhao, X.Y., Moller, G., Nelson, B.D., DePierre, J.W., 2002. Potent suppression of the adaptive immune response in mice upon dietary exposure to the potent peroxisome proliferator, perfluorooctanoic acid. Int. Immunopharmacol. 2, 389–397.

Yang, C. H., Glover, K. P., and Han, X., 2009. Organic anion transporting polypeptide (Oatp) 1a1-mediated perfluorooctanoate transport and evidence for a renal reabsorption mechanism of Oatp1a1 in renal elimination of perfluorocarboxylates in rats. Toxicol. Lett. 190, 163–171.

Yang, C.H., Glover, K.P., Han, X., 2010. Characterization of cellular uptake of perfluooctanoate via organic anion-transporting polypeptide 1A2, organic anion transporter 4, and urate transporter 1 for their potential roles in mediating human renal reabsorption of perfluorocarboxylates. Toxicol. Sci. 117, 294–302.

Ylinen, M., Hanhijarvi, H., Jaakonaho, J., Peura, P., 1989. Stimulation by oestradiol of the urinary excretion of perfluorooctanoic acid in the male rat. Pharmacol. Toxicol. 65, 274–277.

Ylinen, M., Kojo, A., Hanhijarvi, H., Peura, P., 1990. Disposition of perfluorooctanoic acid in the rat after single and subchronic administration. Bull. Environ. Contam. Toxicol. 44, 46-53.

Young, C.J., Mabury, S.A., 2010. Atmospheric perfluorinated acid precursors: chemistry, occurrence, and impacts. Rev. Environ. Contam. Toxicol. 208, 1–109.

Zaja, R., Loncar, J., Popovic, M., Smital, T., 2011. First characterization of fish P-glycoprotein (abcb1) substrate specificity using determinations of its ATPase activity and calcein-AM assay with PLHC-1/dox cell line. Aquat. Toxicol. 103, 53–62.

Zaja, R., Munic, V., Klobucar, R.S., Ambriovic-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquat. Toxicol. 90, 322–332.

Zhang, T., Sun, H.W., Wu, Q., Zhang, X.Z., Yun, S.H., Kannan, K., 2010. Perfluorochemicals in meat, eggs and indoor dust in China: assessment of sources and pathways of human exposure to perfluorochemicals. Environ. Sci. Technol. 44, 3572–3579.

Zhang, Y., Beesoon, S., Zhu, L., Martin, J.W., 2013. Biomonitoring of perfluoroalkyl acids in human urine and estimates of biological half-life. Environ. Sci. Technol. 47, 10619-10627.

Zhao, W., Zitow, J.D., Weaver, Y., Ehresman, D.J., Chang, S-C., Butenhoff, J.L., Hagenbuch, B., 2017. Organic anion transporting polypeptides contribute to the disposition of perfluoroalkyl acids in humans and rats. Toxicol. Sci. 156(1), 84-95.

Appendix 1

Supplemental data for Chapter 2:Toxicokinetics of perfluorooctanoate (PFOA) in rainbow trout (*Oncorhynchus mykiss*)

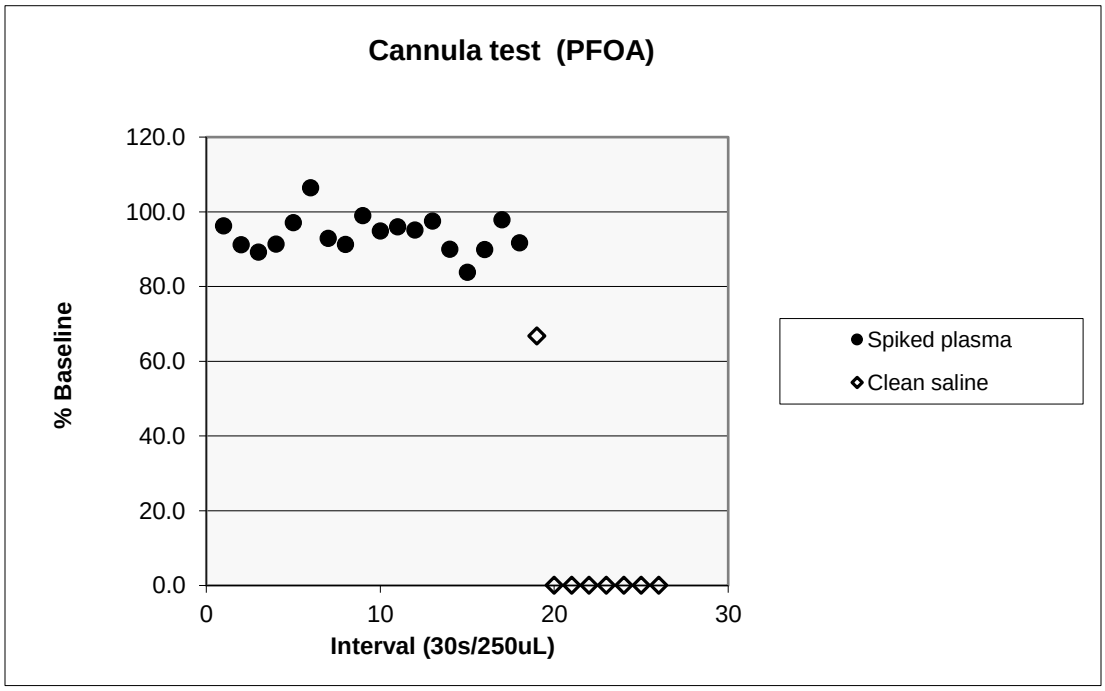

Fig. S2-1. Adsorption of PFOA to polyethylene tubing (Intramedic PE50) used for chronic cannulation of rainbow trout. Measured concentrations of PFOA in solutions (plasma or physiological saline) exiting the cannula are shown as individual points. The pumping rate was 1 mL/min and the nominal concentration of PFOA spiked into plasma was 25 ng/mL. Additional details pertaining to the experimental design are provided in the Section 2.7, Quality controls.

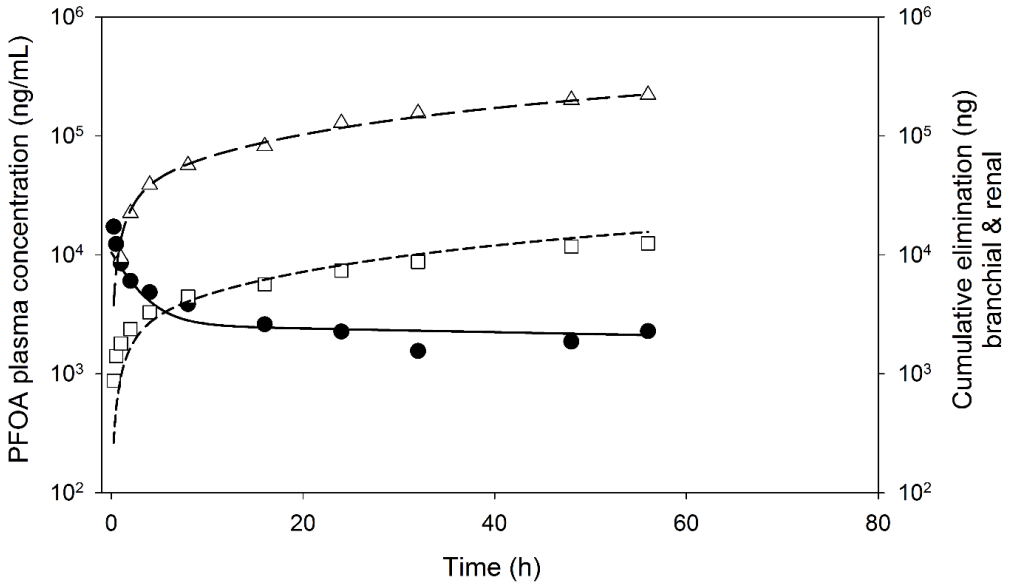

Fig. S2-2. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 2. In this and the following seven figures (S2-2 to S2-9), measured concentrations in plasma are shown as solid dots. Open triangles and open squares denote the cumulative mass of PFOA eliminated in urine and expired water, respectively. Lines show the optimized fit of model simulations to measured values: solid line – plasma; short dashes – expired water; long dashes – urine. Sampling was terminated after 56 h due to failure of the exposure system.

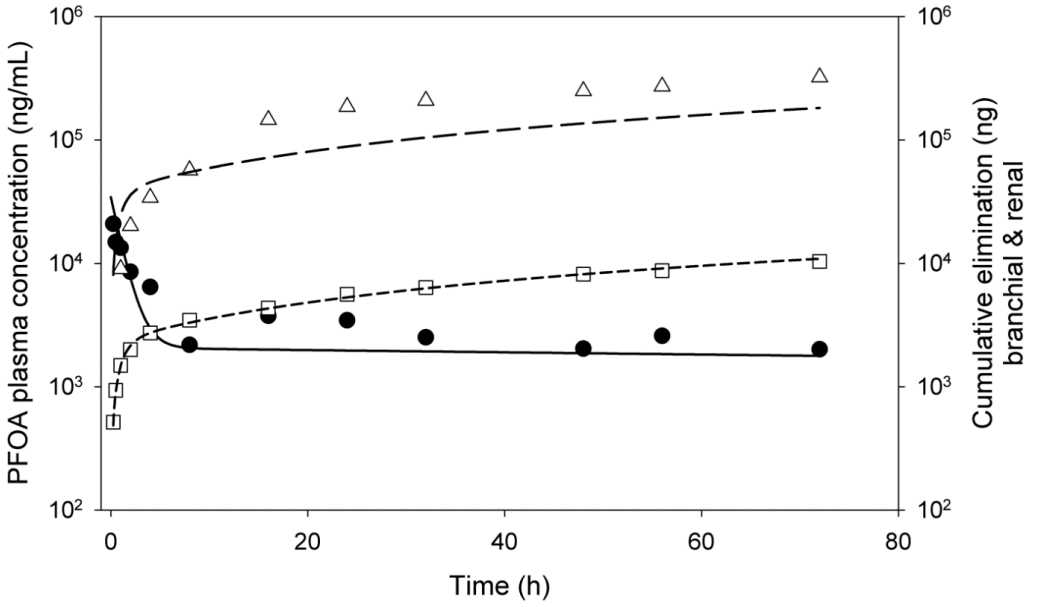

Fig. S2-3. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 3.

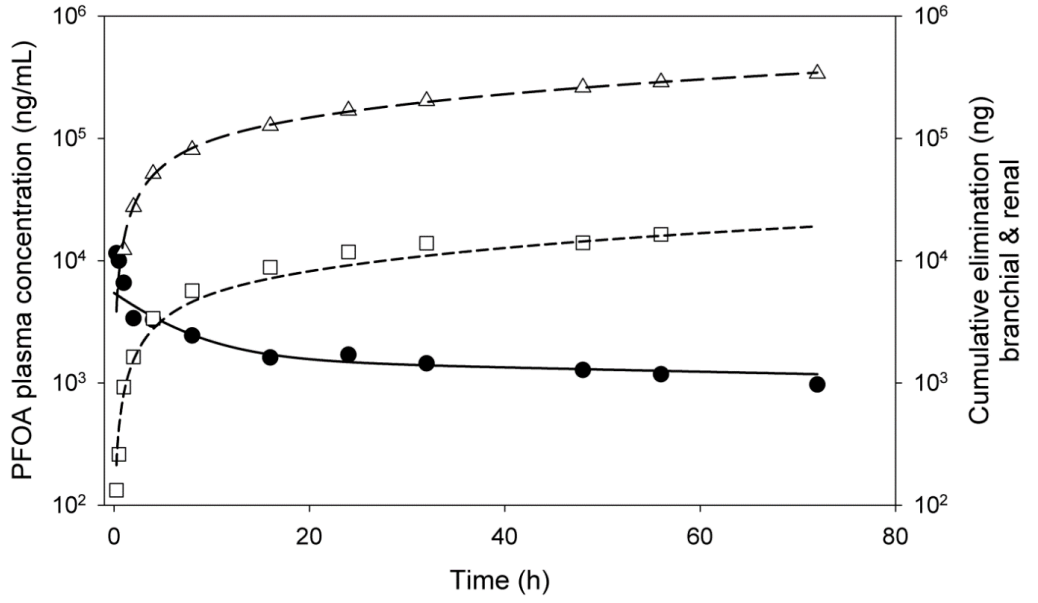

Fig. S2-4. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 4.

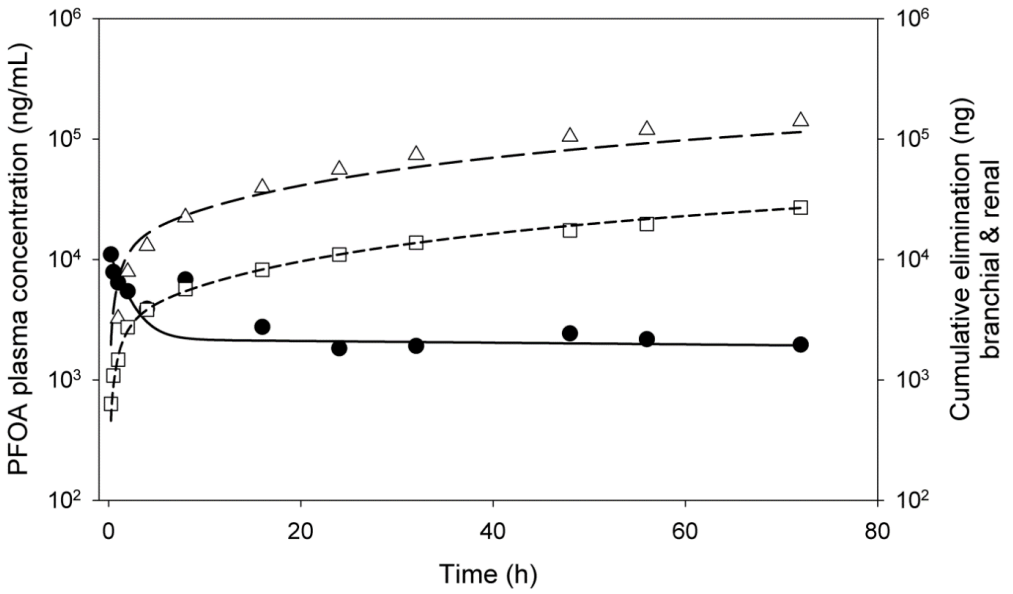

Fig. S2-5. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 5.

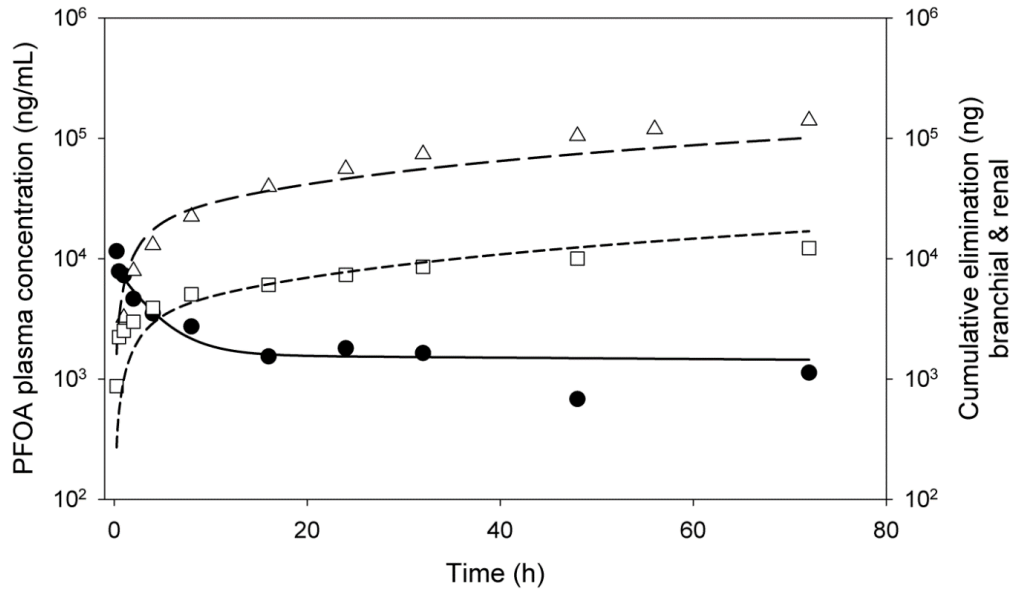

Fig. S2-6. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 6.

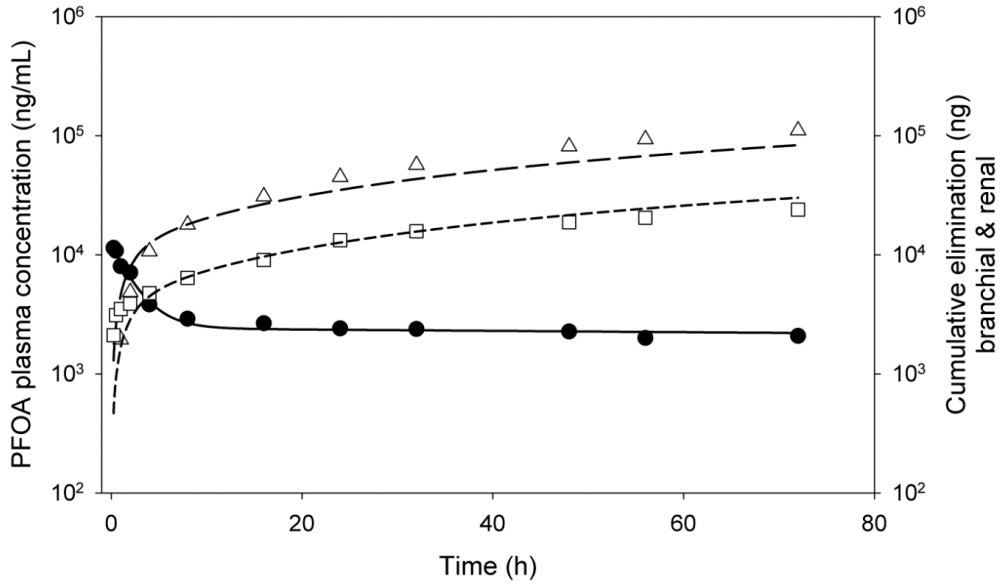

Fig. S2-7. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 7.

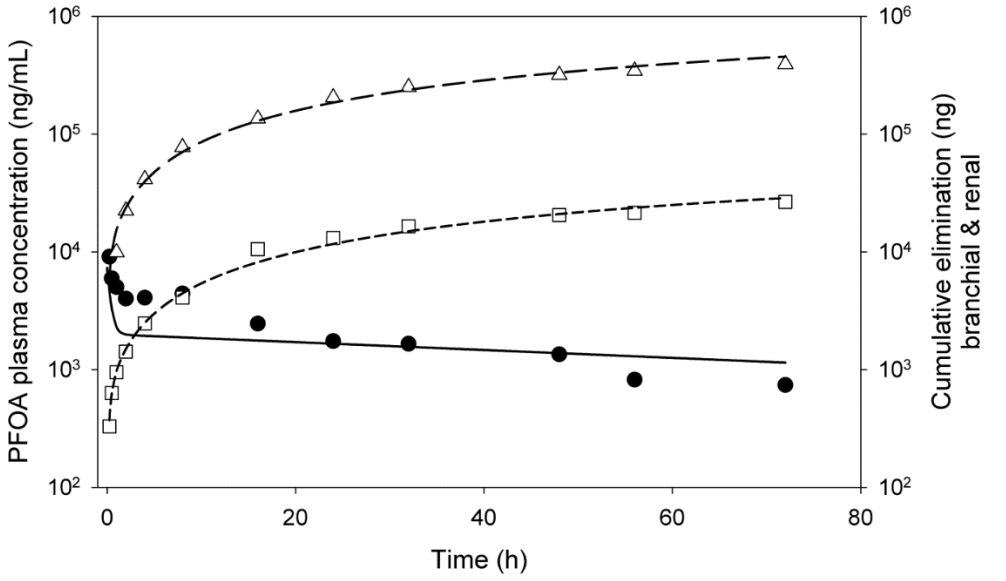

Fig. S2-8. Kinetics of PFOA elimination from trout following a bolus intra-arterial injection – Fish 8.

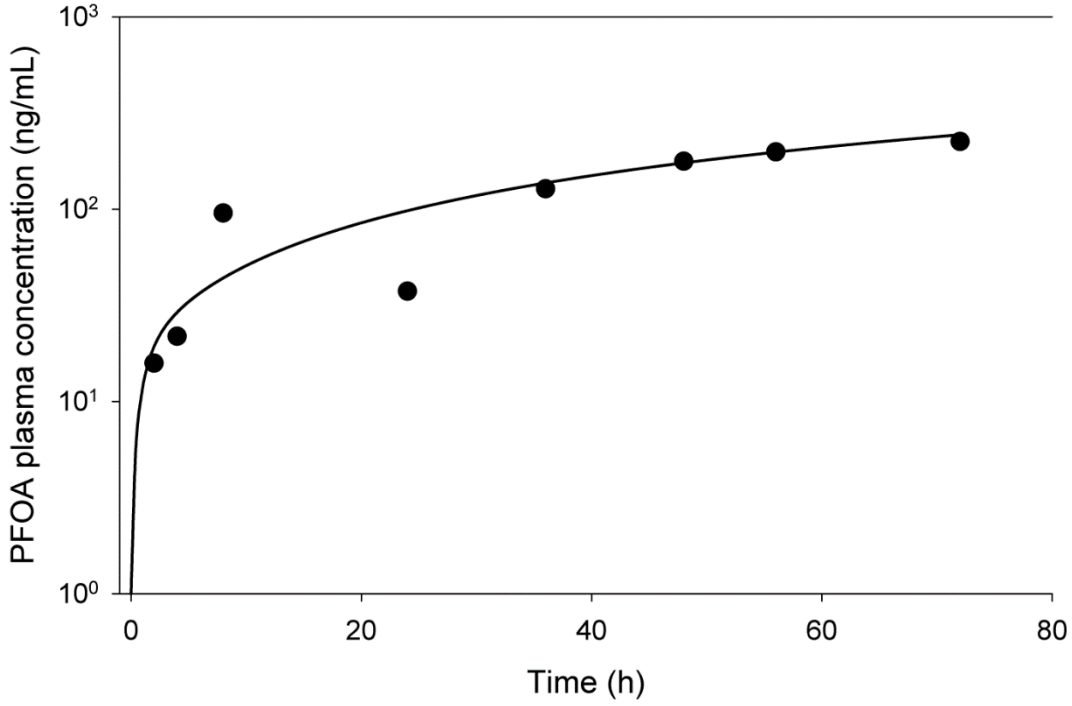

Fig. S2-9. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 2. Measured values in this and the follow six figures (S2-9 to S2-15) are shown as individual points. The fitted model simulation is shown as a solid line.

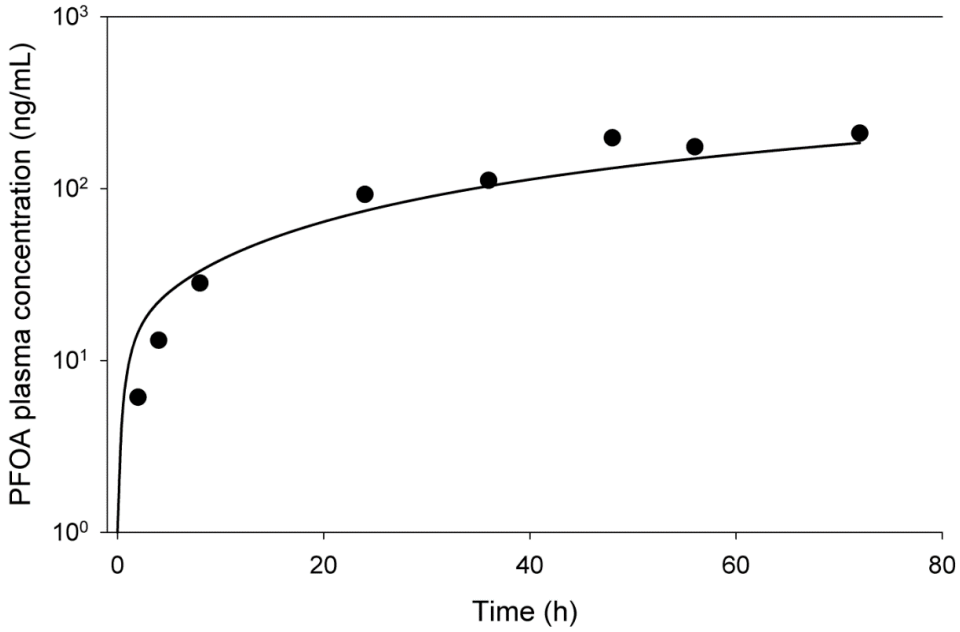

Fig. S2-10. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 3.

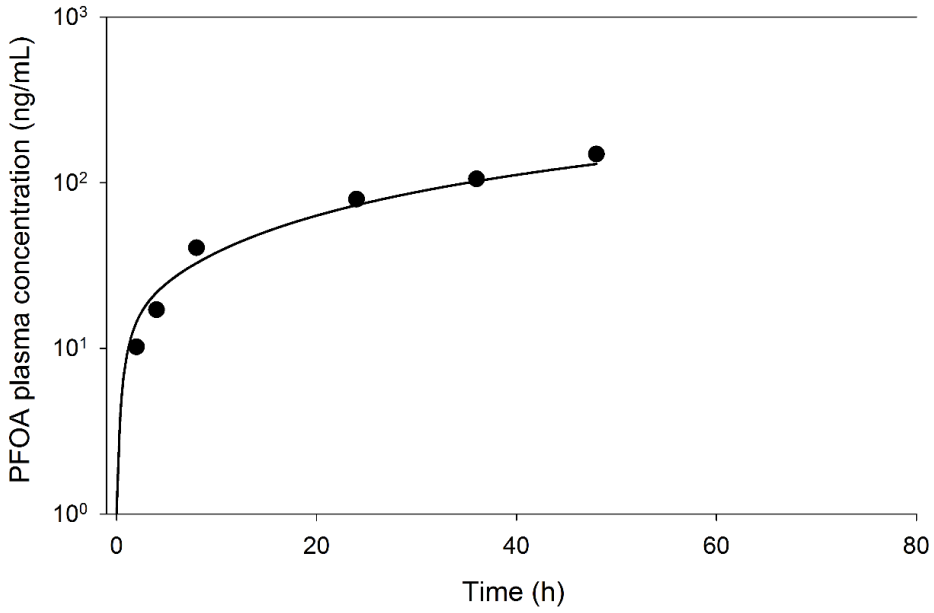

Fig. S2-11. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 4. Sampling was terminated at 48 h due to unexplained death of the animal.

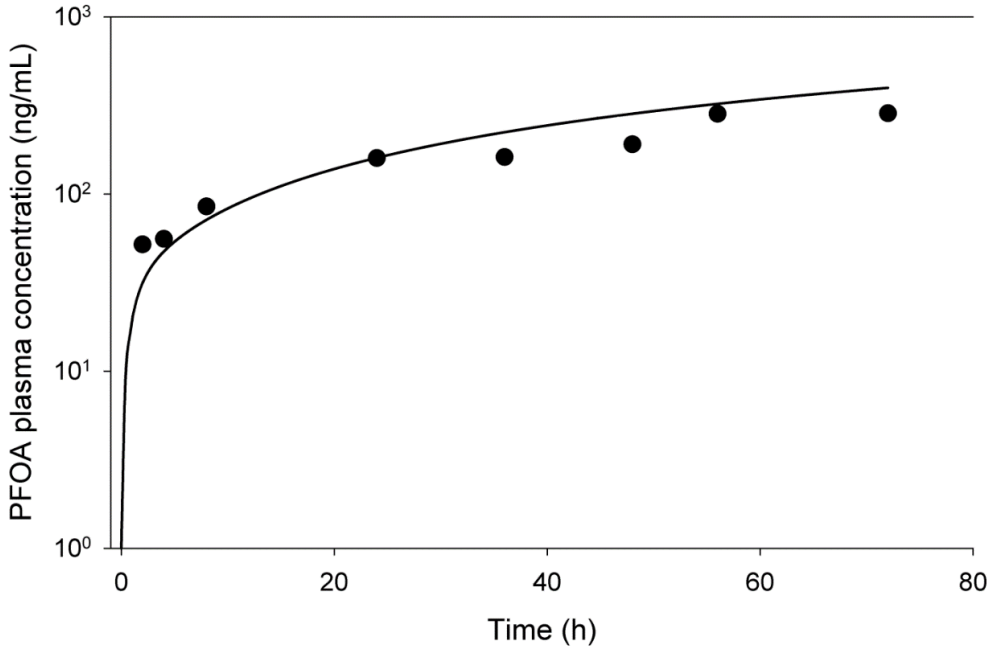

Fig. S2-12. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 5.

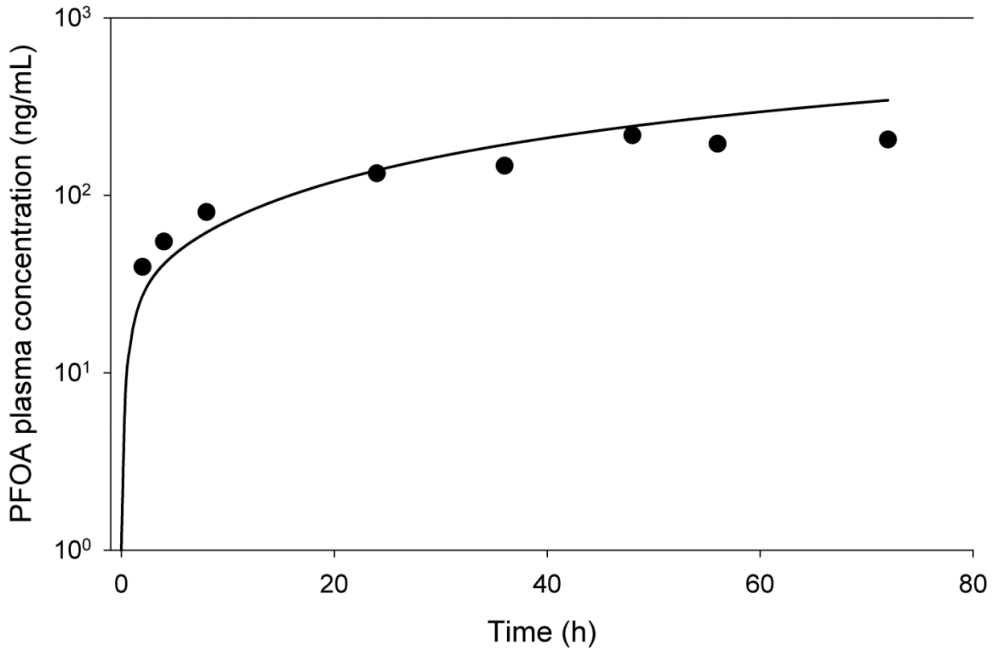

Fig. S2-13. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 6.

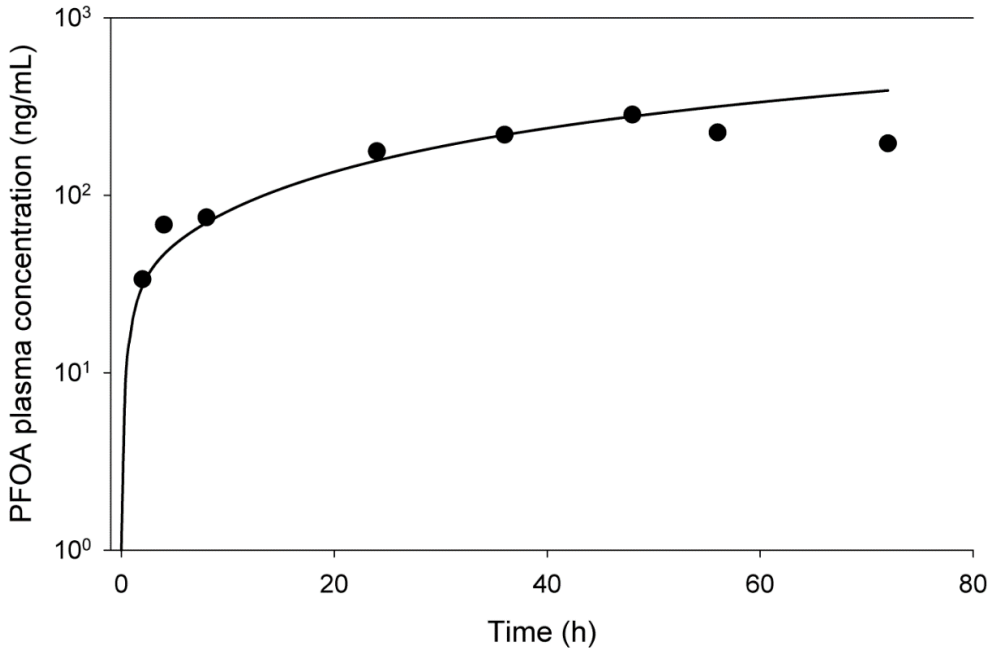

Fig. S2-14. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 7.

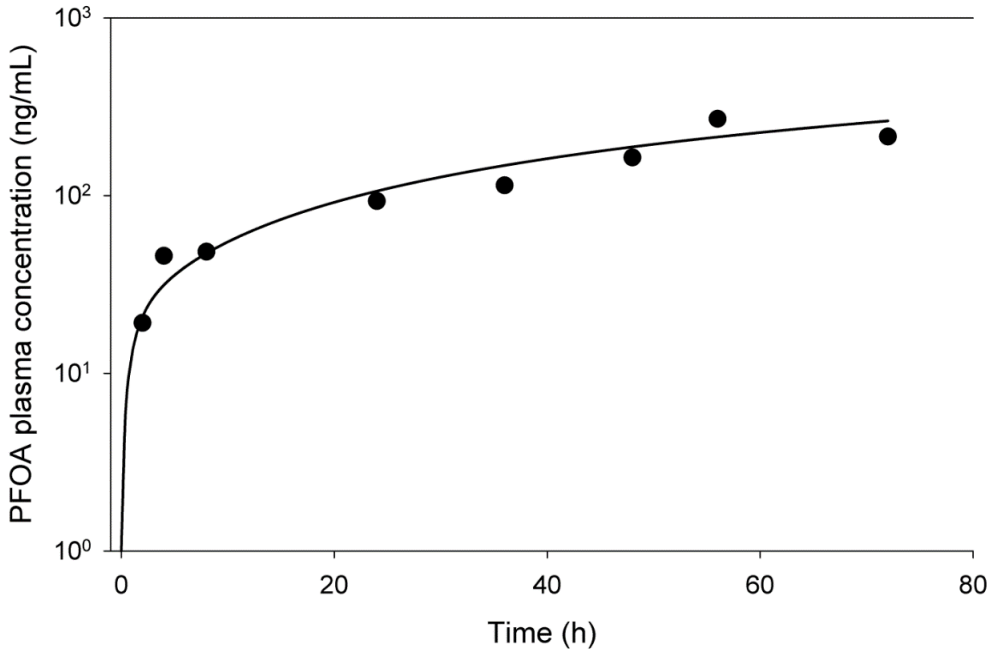

Fig. S2-15. Kinetics of PFOA in trout plasma during a continuous waterborne exposure – Fish 8.

Appendix 2

Supplemental data for Chapter 3: Toxicokinetics of perfluorooctane sulfonate in rainbow trout (*Oncorhynchus mykiss*)

Table S3-1. Kinetic parameters and tissue/plasma concentration ratios for fish used in renal elimination studies, determined using a "renal plus other" model

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Mean (SD)
Gender	♀	♀	♂	♂	♂	♀	
Weight (g)	1346	814	1400	1033	934	694	1037 (285)
Kinetic parameters							
V_C (mL/kg)	29.9	46.3	54.6	74.5	45.6	36.8	47.9 (15.5)
k_{12} (1/d)	0.2051	0.1720	0.3329	0.1151	0.1748	0.4246	0.237 (0.117)
k_{21} (1/d)	0.6995	0.6348	1.4817	0.2671	0.6186	1.1402	0.807 (0.432)
k_{31} (1/d)	0.00038	0.00028	0.00018	0.00013	0.00038	0.00038	0.00029 (0.00011)
k_{41} (1/d)	0.0141	0.0216	0.0583	0.0259	0.0134	0.0145	0.025 (0.017)
V_{SS} (mL/kg)	132	217	297	247	207	135	206 (64)
CL_R (mL/d/kg)	0.27	0.31	0.24	0.24	0.42	0.33	0.30 (0.07)
CL_T (mL/d/kg)	10.36	24.37	76.60	46.51	15.12	13.11	31.01 (25.93)
$T_{1/2}$ (d)	48.0	31.6	11.8	26.6	50.1	46.6	35.8 (15.1)
AIC[a]	7.89	7.31	7.37	7.60	8.51	6.64	7.55 (0.63)
BIC[b]	8.06	7.49	7.54	7.77	8.68	6.81	7.73 (0.62)
Tissue/plasma concentration ratios at takedown							
Liver	0.42	NS[c]	0.83	1.71	0.70	0.70	0.87 (0.49)
Kidney	0.22	NS[c]	0.49	0.75	0.27	0.29	0.41 (0.2)
Muscle	0.06	NS[c]	0.05	0.04	NS[c]	NS[c]	0.05 (0.01)

[a]Akaike information criterion (AIC)
[b]Bayesian information criterion (BIC)
[c]NS = Sample lost or not collected

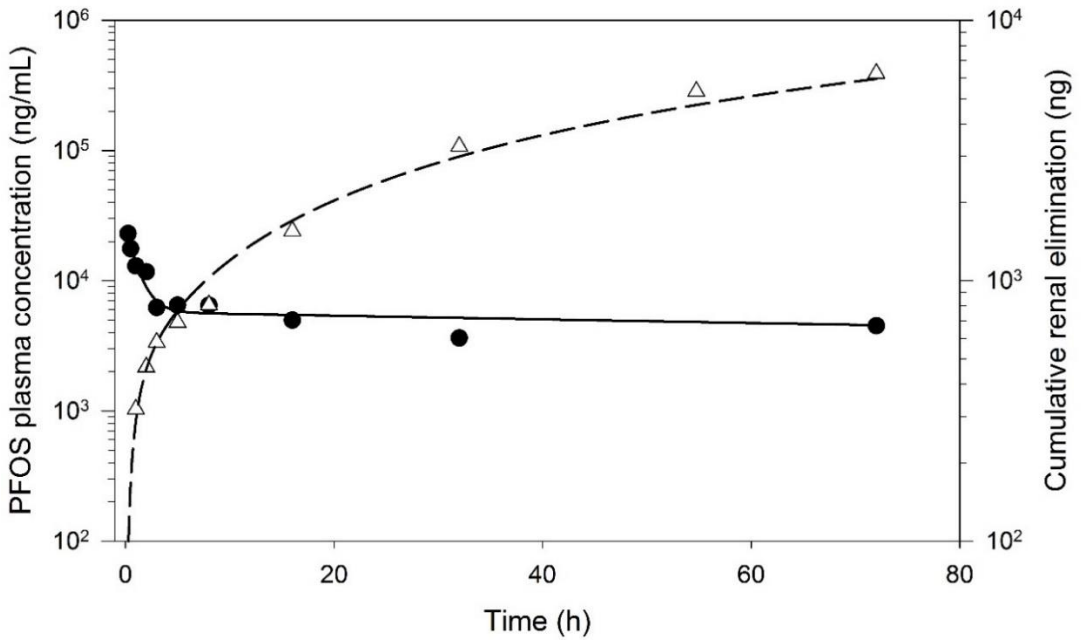

Figure S3-1. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 1. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$. In this and Figures S3-2—S3-6 measured concentrations in plasma are shown as solid dots, while open triangles denote the cumulative mass of PFOS eliminated in urine. Lines show the optimized fit of model simulations to measured values: solid line – plasma; dashed line – urine.

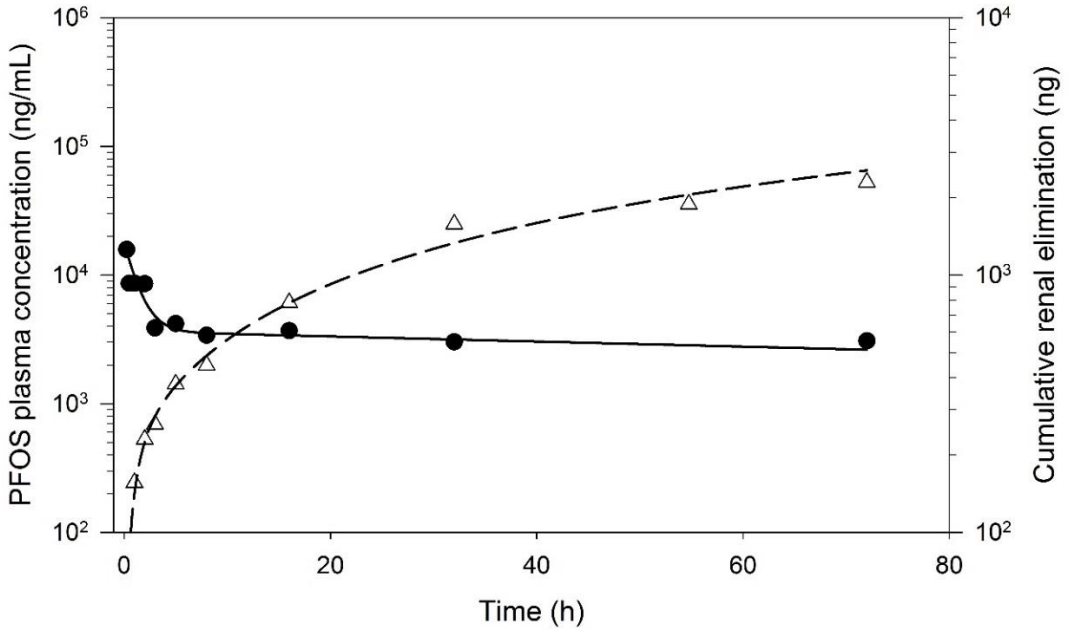

Figure S3-2. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 2. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$.

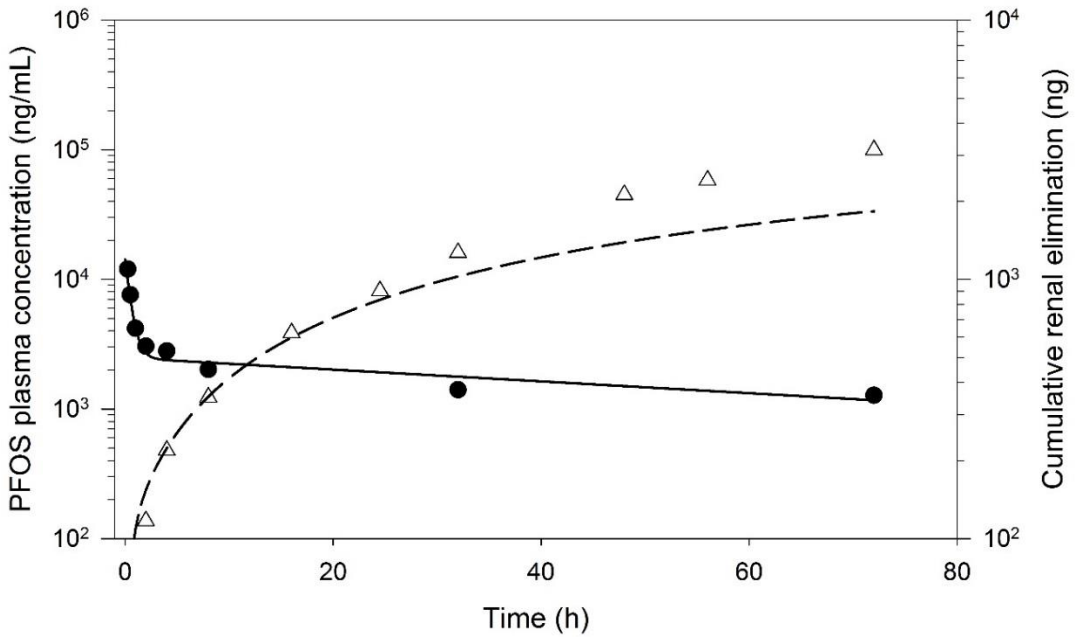

Figure S3-3. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 3. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$.

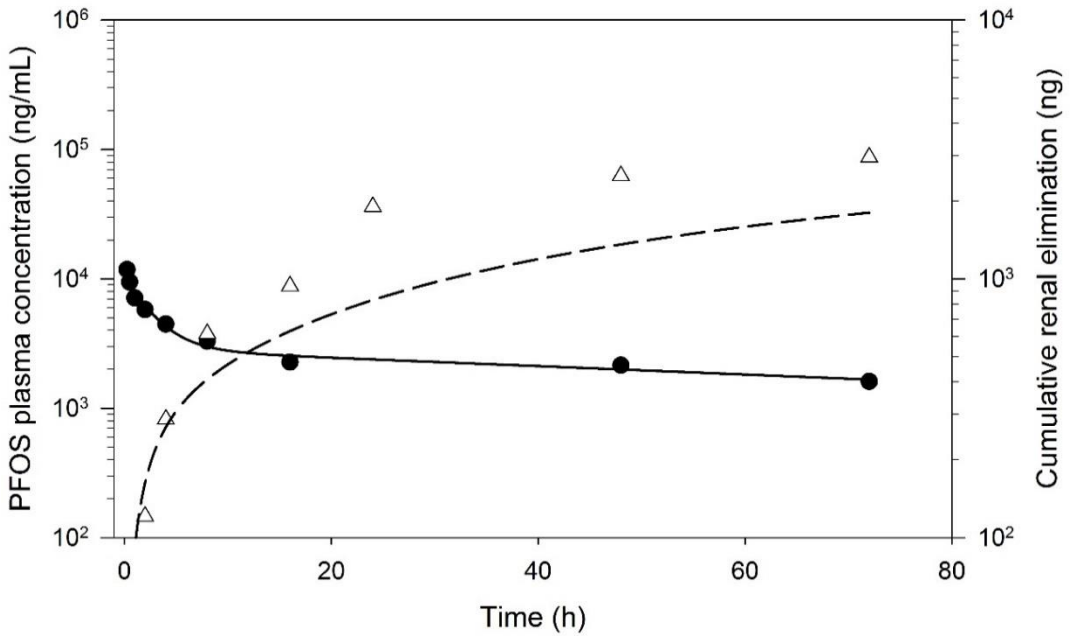

Figure S3-4. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 4. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$.

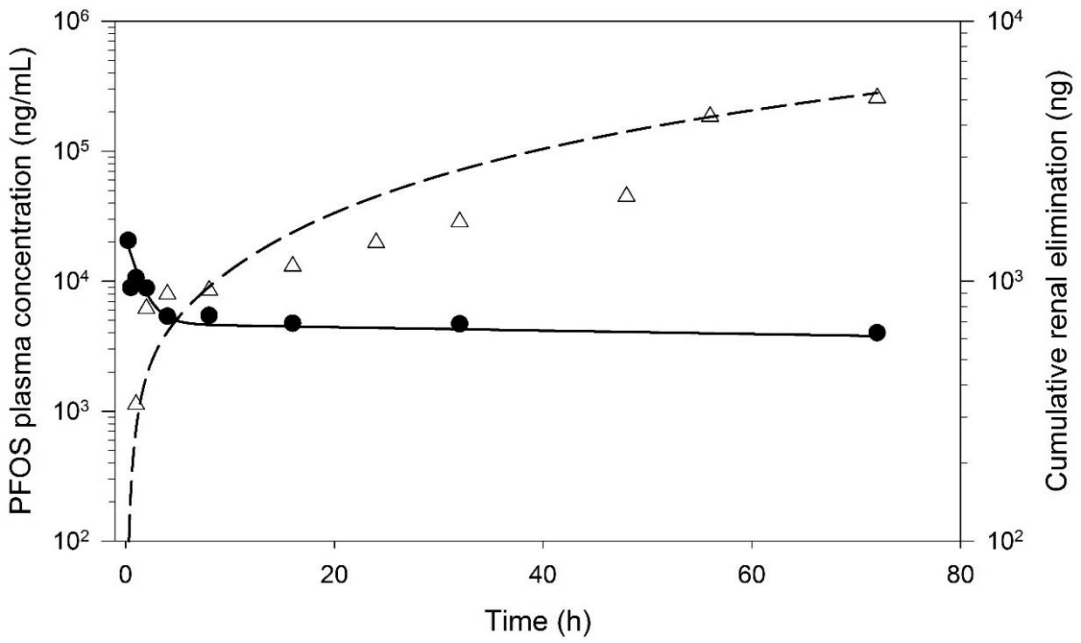

Figure S3-5. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 5. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$.

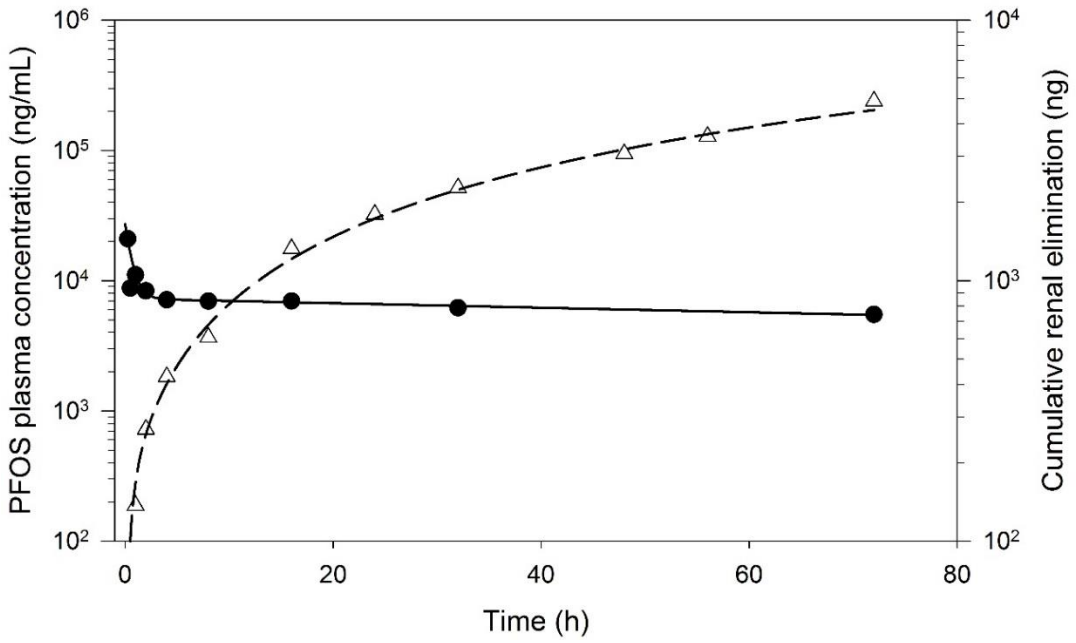

Figure S3-6. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 6. Simulations were obtained using a model that includes a fitted "other" elimination term $k_{4,1}$.

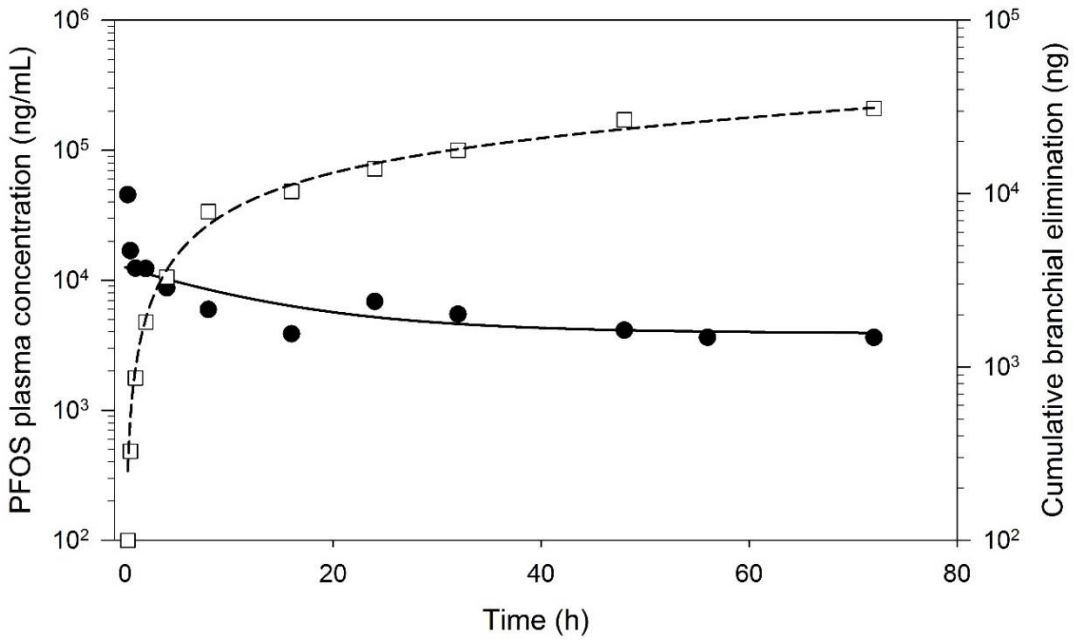

Figure S3-7. Kinetics of PFOS elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 8. In this and Figures S3-8—S3-11 measured concentrations in plasma are shown as solid dots, while open squares denote the cumulative mass of PFOS eliminated to expired water. Lines show the optimized fit of model simulations to measured values: solid line – plasma; dashed line – water.

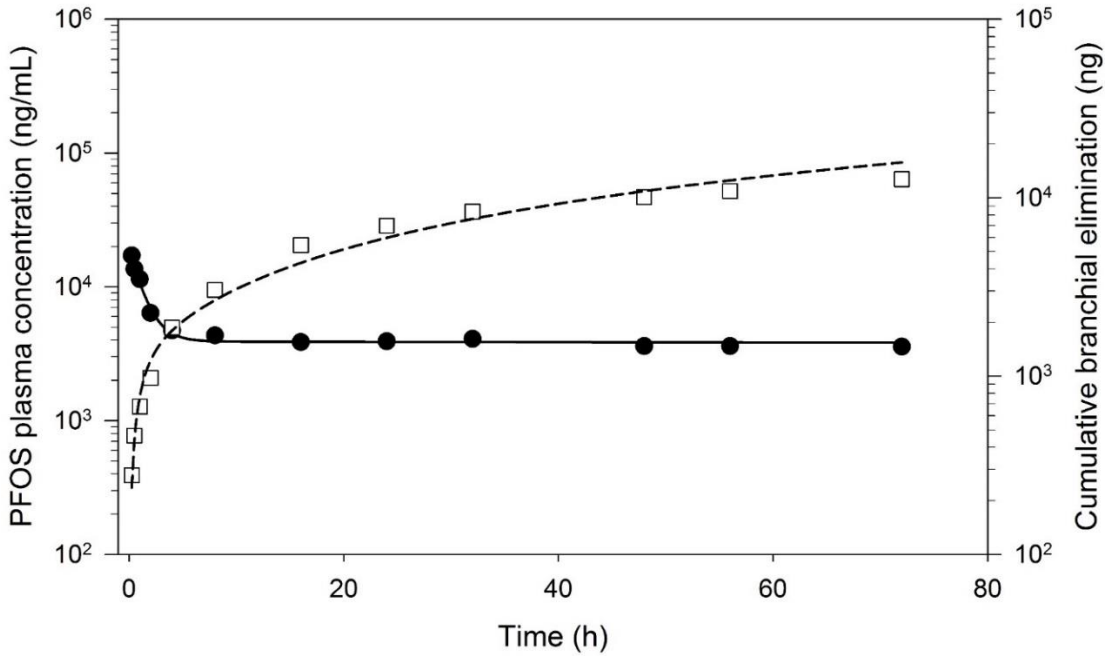

Figure S3-8. Kinetics of PFOS elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 9.

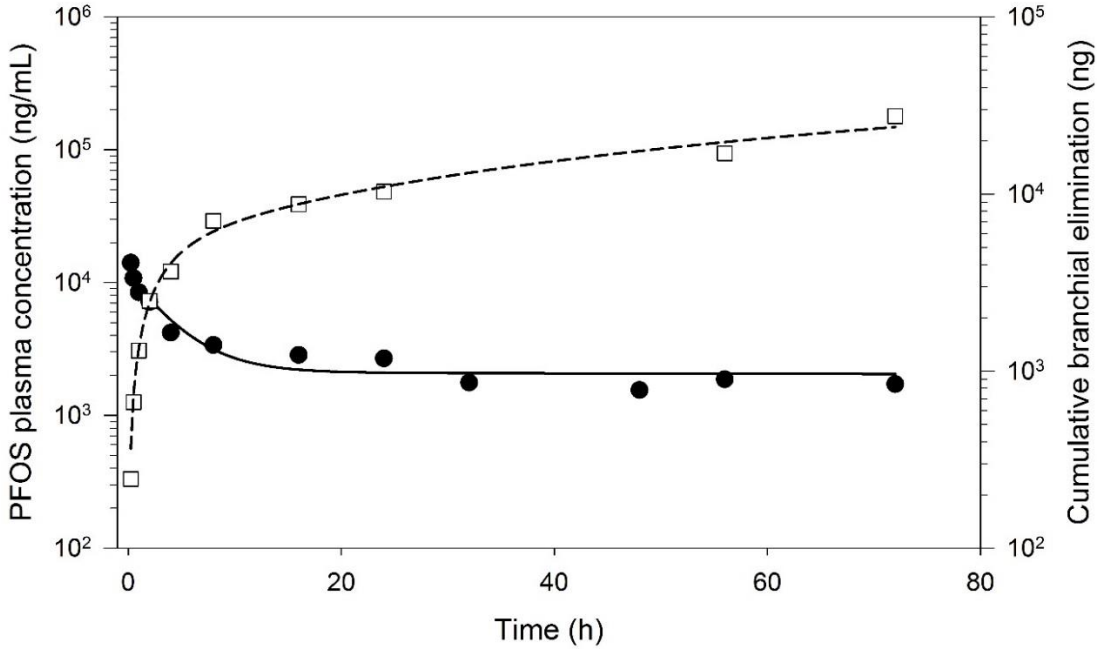

Figure S3-9. Kinetics of PFOS elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 10.

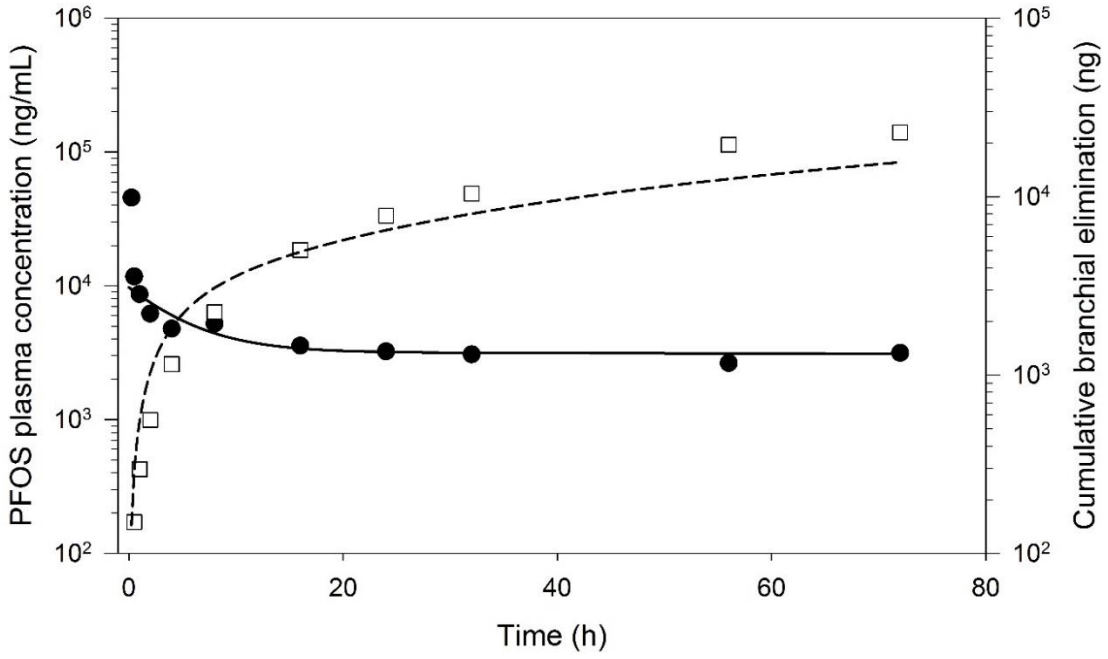

Figure S3-10. Kinetics of PFOS elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 11.

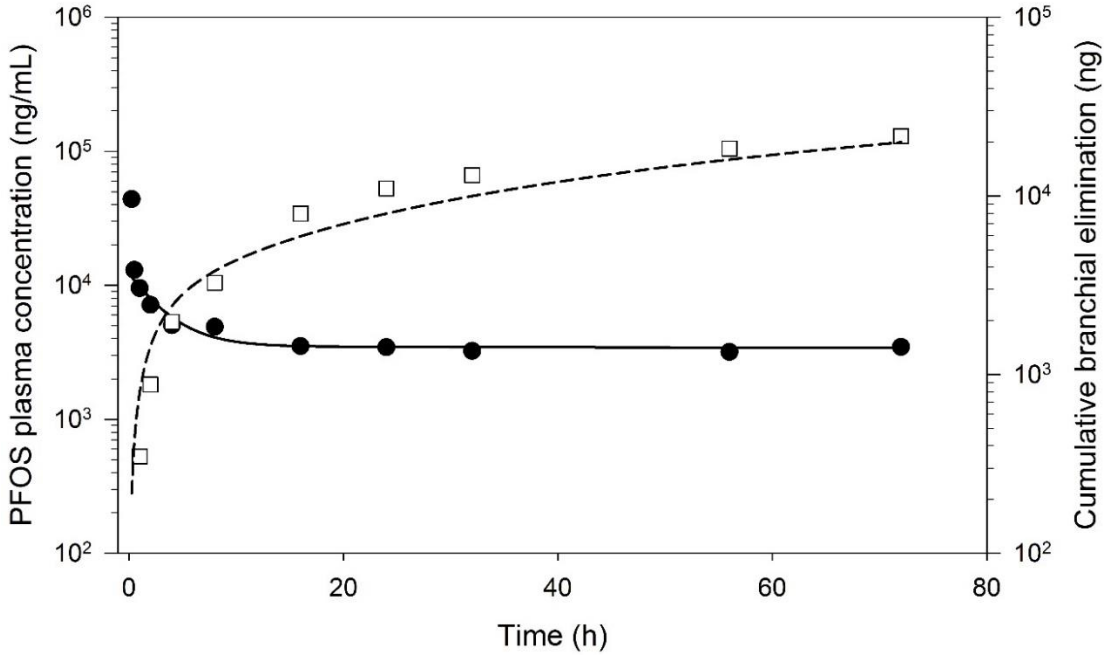

Figure S3-11. Kinetics of PFOS elimination to expired branchial water following bolus intra-arterial injection. Data and models simulations are shown for Fish 12.

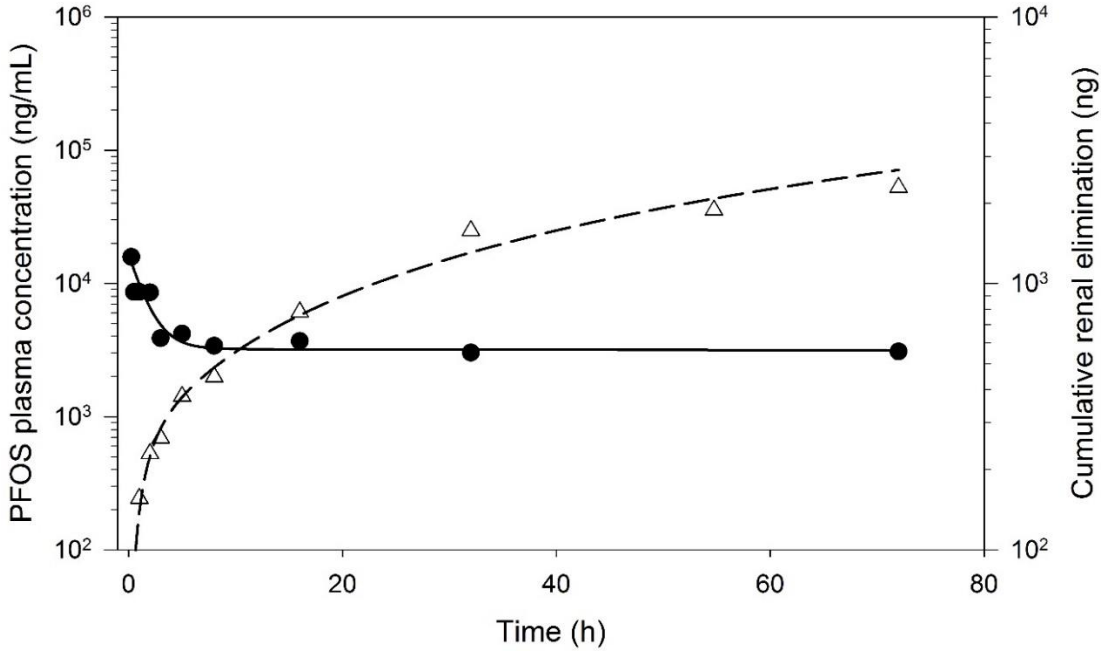

Figure S3-12. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 2. Simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as the elimination rate constant $k_{4,1}$. In this and Figures S13-S16 measured concentrations in plasma are shown as solid dots, while open triangles denote the cumulative mass of PFOS eliminated in urine. Lines show the optimized fit of model simulations to measured values: solid line – plasma; dashed line – urine.

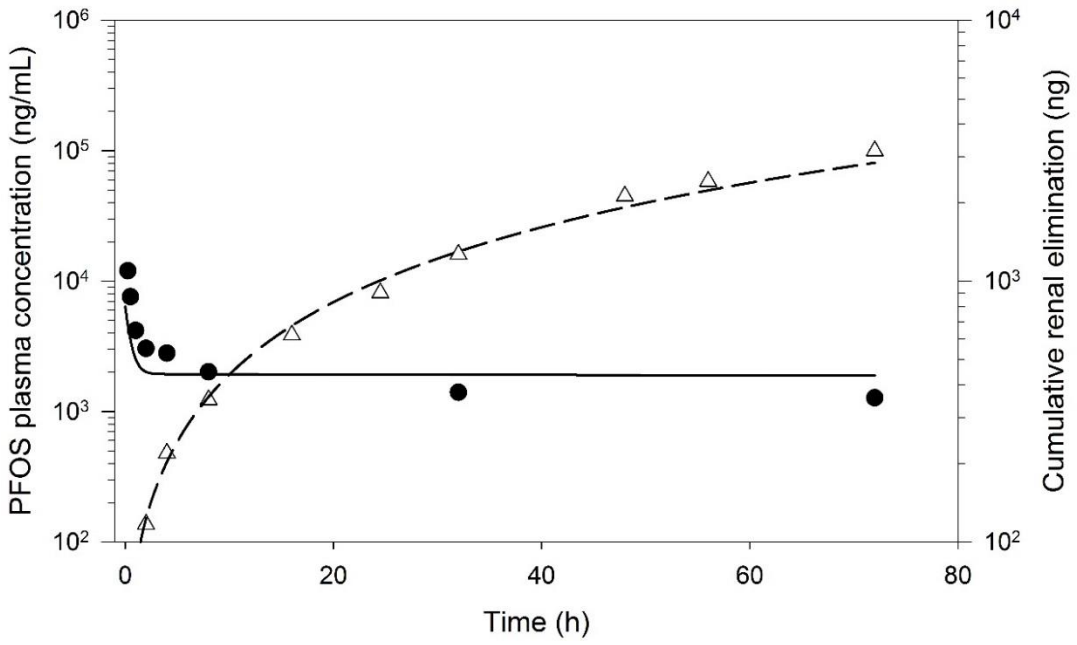

Figure S3-13. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 3. Simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as the elimination rate constant $k_{4,1}$.

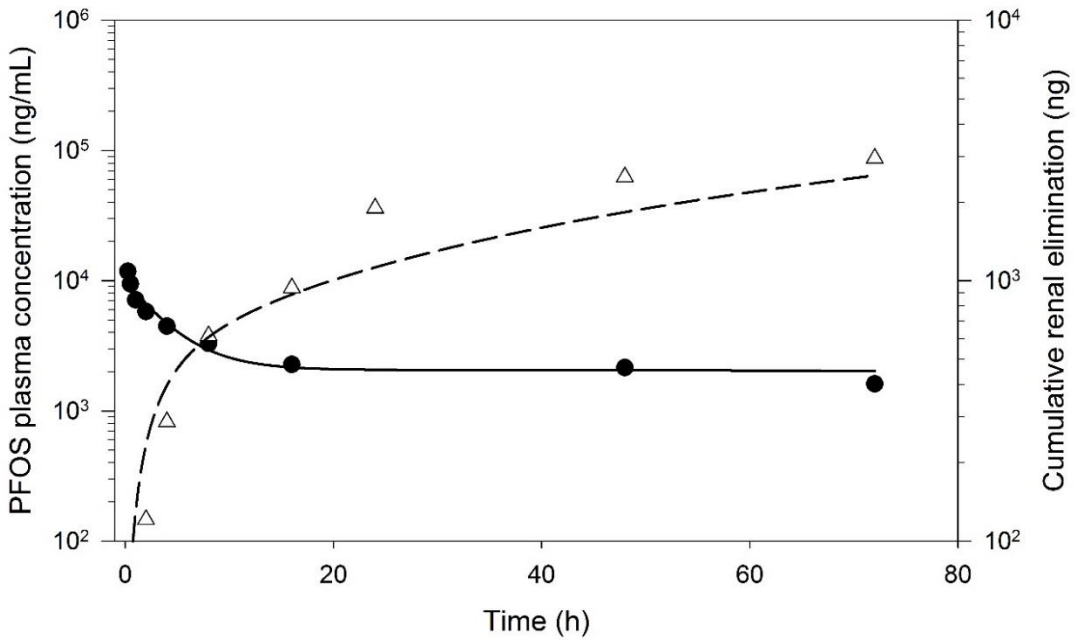

Figure S3-14. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 4. Simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as the elimination rate constant $k_{4,1}$.

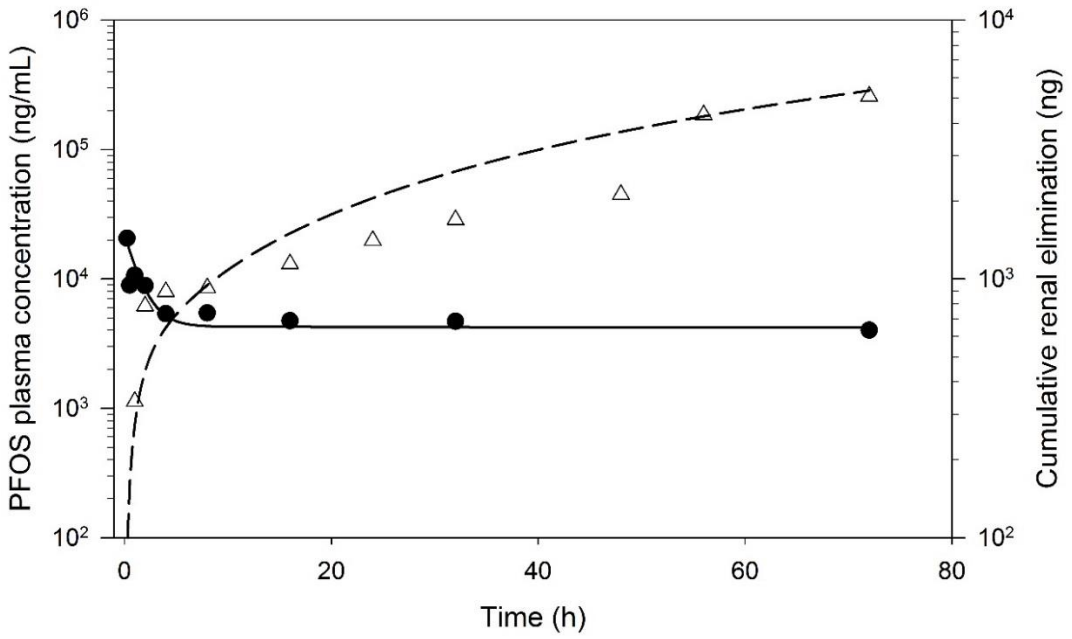

Figure S3-15. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 5. Simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as the elimination rate constant $k_{4,1}$.

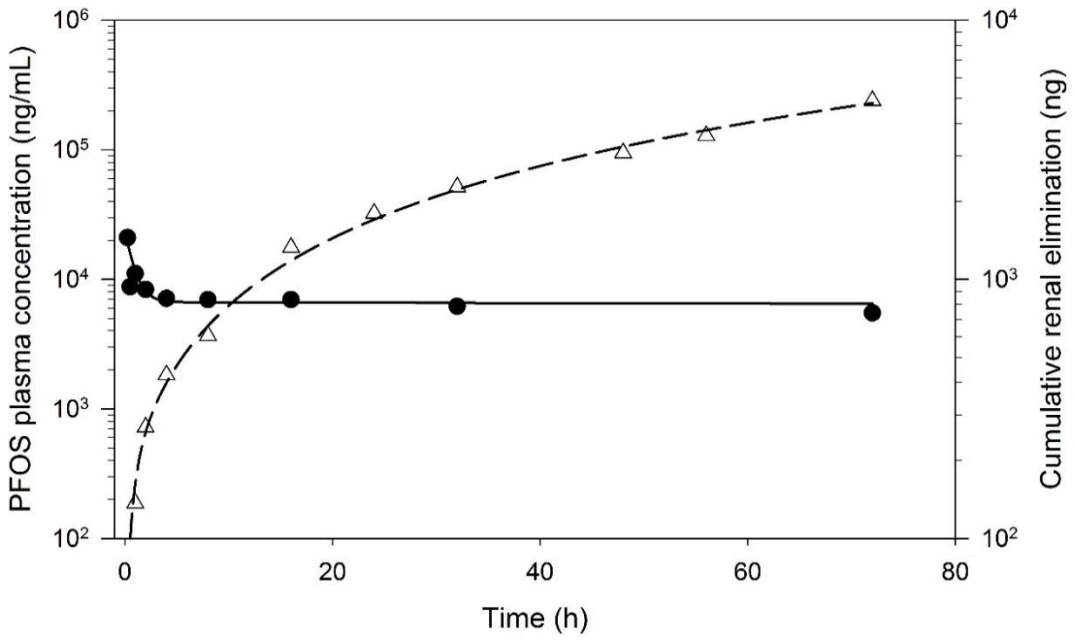

Figure S3-16. Kinetics of PFOS elimination to urine following a bolus intra-arterial injection. Data and model simulations are shown for Fish 6. Simulations were generated by adopting the average branchial elimination rate constant from branchial elimination studies as the elimination rate constant $k_{4,1}$.

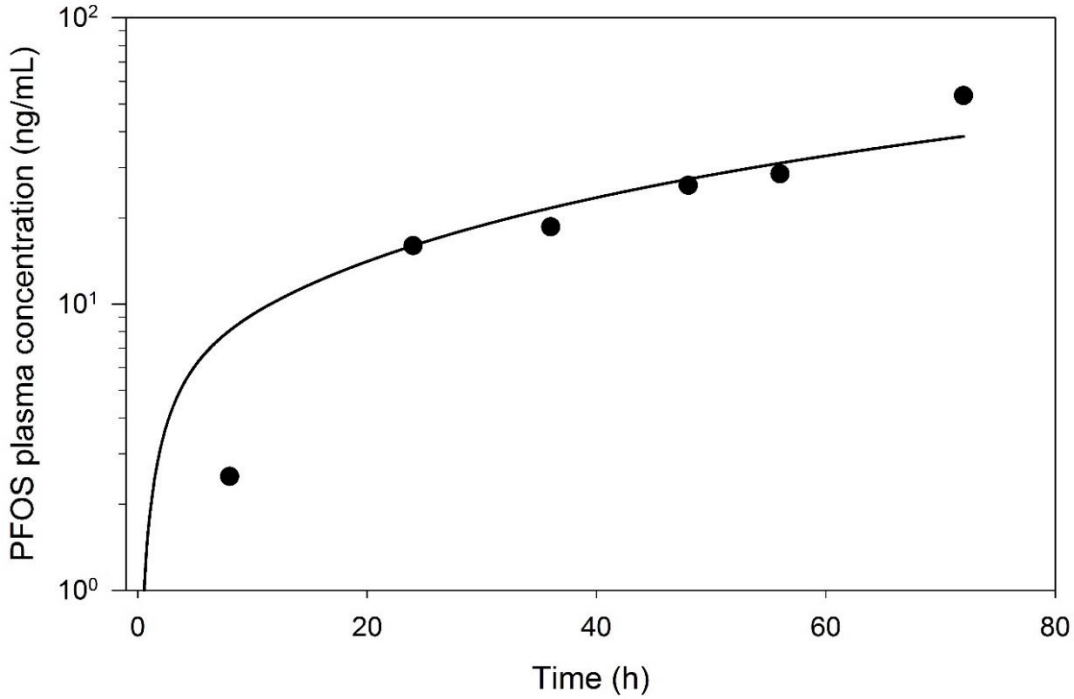

Figure S3-17. Kinetics of PFOS in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 14. In this and Figures S3-18—S3-21 measured values are shown as individual points. The fitted model simulation is shown as a solid line.

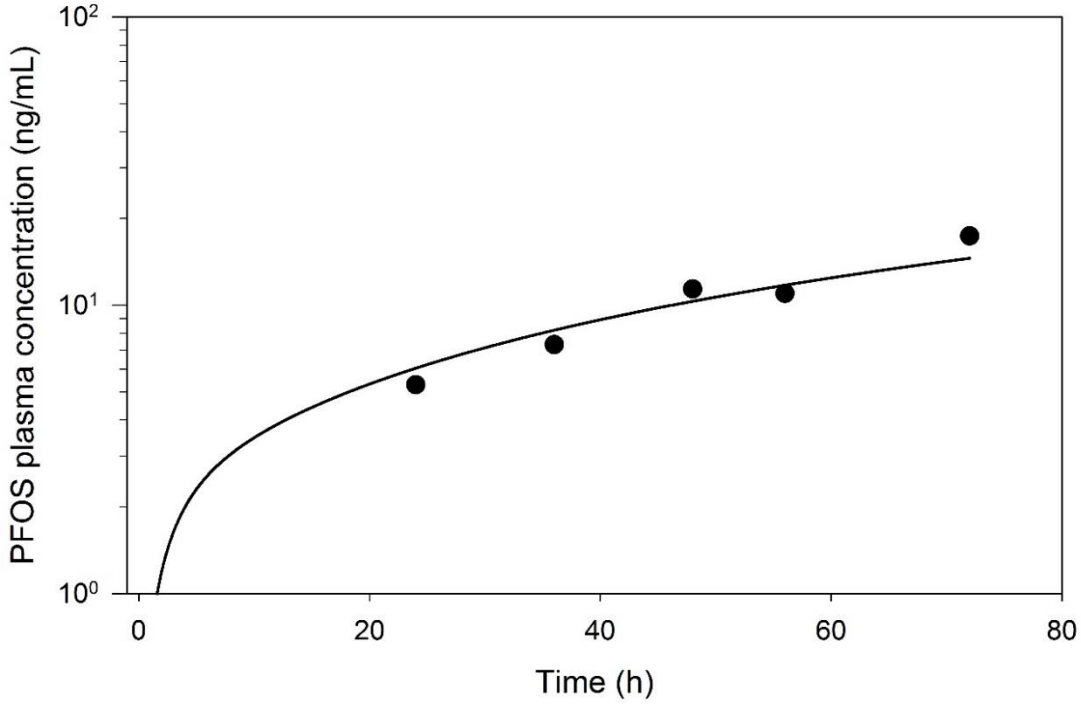

Figure S3-18. Kinetics of PFOS in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 15.

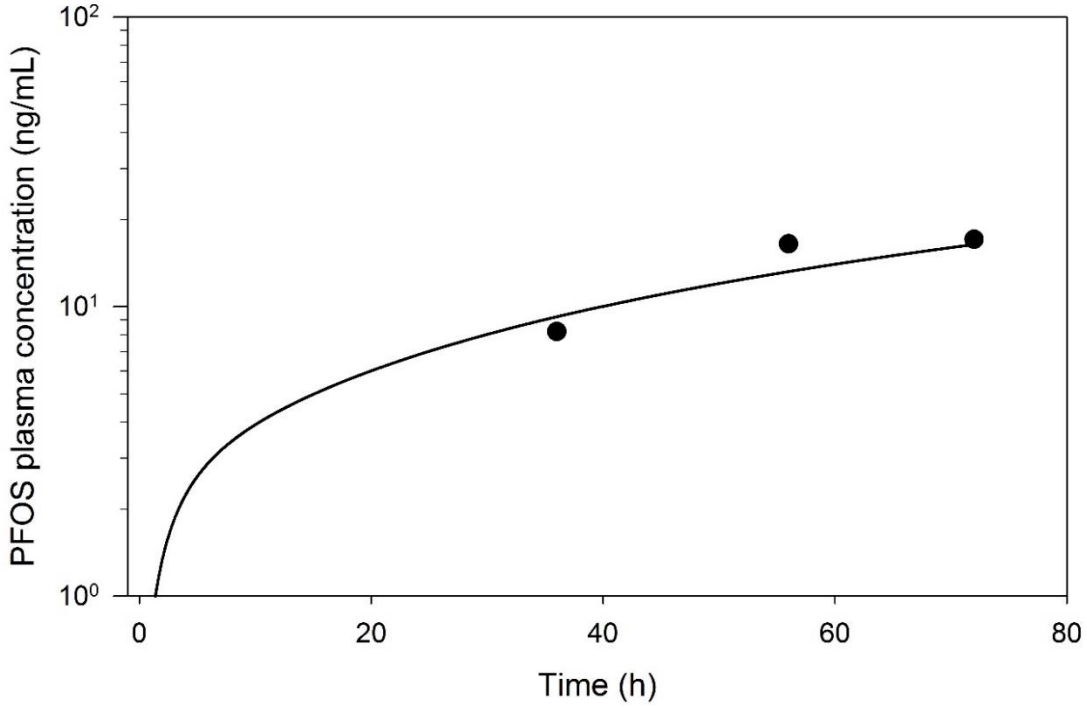

Figure S3-19. Kinetics of PFOS in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 16.

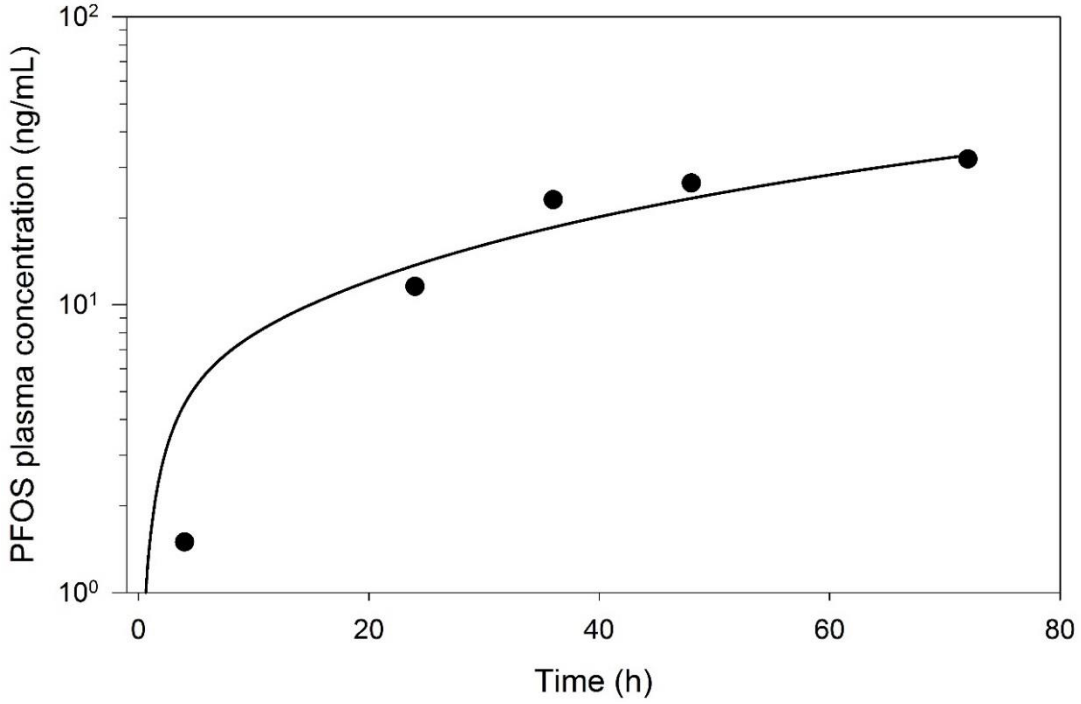

Figure S3-20. Kinetics of PFOS in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 17.

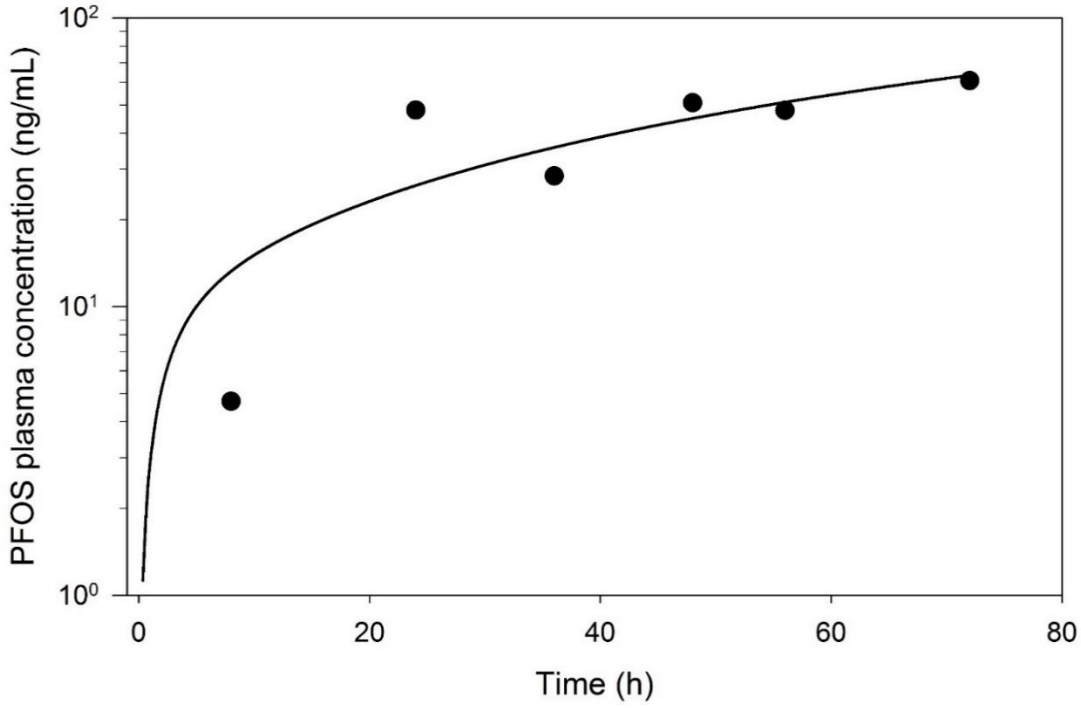

Figure S3-21. Kinetics of PFOS in trout plasma during a continuous waterborne exposure. Data and model simulations are shown for Fish 18.