Bioconcentration, elimination and effects of fire foam-related poly- and perfluoroalkyl substances in brown trout (*Salmo trutta*)

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Abstract

Poly- and perfluoroalkyl substances (PFASs) are anthropogenic and persistent chemicals used as surfactants in many applications, including aqueous film-forming foams (AFFFs, fire foams). Regular training exercises utilising AFFFs have led to the direct releases of PFASs into the environment and subsequently a global distribution of the compounds. PFAS contamination of soil and water has been reported around multiple Norwegian airports such as in the lake Lavangsvatnet near Harstad/Narvik Airport Evenes. This study aimed to quantify the bioconcentration, elimination and effects of selected fire foam-related PFASs on biomarkers for the induction of metabolic enzymes (ethoxyresorufin *O*-deethylase), oxidative damage (lipid peroxidation and peroxisomal acyl-CoA oxidase) and liver damage (alanine aminotransferase). Juvenile brown trout (*Salmo trutta*) were exposed through water to two levels of a PFAS mixture (PFOS, PFHxS, 6:2 FTS, PFPeA, PFHxA and PFOA) for three weeks. The "low" PFAS concentrations (10 µg/L total PFASs) were made to resemble the levels of contamination detected in Lavangsvatnet. The exposure period was followed by a depuration period of ten weeks. Only PFOS and PFHxS were detected in a large proportion of the trout sampled during exposure and depuration. Both the compounds bioconcentrated significantly in trout exposed to the "high" concentration (785 µg/L total PFASs); however, only PFHxS were significantly increasing during the exposure period in fish from the "low" treatment. Bioconcentration factors were calculated to be 244 and 185 L/kg for PFOS, and 6.34 and 11.2 L/kg for PFHxS in trout from the "high" and "low" treatments, respectively. PFOS did not appear to be eliminated following the ten weeks of depuration. The concentrations of PFHxS decreased significantly during the depuration period in trout from the "high" treatment, and the half-life of this compound was estimated to 2.3 weeks. Activity of the CYP1A enzyme (by the analysis of ethoxyresorufin *O*-deethylase activity) in gills was increased in trout exposed to the "low" concentration of PFASs ten weeks after terminated exposure. Neither biomarkers for oxidative damage nor liver damage were significantly affected in the trout. Differences in the sensitivity to PFOS and PFHxS have previously been documented between various species. Based on the results presented in this study, the PFAS contamination in Lavangsvatnet would not be expected to cause oxidative damage or liver damage in the population of trout inhabiting this lake. PFOS would, however, be expected to reside in the trout and induce the activity of CYP1A in gills a while after terminated exposure.

Abbreviations

6:2 FTS – 6:2 Fluorotelomer sulfonate

AFFF – Aqueous film-forming foam

ALAT – Alanine aminotransferase

ANOVA – Analysis of variance

AOX – Acyl-CoA oxidase

BCF – Bioconcentration factor

CI – Confidence interval

CYP1A – Cytochrome P450, family 1, subfamily A

DDT – p,p'-Dichlorodiphenyltrichloroethane

DL – Detection limit

DMSO – Dimethyl sulfoxide

EROD – Ethoxyresorufin *O*-deethylase

H_2O_2 – Hydrogen peroxide

HC – Hepes Cortland

HCB – Hexachlorobenzene

MDA – Malondialdehyde

OD – Optical density

PAH – Polycyclic aromatic hydrocarbon

PBDE – Polybrominated diphenyl ether

PC – Principal component

PCA – Principal component analysis

PCB – Polychlorinated biphenyl

PFBA – Perfluorobutanoic acid

PFBS – Perfluorobutane sulfonate

PFDA – Perfluorodecanoic acid

PFHpA – Perfluoroheptanoic acid

PFHxA – Perfluorohexanoic acid

PFHxS – Perfluorohexane sulfonate

PFNA – Perfluorononanoic acid

PFOA – Perfluorooctanoic acid

PFOS – Perfluorooctane sulfonate

PFPeA – Perfluoropentanoic acid

POP – Persistent organic pollutant

SD – Standard deviation

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TCA – Trichloroacetic acid

TCDD – 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

β-NADH – β-nicotinamide adenine dinucleotide

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1. Introduction

Originally, the definition of persistent organic pollutants (POPs) was limited to persistent and lipophilic compounds (Jones and de Voogt 1999). Some of the newer POPs are, however, less lipophilic and included in the POP-definition due to their global distribution, high resistance against degradation and their potential to cause deleterious effects in humans, animals and the environment. In recent years, the attention has gradually shifted from the originally defined POP chemicals (e.g. PCBs, DDTs, HCBs, PBDEs, etc.) to the fluorinated organic compounds (Muir and de Wit 2010, Ding and Peijnenburg 2013). In 2009 the Stockholm Convention on Persistent Organic Pollutants was expanded to include newer POPs such as perfluorooctane sulfonate, a well-known and widely used poly- and perfluoroalkyl substance (UNEP 2009, Muir and de Wit 2010).

Once introduced in the environment, the distribution and fate of a compound is determined by its physiochemical properties, which also affects the bioavailability of the compound to biota (Ding and Peijnenburg 2013). The aquatic environment is known to be a reservoir for most POPs (including poly- and perfluoroalkyl substances) (Paul *et al.* 2009), and gill-breathing organisms are at risk of being highly exposed to chemicals present in the water. Direct uptake and accumulation of chemicals from water is known as bioconcentration (Barron 1990, Arnot and Gobas 2006). This may yield pronounced toxicological effects in the organism or its offspring; however, bioconcentration without effects should still be regarded as a hazard in the risk assessment process (van der Oost *et al.* 2003). In order to assess the risk factors related to the use of legacy and newly included persistent chemicals, it is important to assess toxicant effects and apply environmentally relevant model organisms, compounds and concentrations. To the author's knowledge, this is the first study to conduct a controlled laboratory experiment assessing the bioconcentration and effects in brown trout (*Salmo trutta*) utilising an environmentally relevant mixture of PFASs.

1.1 Poly- and perfluoroalkyl substances

The family of poly- and perfluoroalkyl substances (PFASs) comprises a large number of anthropogenic POPs (Giesy and Kannan 2002). PFASs have been produced since the 50's, and have at least one (polyfluoroalkyl) or all (perfluoroalkyl) carbon-fluorine (C-F) bonds in

the alkyl chain (Buck *et al.* 2011). Despite their long time of usage, they were not recognized as global environmental pollutants until their presence was reported in wildlife (Giesy and Kannan 2001) and humans (Kannan *et al.* 2004) early in the 21st century.

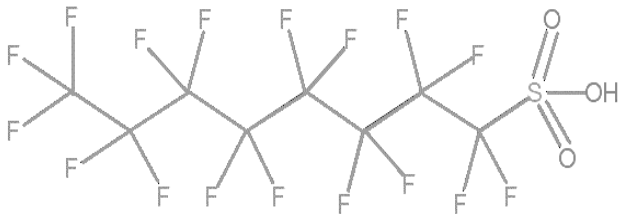
PFASs are amphipathic, and the strong C-F bonds result in a uniquely high chemical and thermal stability (Kissa 2001, Buck *et al.* 2012). The production of PFASs is either conducted by electrochemical fluorination or telomerisation yielding both linear and branched fluorocarbon arrangements (De Silva *et al.* 2009, Buck *et al.* 2011, Buck *et al.* 2012). PFASs are utilised in fire-fighting foams, water-, soil- and stain-resistant coatings, electronics, floor polishers, photographic films, ski waxes and as insecticides (Renner 2001, Lau *et al.* 2004, Buck *et al.* 2012), and they enter the environment through direct emission (the usage of products containing PFASs) or indirectly by degradation from other compounds (Prevedouros *et al.* 2006, Buck *et al.* 2011). The unique persistence has resulted in that PFASs have become global environmental problems (Kissa 2001, Ding and Peijnenburg 2013).

Unlike the lipophilic POPs, PFASs predominantly bind to proteins (Han *et al.* 2003, Jones *et al.* 2003, Qin *et al.* 2010) and consequently accumulate in blood and blood-rich tissues such as the liver and kidneys (Martin *et al.* 2003a, Martin *et al.* 2003b, Goeritz *et al.* 2013, Falk *et al.* 2015). This binding can possibly interfere with the affinity of endogenous ligands to proteins (Luebker *et al.* 2002) or induce changes in the protein structure (Qin *et al.* 2010). Sulfonated PFASs have been shown to be more rapidly taken up and more slowly eliminated than PFASs with carboxylate functional groups of the same C-F chain length (Martin *et al.* 2003a, Conder *et al.* 2008, Inoue *et al.* 2012, Falk *et al.* 2015). Furthermore, the uptake of carboxylated PFASs have been shown to be positively correlated with the fluoroalkyl chain length until 13 or more fluorinated carbons (Martin *et al.* 2003a, Labadie and Chevreuil 2011).

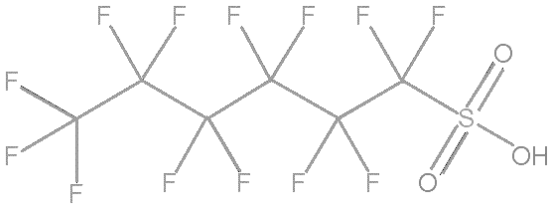
Perfluorooctane sulfonate (PFOS) is one of the most commonly studied PFASs. It has a global distribution (Giesy and Kannan 2001), does not decompose or become biodegraded by microorganisms (Remde and Debus 1996, Key *et al.* 1998) and has the ability to up-concentrate in organisms (Conder *et al.* 2008). Before restrictions were implemented, PFOS was the main fluorochemical surfactant in aqueous film-forming foam (AFFF) formulations (Hagenaars *et al.* 2011, Buck *et al.* 2012). High performance surfactants in AFFFs are mixtures of fluorochemical and hydrocarbon surfactants dissolved in water, and the surfactants containing fluorine are key compounds providing the low surface tension of the

foams (Pabon and Corpart 2002, Buck *et al.* 2011, Buck *et al.* 2012). AFFFs are applied on industrial and petroleum fires (Prevedouros *et al.* 2006, Kishi and Arai 2008), and regular training exercises have led to direct releases of the AFFFs to soil and water and a subsequently widespread environmental input of PFASs (Moody and Field 2000, Moody *et al.* 2002, Moody *et al.* 2003, Hagenaars *et al.* 2011).

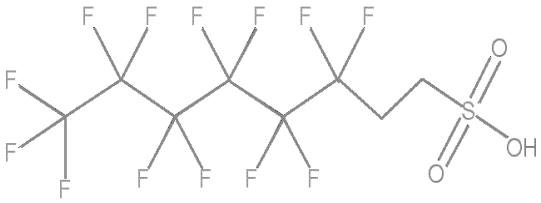
The use of AFFF containing PFASs was terminated in 2012 at the majority of Norwegian airports, but high concentrations are still found at, and around, training platforms for fire-fighting (Sweco 2012, Norconsult and Sweco 2015). At Harstad/Narvik Airport Evenes in northern Norway, leachate from a fire-training platform has led to the accumulation of PFASs in a nearby swamp area draining into a lake, Lavangsvatnet (Cowi and Sweco 2012, Norconsult and Sweco 2015). Substances used in this study (Figure 1) were chosen to resemble PFASs detected in water from this lake (sampled at the outlet of a small stream draining the swamp area).



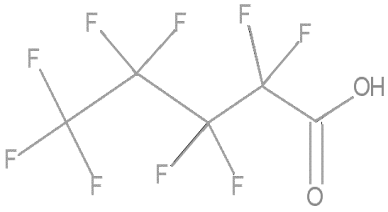
Perfluorooctane sulfonate (PFOS)



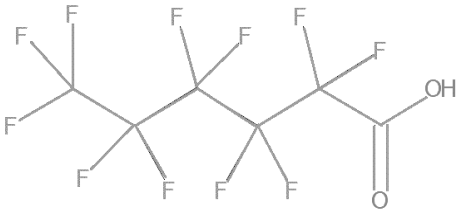
Perfluorohexane sulfonate (PFHxS)



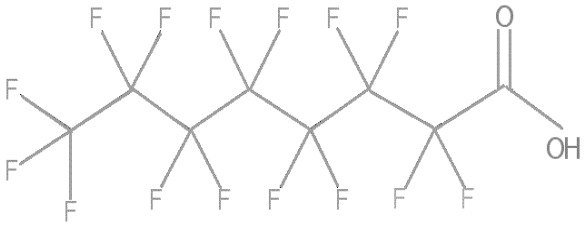
6:2 Fluorotelomer sulfonate (6:2 FTS)



Perfluoropentanoic acid (PFPeA)



Perfluorohaxanoic acid (PFHxA)



Perfluorootanoic acid (PFOA)

Figure 1. Selected poly- and perfluoroalkyl substances.

1.2 Effects of PFASs

Several laboratory tests have linked PFASs to changes and toxic effects in the liver (Hoff *et al.* 2003a, Du *et al.* 2009, Fair *et al.* 2013), oxidative damage (Berthiaume and Wallace 2002, Arukwe and Mortensen 2011, Liu *et al.* 2014a) and induction of various metabolic enzymes (Liu *et al.* 2007, Han *et al.* 2012, Liu *et al.* 2014b). In addition, some PFASs are thought to be tumour promoters (Benninghoff *et al.* 2012, Klaunig *et al.* 2012) and exhibit developmental (Lau *et al.* 2004, Shi *et al.* 2008), immunotoxic (DeWitt *et al.* 2012, Han *et al.* 2012, Fair *et al.* 2013) and endocrine disrupting responses (Jensen and Leffers 2008, Benninghoff *et al.* 2011, Han *et al.* 2011).

The effects chosen to be investigated in this study (the induction of metabolic enzymes, oxidative damage responses and liver damage) are briefly described below. A biomarker is defined as an alteration in a biological process (from molecular to behavioural) indicating a deviation from the normal status (van der Oost *et al.* 2003). It gives a measurement of exposure and possible toxic effects, rather than mere quantification of the pollutant level, and is important in environmental assessment (Peakall and Walker 1994). In the newly released Madrid Statement on poly- and perfluoroalkyl substances scientists are urged to continue monitoring and investigating PFAS in the environment through the use of biomarkers and analytical methods (Blum *et al.* 2015). The biomarkers used in this study were selected to investigate the effects of a PFAS mixture simulating the level of contamination in Lavangsvatnet from the use of AFFFs at Harstad/Narvik Airport Evenes.

1.2.1 Induction of metabolic enzymes

The analysis of CYP1A enzyme activity, i.e. the activity of ethoxyresorufin *O*-deethylase (EROD), is a well-known indicator for the presence of aryl hydrocarbon receptor agonists (e.g. TCDD, PCBs and PAHs) in fish (Goksøyr and Förlin 1992, Jönsson *et al.* 2003). Although these agonists not necessarily are directly toxic they may be biotransformed to more toxic metabolites in the organism (Whyte *et al.* 2000). In addition, activation of the aryl hydrocarbon receptor may cause oxidative stress (see section 1.2.2) or create reactive oxygen species in the exposed cells (Dalton *et al.* 2002). EROD activity describes the rate of deethylation of 7-ethoxyresorufin, which is mediated by the CYP1A enzyme in the cytochrome P450 monooxygenase enzyme family. This results in the product resorufin, and

the activity of EROD is consequently an indicator for the relative amount of CYP1A in a sample (Whyte *et al.* 2000, van der Oost *et al.* 2003).

EROD activity is commonly measured in the liver, because of its high metabolic capacity and function (Whyte *et al.* 2000); however, measurements of EROD in gills have been shown to be a sensitive biomarker of exposure to waterborne CYP1A inducers (Abrahamson 2007). PFASs are not known as aryl hydrocarbon receptor agonists, but it has been shown that the combined exposure of cells to PFOS and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increases the EROD activity by up to 40% compared to when the cells were only exposed to TCDD (Hu *et al.* 2003). Knowledge on the effect of PFASs on EROD activity in gills is limited. It is, thus, of interest to investigate the changes in EROD after exposure to environmentally relevant mixtures and concentrations of PFASs.

1.2.2 Oxidative damage responses

Oxidative stress occur when there is an overproduction and imbalance between pro-oxidants (e.g. reactive oxygen species) and antioxidant defence systems. This can have diverse deleterious effects in a cell such as membrane damage and enzyme dysfunction (Newman and Clements 2008 p. 31). Thiobarbituric acid reactive substances (TBARS), including lipid peroxides and aldehydes (e.g. malondialdehyde (MDA)), increase in concentration due to oxidative stress to unsaturated fatty acids (lipids) (Botsoglou *et al.* 1994). The measurement of MDA through the TBARS method is a common technique for detecting oxidative damage in tissues and biological systems (Valenzuela 1991, Halliwell and Chirico 1993, Dawn-Linsley *et al.* 2005).

PFOS has been shown to cause oxidative stress in fish; however, the amount of lipid peroxidation (TBARS) seems to differ between various species (Oakes *et al.* 2005, Shi and Zhou 2010, Arukwe and Mortensen 2011, Feng *et al.* 2015). As with EROD activity, TBARS is usually measured in the liver, and to the author's knowledge only one previous study has investigated the effect of PFASs on lipid peroxidation in the gills of fish (Munyemera 2014). Thus, it is of interest to assess how TBARS in gills are affected when exposed to PFASs. In addition, it is of importance to investigate the possible lipid peroxidation of hepatic tissues and subsequently elevated TBARS levels after exposure to a mixture of PFASs simulating contamination from the use of AFFFs.

Another oxidative damage response is the proliferation of peroxisomes in cells. This implies an increase in volume and number of peroxisomes (single membrane-bound organelles in cells), and is usually followed by an induction of various enzymes such as peroxisomal acyl-CoA oxidase (AOX) (Cajaraville *et al.* 2003). AOX is the first rate-limiting enzyme in the β-oxidation pathway and oxidizes fatty acids accompanied by the release of H_2O_2 (a reactive oxygen species) (Lock *et al.* 1989, Cancio and Cajaraville 2000). The induction of AOX is used as an indicator for the exposure to peroxisome proliferators (Cajaraville *et al.* 2003), and is conducted by detecting the H_2O_2-dependent oxidation of leuco-dichlorofluorescein (in this study: 2'7'-dichlorofluorescein) by exogenous peroxidases in a sample (Small *et al.* 1985).

It has been shown that PFASs act as peroxisome proliferators and to increase the activity of hepatic AOX in rats (Berthiaume and Wallace 2002, Elcombe *et al.* 2012), but with lower levels of exposure the induction have been shown to be small or non-significant (Seacat *et al.* 2003, Elcombe *et al.* 2012). Notable species differences have been reported in the hepatic AOX activity of fish after exposure to PFASs (Hoff *et al.* 2003b, Oakes *et al.* 2005, Bilbao *et al.* 2010, Oakes *et al.* 2010). Thus, it is of interest to investigate how AOX activity in brown trout is affected when exposed to environmentally relevant concentrations of PFAS.

1.2.3 Liver damage

Increased enzyme activities (e.g. of aminotransferases) in plasma are commonly used as biomarkers for tissue damage (van der Oost *et al.* 2003), and frequently applied as a diagnostic tool in human medicine (Reichling and Kaplan 1988, Kim *et al.* 2008). The enzyme alanine aminotransferase (ALAT) is considered to be specific for detecting damage and diseases in the liver (van der Oost *et al.* 2003). However, the use of ALAT as an indicator of liver toxicity can be problematic due to the potential inhibition of enzyme synthesis or activity, or failure to detect hepatic damage when the majority of the cells have undergone necrosis (Reichling and Kaplan 1988, Bucher and Hofer 1990).

ALAT (and the cofactor pyridoxal-5'-phosphate) catalyses the transfer of an amino group from the amino acid alanine to α-ketoglutaric acid resulting in the production of pyruvate and L-glutamate (Reichling and Kaplan 1988). Results from field studies have shown that PFOS correlate significantly with the ALAT activity in plasma of fish and dolphins (Hoff *et al.* 2003b, Hoff *et al.* 2005, Fair *et al.* 2013). The effects of PFOS on ALAT have also been

shown in laboratory studies (Seacat *et al.* 2003, Hoff *et al.* 2003a); however an increased activity of ALAT does not occur in experiments conducted on monkeys or in some studies using rats (Seacat *et al.* 2002, Elcombe *et al.* 2012). To the author's knowledge, no detailed method for measuring ALAT in small volumes of plasma has been published. In this study, a modification of the method described by Bergmeyer *et al.* (1986) is described and applied to plasma of brown trout exposed to a mixture of PFASs.

1.3 Brown trout as a model organism in aquatic toxicology

Brown trout (*Salmo trutta*) is a highly relevant study organism due to its wide geographical distribution and it being indigenous to freshwater lakes all over Europe, including Norway (Frost and Brown 1967 p. 55, Kottelat and Freyhof 2007 p. 409, Filipe *et al.* 2013) . Trout has been targeted in sport fishing from as early as the mid-1400, and rearing of trout in captivity before their release into the wild is used for its recreational value (Frost and Brown 1967 p. 15).

Leaching of PFASs from contaminated fire-training areas have been shown to occur in Lavangsvatnet close to Harstad/Narvik Airport Evenes (Sweco 2012), which has a natural population of brown trout. The PFASs can bioconcentrate in these trout (as well as in additional fish populations in Lavangsvatnet or in other PFAS polluted lakes) and possibly lead to effects even after the input and exposure to PFASs have been terminated. Such effects are usually more pronounced in the sensitive life-stages of organisms (e.g. the juvenile stage). The brown trout in Lavangsvatnet are also a source of food for locals indicating a possible route of human exposure to PFASs.

1.4 Objectives

The aim of this study was to quantify the bioconcentration, elimination and effects of a mixture of fire foam-related PFASs in juvenile brown trout (*Salmo trutta*). Effects were assessed by investigating the induction of metabolic enzymes, oxidative damage responses and liver damage.

Five null-hypotheses (H_0) were formulated within two objectives:

Objective one: quantify the bioconcentration and elimination of PFASs in fish.

1. There was no significant bioconcentration of the selected PFASs in trout.
2. There was no significant elimination of the selected PFASs from trout.

Objective two: quantify the effects of two PFAS concentrations on selected biomarkers during and after ended exposure.

3. There was no significant response in the induction of metabolic enzymes in gills.
4. There was no significant effect on oxidative damage responses in gills and liver.
5. There was no significant liver damage.

The objectives were addressed using experimental exposure to a mixture of PFOS, perfluorohexane sulfonate (PFHxS), 6:2 fluorotelomer sulfonate (6:2 FTS), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA) and PFOA chosen to resemble the PFAS contamination in Lavangsvatnet near Harstad/Narvik Airport Evenes. A parallel study by Stine Hellstad (Hellstad 2015) investigated the effects on gene expression in gills and liver from the same trout exposed to the fire foam-related PFAS mixture. Those results can be compared with the responses in biomarkers reported here and are discussed below.

2. Materials and methods

2.1 Chemicals

Perfluorooctane sulfonate (PFOS), perfluorohexane sulfonate (PFHxS), 6:2 fluorotelomer sulfonate (6:2 FTS), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA) and perfluorooctanoic acid (PFOA) were purchased from Chiron. All other chemicals were purchased from Sigma-Aldrich if not otherwise stated. Eppendorf tubes (1.5 and 0.5 mL), cryo-tubes (2 mL) and 96-well microtitre blank, sterile plates (Nunclon^{TM}) were purchased from VWR. A Synergy MX (BioTek) multi-well plate reader with Gen5 software was used for all measurements of absorbance and fluorescence.

2.2 Experimental design

2.2.1 Brown trout

Juvenile brown trout (*Salmo trutta*) of the same age and weighing 7-15 grams were purchased from Bjørkelangen Settefiskanlegg (Aurskog, Norway) mid-November 2013. The fish were maintained in flow-through freshwater tanks (water temperature 8.8°C ± 0.8°C (SD)) at the University of Oslo (UiO), Kristine Bonnevies hus, Blindern until mid-January 2014. All fish were placed in aquaria for acclimatization on the same day, 11 and 12 days prior to week zero sampling (reference), and 18 and 19 days prior of PFAS exposure initiation. The photoperiod was set to 12:12 hours light:dark. Fish were fed daily with pellets (Tetrapond Pond Sticks) before acclimatization and twice a week with boiled shrimp (0.6 mg/kg/fish, purchased frozen at REMA 1000 Oslo) during the acclimatization and the experimental period.

2.2.2 Experimental setup

The experiment was conducted in Kristine Bonnevies hus, UiO. Three main header tanks received filtered tap water delivered from Oset water treatment facility (water from Maridalsvannet). Water was distributed randomly by gravity from the header tanks through individual tubes (18x22 mm PVC, VWR) and to the 15-L all-glass aquaria (flow 53 ± 5.9 mL/min (SD)). Excess water was drained through a cut-out at the top of each aquarium

(keeping a constant water level), and collected in two water baths surrounding the aquaria. Subsequently, the collected water was filtrated through an activated carbon filter (VWR) to remove the remaining PFASs before discharge. Transparent hard plastic plates were placed on top of the aquaria, and the cut-out was covered by plastic netting to prevent fish from escaping. Each aquarium was isolated with black hard plastic plates, and equipped with individual air bubbling systems (Schego airpump prima). A total of 150 brown trout were randomly distributed among the 15 aquaria, each receiving ten fish.

The exposure setup is illustrated in Figure 2.

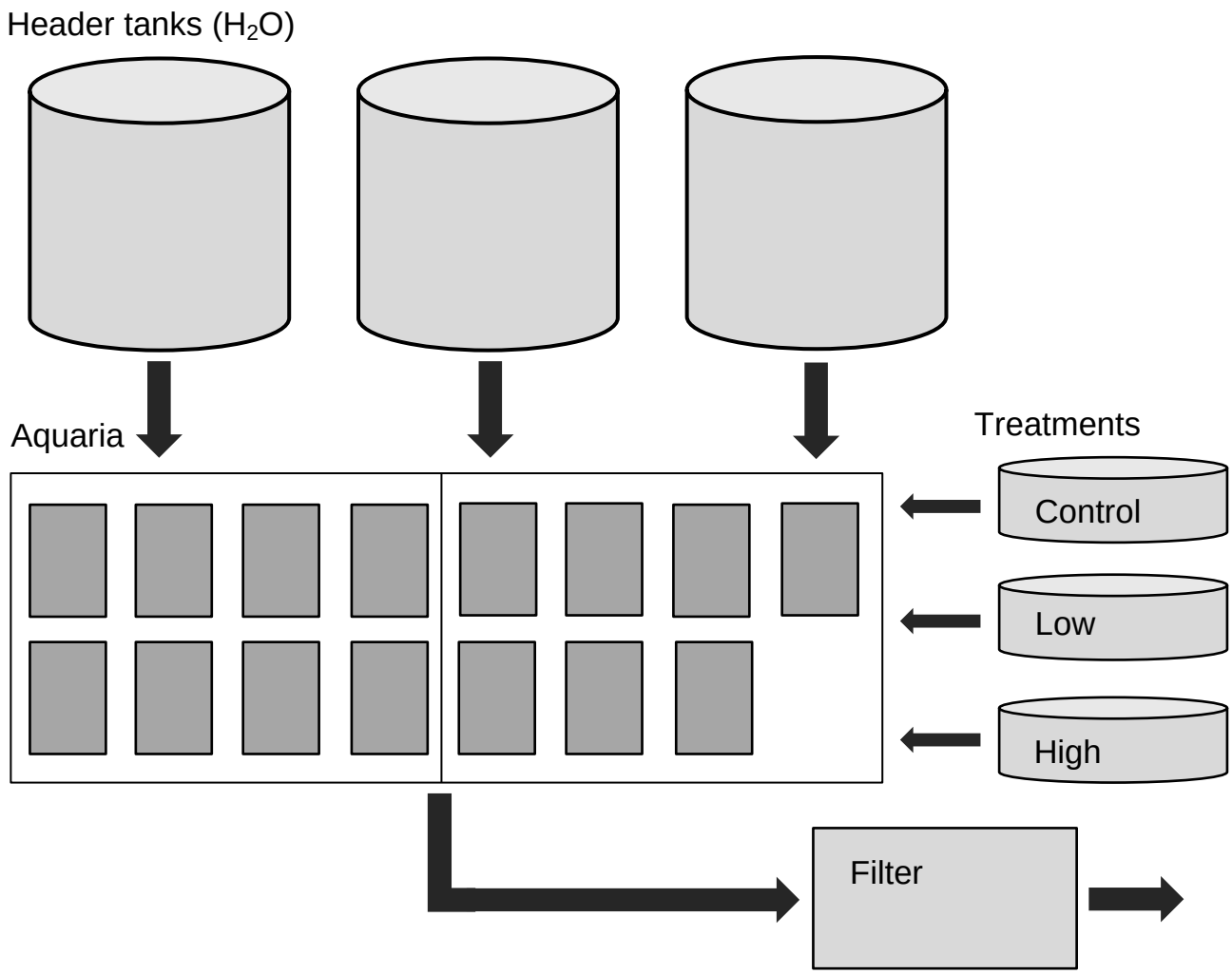


Figure 2. Experimental setup of the aquatic exposure to poly- and perfluoroalkyl substances (PFASs). Treatments and filtered water from the header tanks were randomly distributed between 15 aquaria. After drainage from the aquaria, water was led through an activated carbon filter before being discharged. Concentrations of total PFASs were 0, 10 and 785 µg/L in the “control”, “low” and “high” treatments, respectively. All treatment solutions contained 7% acetone (solvent).

2.2.3 PFAS exposure

Treatment solutions of the PFASs exposures and "control" treatment were prepared firstly in a 10-L hard plastic container and thereafter in a 20-L container deemed sufficient for the remainder of the exposure period. "Low" and "high" treatments contained a mixture of PFOS, PFHxS, 6:2 FTS, PFPeA, PFHxA and PFOA at the concentrations shown in Table 1. Seven percent acetone (VWR, 0.03% in each aquarium) were used to ensure complete dissolution of the chemicals in distilled H_2O, and the control treatment solution only consisted of H_2O and acetone.

Polyethylene tubes (0.5x1 mm, VWR) and two Watson-Marlow 500 series process pumps were used to transfer the appropriate amount of treatment solution to the aquaria. Five aquaria were randomly chosen for each of the exposure regimes, and the concentrations were maintained in each aquarium for 20 days (note: fish were sampled at day 21 and denoted as week three of exposure). After termination of the exposure, the remaining fish were kept in filtered tap water in the same aquaria for ten more weeks (depuration).

Table 1. Nominal concentrations of PFASs in the "low" and "high" treatments.

Poly- and perfluoroalkyl substance (PFAS)	Low (µg/L)	High (µg/L)
Perfluorooctane sulfonate (PFOS)	5.80	455
Perfluorohexane sulfonate (PFHxS)	1.60	125
6:2 Fluorotelomer sulfonate (6:2 FTS)	1.30	102
Perfluoropentanoic acid (PFPeA)	0.60	47.0
Perfluorohexanoic acid (PFHxA)	0.40	31.3
Perfluorooctanoic acid (PFOA)	0.30	23.5
Total nominal concentration	10.0	785

The selected PFASs in the "high" and "low" treatments were derived from chemical analyses of water collected from the lake Lavangsvatnet (by the outlet of a small stream draining water from the swamp area) close to Harstad/Narvik Airport Evenes in October 2013. The collection was performed in collaboration with Stine Hellstad (Department of Biosciences, UiO), Gro Dehli Villanger (Norwegian Institute of Public Health), Beate Løland (Department of Geology, UiO) and Gijs Breedveld (Norwegian Geotechnical Institute). Water analyses were performed by Beate Løland and Gijs Breedveld (Løland 2014, Norconsult and Sweco 2015). The "low" PFAS concentrations (Table 1) were made to resemble the concentrations found in Lavangsvatnet at the time of measurement. The concentrations of PFASs in the

"high" treatment were approximately 80-fold higher than the levels in the "low" treatment, and chosen on a basis for comparison with previous studies.

2.2.4 Sampling and storage

The use of test animals was approved by the Animal Research Authority (Forsøksdyrutvalget) for the appropriate treatments and number of fish. All trout to be sacrificed were randomly chosen for chemical or biomarker analyses. The weight and length of fish used in biomarker analyses are presented in Table 2 and 3. Trout sampled from the "low" and "high" treatments at week ten of depuration (week 13) were relatively heavier and longer than fish from the "control". This was possibly due to an uneven distribution of food between the fish in each aquarium during feeding. All samples were collected on the same day, except for the reference samples where aquaria 1-8 and 9-15 were sampled on the 20th and 21st of January, respectively. The fish sacrificed at week three of exposure and at week one of depuration were sampled one and eight days after ended exposure to PFASs. One fish from the "control" treatment died during the exposure period. A total of nine trout died during the ten weeks of depuration, five of which were from the aquaria previously treated with the "high" concentration of PFASs. One fish in each aquaria was observed to act dominant and aggressively towards the others, and was possibly the cause of death for many of the smaller trout during the depuration period.

Table 2. Weight (g w.w.) before removal of gills and intestines of trout exposed to different treatments ("control", "low" and "high") of PFASs sampled at various times after initiation of exposure (exposure termination at week 3) (n=5 if not otherwise stated).

Week	Control		Low		High	
	Range	Median	Range	Median	Range	Median
0	9.12 - 17.3	11.7	7.09 - 19.9	12.2	7.92 - 16.5	10.5
1	7.73 - 16.0	13.8	7.1 - 16.5 (n=4)	11.1	11.0 - 24.6	16.4
3	8.99 - 20.0	11.1	13.7 - 25.1	18.3	8.33 - 19.0	13.9
13	13.2 - 24.6	19.9	16.7 - 47.1	33.0	16.4 - 35.6	27.5

Table 3. Length (cm) of trout exposed to different treatments ("control", "low" and "high") of PFASs sampled at various times after initiation of exposure (exposure termination at week 3) (n=5).

Week	Control		Low		High	
	Range	Median	Range	Median	Range	Median
0	10.0 - 11.8	11.0	9.4 - 12.5	11.1	9.4 - 12.0	10.6
1	9.5 - 11.9	11.5	9.6 - 11.9	11.1	11.0 - 13.0	11.6
3	10.4 - 12.6	10.6	11.0 - 13.4	12.5	9.90 - 12.7	11.3
13	11.3 - 14.0	12.8	12.2 - 16.7	14.9	12.1 - 16.2	14.1

Fish were sacrificed by a blow to the head before blood was retrieved from the caudal vein using a syringe (1-mL insulin syringe, 12 mm, VWR) rinsed in heparin (10 000 Units/mL) to prevent the blood from clotting. The blood was centrifuged at 2000 g (Spectrafuge^{TM} Mini Laboratory Centrifuge, Labnet International) for approximately five minutes, and plasma was distributed in cryo-tubes for analysis of liver damage (alanine aminotransferase activity). Gills and liver were rapidly collected while the fish was kept on a cooled metal plate covered in aluminium foil to prevent degradation of molecules in the tissues. All samples were cleared of excess blood and blood clots in Hepes-Cortland (HC) buffer (see section 2.5) before being snap-frozen by liquid nitrogen and stored at -80°C in cryo-tubes.

The first pharyngeal arch from the left side of trout was stored in ice-cold HC buffer before analysis of the metabolic enzyme ethoxyresorufin *O*-deethylase. Severed gill filaments from the second, third, and fourth pharyngeal arches were transferred to cryo-tubes containing 200 µL 0.1 M K-PO_4 buffer (pH 7.4) with 0.1% Triton X-100 and 18.2 nM butylated hydroxytoluene in methanol (see section 2.6) for analysis of lipid peroxides (thiobarbituric acid reactive substances). The liver was excised and divided into three approximately equal pieces with a T-shaped cut. The anterior part was stored in a cryo-tube for analysis of peroxisome proliferation (peroxisomal acyl-CoA oxidase activity). The upper section was transferred to another cryo-tube containing 200 µL 0.1 M K-PO_4 buffer (pH 7.4) with 0.1% Triton and 18.2 nM butylated hydroxytoluene in methanol for analysis of lipid peroxides.

2.3 Validation of experimental setup and exposure

2.3.1 Temperature, oxygen and nitrite

Water temperature and oxygen levels were measured twice a week during and after ended exposure in all aquaria (mean values of 8.8°C ± 0.8°C (SD) and 96% ± 2.1% (SD),

respectively). Nitrite levels were measured at four different times during the experiment (one time before, two times during, and one time after ended exposure). All aquaria had levels below 0.3 mg/L except in three aquaria from the "control", one aquaria from the "low" and two aquaria from the "high" treatments measured 91 days after initiation of the exposure (nitrite=0.3 mg/L) (see Appendix 3).

2.3.2 PFASs in water

Approximately 100 mL water was collected at three, six, ten, 15 and 20 days during exposure, and seven days post exposure. Samples were taken from one randomly chosen aquarium of all treatments ("control", "low" and "high") at each time, and stored at -20°C before transport to Eurofins Environment Testing Norway AS for chemical analysis by LC/MS-MS (see section 2.4). The three aquaria sampled post exposure were the only aquaria sampled twice.

All added PFASs (PFOS, PFHxS, 6:2 FTS, PFPeA, PFHxA and PFOA) were detected in water samples from the "high" and "low" treatments during the exposure period (Table 4). Perfluorobutane sulfonate (PFBS, DL 0.02 µg/L and 0.8 µg/L) was detected in water from one "high" treatment aquaria at 15 days of exposure (0.9 µg/L), and one "low" treatment aquaria at 20 days of exposure (0.02 µg/L). Perfluoroheptanoic acid (PFHpA, DL 0.0100 µg/L and 0.500 µg/L) was detected in all "high" and "low" treatments during exposure with median concentrations of 1.6 µg/L (0.9-2.0 µg/L) and 0.01 µg/L (0.01-0.03 µg/L) for the "high" and "low" treatments, respectively. No PFASs were detected in water from the "control" treatment aquaria, as well as in samples collected six days post exposure (Appendix 2).

Table 4. Range and median PFAS concentrations (µg/L) in water from the "low" and "high" treatments during the three weeks of exposure (n=5).

Poly- and perfluoroalkyl substance (PFAS)	Low		High	
	Range	Median	Range	Median
Perfluorooctane sulfonate (PFOS)	0.500 - 14.8	3.30	38.1 - 413	284
Perfluorohexane sulfonate (PFHxS)	0.500 - 2.10	1.30	54.2 - 126	122
6:2 Fluortelomere sulfonate (6:2 FTS)	0.400 - 2.00	1.40	30.4 - 121	108
n-Perfluoropentanoic acid (PFPeA)	0.500 - 1.20	0.700	36.4 - 107	57.2
Perfluorohexanoic acid (PFHxA)	0.300 - 0.700	0.400	23.1 - 67.4	37.8
Perfluorooctanoic acid (PFOA)	0.0900 - 0.500	0.300	8.4 - 28.8	27.5

2.4 Chemical analysis of PFASs

One trout from each aquarium was sacrificed for analysis of PFAS concentrations in whole fish homogenate. Fish were rinsed in filtered tap water, packed in aluminium foil, and stored at -20°C before transport on dry ice to Eurofins Environment Testing Norway AS (analysis was conducted by Eurofins GfA Lab Service Gmbh in Hamburg). Determination of compound concentrations were conducted by solid phase extraction, and analysed by means of liquid chromatography coupled with mass spectrometry (LC/MS-MS). The addition of radioactive internal standard substances ($^{13}C_4$-PFBA, $^{13}C_2$-PFHxA, $^{13}C_8$-PFOA, $^{13}C_5$-PFNA, $^{13}C_2$-PFDA, $^{13}C_2$-PFUnA, $^{13}C_2$-DoA, $^{18}O_2$-PFHxS and $^{13}C_4$-PFOS) was conducted prior to the analysis, followed by extraction by methanol or acetonitrile for water and fish samples, respectively. A recovery standard ($^{13}C_4$-PFOA) was used to quantify the accuracy of the method. The limit of detection resulted from the background noise of the analysis and was preferred to be 1.0 µg/kg (w.w.). Higher limits were, however, reported for samples of smaller size or lower quality.

The measured concentrations and detection limits of PFOS, PFHxS, PFHxA, PFPeA, 6:2 FTS, and PFOA in fish can be found in Appendix 1. Table 5 show PFASs detected in the reference samples (denoted as week zero). Perfluorobutanoic acid (PFBA) was not included in the selected PFAS mixture (Table 1) but was detected in seven trout from the reference group. Samples with non-detected concentrations were set to half the detection limit when investigating the accumulation and elimination of PFOS and PFHxS in trout.

Table 5. PFASs detected in trout from the reference group (week zero, ng/kg w.w.). Samples marked with < are not detected. Concentrations after < are detection limits (varying with sample volume and sensitivity of analysis).

Sample	Perfluorobutanoic acid (PFBA)	Perfluorooctane sulfonate (PFOS)
1	150	350
2	< 58.2	204
3	74.2	185
4	102	226
5	87.0	671
6	115	138
7	< 50.7	149
8	< 62.6	169
9	< 64.3	< 64.3
10	< 54.5	143
11	81.8	70.2
12	< 62.7	120
13	< 66.1	272
14	< 70.2	104
15	134	92.5

2.5 Ethoxyresorufin *O*-deethylase activity in gills

Measurements of the ethoxyresorufin *O*-deethylase (EROD) activity were performed to investigate whether PFASs affected the induction of this metabolic enzyme and possibly changed the biotransformation capacity in gills of trout during the exposure and depuration periods. The method was conducted according to the procedure described by Jönsson *et al.* (2002).

The gills were excised and stored in ice-cold HC buffer, pH 7.7 (0.38 g/L KCl Merck, 7.74 g/L NaCl VWR, 0.23 g/L $MgSO_4*7H_2O$ Merck, 0.17 g/L $CaCl_2$, 0.33 g/L $NaH_2PO_4*H_2O$, 1.43 g/L Hepes Applichem, and 1.0 g/L Glucose) for a maximum of eight hours before analysis. Reaction buffer was made by mixing 35.0 mL HC buffer, 35.0 µL dicumarol (10 mM) and 13.7 µL 7-ethoxyresorufin (2.6 mM) dissolved in dimethyl sulfoxide (DMSO). The resorufin standard (6.75-200 nM) was prepared from a stock solution (10 mM) by dilution, initially in DMSO and thereafter in reaction buffer. Because both ethoxyresorufin and resorufin are degraded when exposed to light, all work was performed without direct light exposure.

The filaments were cut directly above the septum using a scalpel and tweezers resulting in approximately 2 mm long filament pieces (minimum 20 filaments per fish and replicate), and put in a 24-well tissue culture plate (Falcon, VWR). Pre-incubation of the filaments (2-10 minutes) was conducted by adding 0.50 mL room-tempered reaction buffer to each well. To start the assay the buffer was replaced by 0.70 mL fresh room-tempered reaction buffer and start time was noted. After 45 minutes (60 minutes for control and reference samples), 0.20 mL aliquots were transferred to a 96-well tissue culture plate (Falcon, VWR) in triplicates. For the resorufin standard solutions, duplicate 0.20 mL aliquots (reaction buffer for blanks) were added to the 96-well plate. Fluorescence was determined at 530 nm (excitation) and 590 nm (emission) (slit opening at 17 nm and reader sensitivity set to auto). A linear curve from the standard dilution was constructed and applied to calculate the EROD activity in each sample. The activity was expressed as picomoles produced resorufin per filament and minute.

2.6 Thiobarbituric acid reactive substances in gills and liver

The determination of thiobarbituric acid reactive substances (TBARS) was conducted to analyse for oxidative damage to unsaturated fatty acids (lipid peroxidation) in gills and liver caused by the exposure to PFASs. The method was carried out according to Ohkawa *et al.* (1979).

Liver tissue and gill filaments (cut directly above the septum as described in section 2.5) were added to 200 µL 0.1 M K-PO_4 homogenization buffer, pH 7.4 (2.59 g/L KH_2PO_4 and 18.5 g/L K_2HPO_4) with 0.1% Triton X-100 and 18.2 nM butylated hydroxytoluene (dissolved in methanol) to avoid further oxidation of lipids. The samples were immediately frozen by liquid nitrogen and stored at -80°C until analysis.

Malondialdehyde (MDA) tetrabutylammonium salt was diluted in distilled H_2O creating a 2x standard dilution series (0.40-25.6 µM). Samples were thawed on ice (kept on ice during analysis) and homogenized using Precellys ®24 (Bertin technologies, 1x10 seconds at 6000 rpm) with four homogenization beads in each tube. The homogenate was centrifuged at 9391 g for one minute (Eppendorf Centrifuge 5425), and 100 µL of the supernatant was added to an eppendorf tube (1.5 mL). The standard dilutions (distilled H_2O for blanks) were added to individual eppendorf tubes. All containers received 0.40 mL 60 mM Tris buffer (7.93 g/L Trizma HCl and 1.16 g/L Trizma Base, pH 7.4 at 25°C) containing 0.10 mM

diethylenetriaminepentaacetic acid, 0.50 mL 0.051 M thiobarbituric acid (TBA), and 0.50 mL 0.73 M trichloroacetic acid (TCA, Merck). A small hole was made on the top of the tubes to prevent spilling of the samples during incubation. Samples, standards and blanks were incubated for 60 minutes at 95°C in a Termaks T1056 UV incubator, and cooled to room temperature on ice followed by centrifugation at 12 000 g for five minutes (Eppendorf Centrifuge 5424). The supernatants were diluted 2x with distilled H_2O and 250 µL of this solution was added to a 96-well microtitre plate in duplicates. Absorbance was determined at 532 nm, and a linear curve from the standard dilution was constructed and applied to calculate MDA concentrations in each sample.

A reference sample was included in all 96-well plates, enabling the validation of plates in terms of human and instrumental inaccuracy. Total protein concentration of samples were determined using the method developed by Lowry *et al.* (1951) described in section 2.8. TBARS were expressed as nmol MDA/mg protein.

2.7 Peroxisomal acyl-CoA oxidase activity in liver

The measurement of peroxisomal acyl-CoA oxidase (AOX) activity was performed to investigate if PFASs cause peroxisome proliferation and possible oxidative damage in liver tissues, consequently affecting the activity of this enzyme. The method was conducted according to the procedure described by Small *et al.* (1985).

Samples were thawed on ice and added to eppendorf tubes (1.5 mL) with 200 µL chilled TVBE homogenization buffer pH 7.6 (84 mg/L $NaHCO_3$ Biochrom AG, 10 mL/L Na-EDTA, 1.0 mL/L ethanol, and 1.0 mL/L 10% Triton X-100). Homogenization was conducted (for 1x15 seconds at 6000 rpm) using Precellys ®24 with cryolys (Bertin technologies) cooling system and four homogenization beads in each tube. The homogenates were centrifuged at 500 g and 4.0°C for 15 minutes using an Eppendorf Centrifuge 5702R. Aliquots (50 µL) were diluted 20x in distilled H_2O.

Reaction medium (2.0 mL K-PO_4 buffer 0.50 M pH 7.4, 2.0 mL 2'7'-dichlorofluorescein diacetate 2.6 mM, 1.0 mL horseradish peroxidase 1200 units/mL, 1.0 mL sodium azide 4.0 M, 200 µL 10 % Triton X-100, and 93.8 mL distilled H_2O) was frozen at -20°C in 1.5 mL eppendorf tubes and later thawed in a water bath at 25°C before analysis. Diluted aliquots (50

µL) were added to 0.95 mL reaction medium and incubated in a water bath for five minutes at 25°C. The total solution was divided between four wells in a 96-well microtitre plate (240 µL in each well). Palmitoyl-CoA 3.0 mM solution (5.0 µL) was added to the wells chosen for the positive controls (two wells of each sample) using a multi canal pipette. Absorbance (502 nm) was determined after approximately 30 seconds incubation in room temperature, and new measurements were taken every 45 seconds for ten minutes. 2'7'-dichlorofluorescein diacetate, reaction medium, and 96-well plate containing solution were protected from light using aluminium foil.

Total protein concentration in the samples were determined using the method described by Lowry *et al.* (1951) (see section 2.8). The AOX activity were calculated using equations *2.7i* and *2.7ii*, where ΔOD/Δt was obtained from the absorbance measurements. The activity was expressed as mUnits/mg protein (1 unit = 1 µmol/minute).

$$2.7i) \quad \frac{mUnits}{mL} = \frac{\Delta OD}{\Delta t} \times \frac{Reaction\ volume}{Sample\ volume} \times \frac{Dilution\ factor}{0.091}$$

$$2.7ii) \quad \frac{mUnits}{mg\ protein} = \frac{mUnits}{mL} \div \frac{mg\ protein}{mL}$$

2.8 Protein analysis

The results of the TBARS and AOX analyses were protein normalised. Protein concentrations were measured using a modification of the method described by Lowry *et al.* (1951). This assay enables the detection of proteins using absorbance measurements of the blue colour developed from the reaction between an alkaline copper tartrate solution and a Folin reagent reacting with proteins in the sample.

The chosen dilution factors where 50x and 20x for liver and gill homogenates, respectively. Samples (stored in 0.5-mL eppendorf tubes) were thawed on ice by the addition of distilled H_2O which resulted in the desired dilutions. A bovine serum albumin protein standard was diluted in ice-cold 0.10 M Tris buffer (1.94 g/L Trizma base and 13.2 g/L Trizma HCl, pH 8.0 at 4.0°C) creating a 2x dilution series (10.4-82.2 mM), and kept on ice during analysis. The diluted samples, standard concentrations, blanks (Tris buffer or H_2O) and reference samples were plated out in triplicates (10 µL in each well) on the bottom of a 96-well microtitre plate.

25 µL of the alkaline copper tartrate solution (Reagent A, Bio-Rad) was added to all wells, followed by 200 µL dilute Folin reagent (Reagent B, Bio-Rad) and careful mixing. Absorbance was read (750 nm) after 15 minutes incubation at room temperature (protected from light by aluminium foil). Samples giving absorbance readings higher or close to the highest value of the standard concentrations were diluted 2x more and measured again. The linear curve from the standard dilution was constructed and applied to calculate the protein concentration in each sample.

As described by Lowry *et al.* (1951) samples should be diluted in 0.10 M Tris buffer. Tests showed, however, that a standard curve diluted in H_2O was approximately equal to a curve diluted in Tris buffer (variation 1.1-11%). The absorbance readings of the samples were corrected for ambient noise with blank H_2O values.

2.9 Alanine aminotransferase activity in plasma

Determination of the alanine aminotransferase (ALAT) activity in plasma was conducted to analyse for possible damage to liver tissues of trout during and after ended exposure to PFASs. The method was conducted according to the procedure described by Bergmeyer *et al.* (1986) modified for the use of 96-well microtitre plates (small sample volumes) and plasma from brown trout. This analysis is based on the reaction catalysed by ALAT (*2.9i*) and the indicator reaction catalysed by lactate dehydrogenase (*2.9ii*). The equilibrium of reaction *2.9ii* is shifted far to the right causing the equilibrium of the reaction *2.9i* to become irrelevant because pyruvate is continuously depleted in reaction *2.9ii*. ALAT activity is determined by measuring the colorimetric product (NAD+), which is proportional to the amount of pyruvate generated by ALAT and thereby also the rate of NADH oxidation in reaction *2.9ii*.

$$2.9i) \quad L\text{-}Alanine + \text{α-Ketoglutaric acid} \xleftrightarrow{Alanine\ aminotransferase} Pyruvate + L\text{-}Glutamate$$

$$2.9ii) \quad Pyruvate + NADH + H^+ \xleftrightarrow{Lactic\ dehydrogenase} L\text{-}Lactate + NAD^+$$

Two 96-well plates were run to test the modified solutions and different volumes of plasma (5.0, 10, 20, 30 and 40 µL). Time of final measurements at room temperature was evaluated to verify that a constant change in absorbance per change in time ($\Delta OD/\Delta t$) was obtained. Time of pre-incubation was also evaluated to confirm the complete saturation of alanine

aminotransferase with pyridoxal phosphate and to allow reactions between NADH and endogenous substances in serum to be fulfilled.

Plasma samples were thawed on ice and centrifuged for one minute at 241 g (Eppendorf Centrifuge 5810R). Plasma was transferred to a 96-well microtitre plate, 15 µL in one well for overall ALAT reaction and 15 µL in a second well for individual sample blank. Each well was brought to a final volume of 20 µL by the addition of 5.0 µL 0.15 M NaCl. For blanks, 20 µL NaCl (0.15 M) was added to eight wells for overall reaction and eight wells for sample blank.

The reaction mixture for overall reaction consisted of 16.4 mL 0.11 M Tris buffer (4.33 g/L Trizma base and 11.7 g/L Trizma HCl, pH 7.8 at 25°C), 19.7 mL Tris/L-alanine solution (0.11 M Trizma base and 0.63 M L-alanine, adjusted to pH 7.8 with 1.0 M HCl), 7.93 mL pyridoxal 5'-phosphate solution (0.63 mM pyridoxal phosphate hydrate in 0.11 M Tris buffer), 5.68 mL β-NADH solution (6.12 mM β-NADH in 0.11 M Tris buffer), and 267 µL lactic dehydrogenase solution (225 Units/L containing 10% glycerol and 90% Tris buffer). In the reaction mixture for individual sample blank, Tris/D-alanine (0.11 M Trizma base and 0.63 M L-alanine, adjusted to pH 7.8 with 1.0 M HCl) was added instead of the Tris/L-alanine solution, which inhibits the reaction by alanine aminotransferase to form pyruvate and L-glutamate.

After addition of plasma and blank samples to the 96-well plates, reagent mixtures for overall reaction (220 µL) and individual sample blank (220 µL) were added to their respective wells. The reagents were mixed by shaking in the plate reader for five seconds at medium intensity. Absorbance (339 nm) was measured every 45 seconds for 15 minutes to ensure that a constant change in absorbance per change in time ($\Delta OD/\Delta t$) was achieved during the pre-incubation period

20 µL Tris/α-ketoglutaric acid (0.11 M Trizma base and 0.18 M α-ketoglutaric acid disodium salt dihydrate, adjusted to pH 7.8 at 25°C with 12 M and 5.0 M HCl) were added to all wells, and the solutions were mixed for 15 seconds at medium level in the plate reader. The change in absorbance ΔOD (339 nm) was measured every 45 seconds for 30 minutes, and the calculated slope values were individually evaluated and corrected when a lag phase occurred at the start of the measurement. All solutions containing pyridoxal 5'-phosphate and β-NADH were protected from light using aluminium foil.

The following equation was used to determine ALAT activity in plasma samples.

$$Catalytic\ concentration\ (Units/L) = \frac{Reaction\ volume\ (L)}{\varepsilon \times l \times Sample\ volume\ (L)} \times \frac{\Delta OD}{\Delta t} \times 60 \times 10^6$$

Where l is the path length of light through the solution in the well (mm), ε is the molar absorbance (m^2/mol) of NADH under the defined conditions of the experiment, and ΔOD/Δt is obtained from the absorbance measurements. The molar absorbance of NADH has been measured to 630 m^2/mol at 340 nm, 25°C and pH 7.8 (McComb *et al.* 1976, Ziegenhorn *et al.* 1976). The path-length when the light passes through a volume of 260 µL in one well (NunclonΔ, cat.no 167008) is 7.41 mm. ALAT activities were expressed in Units/L.

2.10 Statistical analysis

Univariate statistical analyses were performed using R version 3.1.2 (The R foundation for statistical analysis, 2014). The additional packages "lawstat" and "ggplot2" were used to perform Levene's test for homogeneity of variance and to graphically display the results from the chemical and biomarker analyses.

When testing for significance using parametric tests such as analysis of variance (ANOVA), it is assumed that the data are randomly drawn from a population with a normal distribution, and that the variance between groups is uniform. If there is a limited amount of observations within each group (<10), the dataset often does not yield enough power to reject the null hypothesis that the observations are not normally distributed (e.g. using the Shapiro-Wilk test) (Whitlock and Schluter 2009 p. 324). Based on this information, a normal distribution of the data was assumed. Levene's test was used to test the homogeneity of variances between groups (Levene 1960), and data was log-transformed if the groups were found to have heterogeneous variance. If the transformation resulted in homogeneity of variance, data were analysed using one-way ANOVA followed by Tukey's post hoc multiple comparison test (Tukey's HSD) (Tukey 1949). Alternatively, if homogenous variance was not achieved, significance was calculated using the unequal variance Welch test (Welch 1938) followed by Games-Howell post hoc test on non-transformed data. A Dunnett's post hoc test was not used because this test does not analyse for differences between non-control treatments. Student's two-sample t-test was used when comparing two groups with homogenous variance, and Welch two sample t-test was used when the variance was heterogeneous.

When developing the method for ALAT activity in plasma, estimated slope values from the linear regression of the 17 samples added Tris/L-alanine solution were compared with the 17 samples added Tris/D-alanine solution. These data groups deviated from normality (Shapiro-Wilk test), and Levene's test showed significant differences in variation between the groups. A log-transformation of the data was not possible due to both negative and positive values. Thus, a non-parametric Kruskal-Wallis ANOVA (Kruskal and Wallis 1952) was conducted on untransformed data to investigate the difference in slope values between the two groups.

The level of significance to reject H_0 was set to 0.05.

2.10.1 Estimation of half-life

In ecotoxicology, the most commonly used model for uptake and elimination of a compound in an organism is the one-compartment model. Here, the toxicant is assumed to be distributed evenly throughout that one compartment (e.g. whole body). The first order elimination from this compartment is represented by the equation below, where C_i is the concentration of the compound in the compartment and k_e is the first order rate constant (Newman and Clements 2008 p. 119).

$$\frac{\Delta C_i}{\Delta t} = -k_e C_i$$

The k_e can be estimated by fitting a linear model (regression) to $\log(C_i)$ over time (t). The intercept value of the model is equivalent to the initial concentration in the compartment (C_0). The slope value measures how much $\log(C_i)$ changes per unit change in time (Whitlock and Schluter 2009 p. 467), which further can be used as the rate constant (k_e) when calculating the time it takes for the compartment to eliminate 50% of the compound (biological half-life). The equation for half-life ($t_{1/2}$) can be written as below (Newman and Clements 2008 p. 119):

$$t_{1/2} = \frac{\log 2}{k_e}$$

2.10.2 Estimation of bioconcentration factors

A bioconcentration factor (BCF) can be calculated by dividing the chemical concentration in the organism by the corresponding concentration in water, given that a steady state has been reached (Arnot and Gobas 2006, Newman and Clements 2008 p. 121, 737). The BCFs of PFOS and PFHxS were calculated using concentrations measured in fish from the low and high treatments at three weeks, and concentrations in water from the respective aquaria sampled between day three and 20 during exposure to PFASs.

2.10.3 Multivariate data analysis

The multivariate statistical analysis were performed using R version 3.1.2 (The R foundation for statistical analysis, 2014), with the additional package "vegan". A principal component analysis (PCA) was used to explore the variation and relationship between observed responses and biomarker variables. The PCA aims to reduce the dimensions of a dataset such that the principal component axes (PCs) explains the highest possible amount of the variation. PC1 (x axis) account for the largest variation in the dataset, and the subsequent PC2 (y axis) explains the second largest variation. In addition, PC2 is perpendicular and uncorrelated with the first PC (Sparks *et al.* 1999, Bro and Smilde 2014).

The information in a PCA can be presented as a biplot. This type of plot consist of the score plot, which displays how the observations (as points) are arranged in respect to each other, and the loading plot showing how the variables (as arrows) relate to each other. The angles between the arrows indicate possible correlation between the variables, where an angle of 90 degrees represents no correlation (Sparks *et al.* 1999).

The dataset of biomarker responses were standardized (mean subtracted from the observation and divide by SD) and centred (shifting the scale so that the mean equals zero) before analysis. PCA was performed separately on results from each sampling week (week zero, one, three and 13), and over time for the different treatments (low and high). Observations were plotted as points and biomarker variables were presented as arrows. If a variable contained NA-values the whole row was deleted during the PCA, and the observation was not visible in the biplot.

3. Results

The selected PFASs, yielding the results below, were chosen to simulate contamination in Lavangsvatnet originating from the use of fire foams (AFFFs) at Harstad/Narvik Airport Evenes. Results denoted as three weeks of exposure and as one week of depuration to PFASs were sampled one and eight days after termination of the exposure.

3.1 Bioconcentration and elimination of PFASs in trout

3.1.1 Exposure conditions and detection of PFASs

Perfluorooctane sulfonate (PFOS) was detected in all but one trout from the reference group (DL=0.064 µg/kg). Perfluorohexane sulfonate (PFHxS) was detected in 46% of the reference and treatment samples (detected in 59% of trout when not including reference samples, DL from 0.067 µg/kg to 90.1 µg/kg), with the majority of non-detected concentrations originating from the reference group (week zero), control samples, and the samples collected ten weeks after ended exposure. Perfluorohexanoic acid (PFHxA), n-perfluoropentanoic acid (PFPeA) and 6:2 fluorotelomer sulfonate (6:2 FTS) were not detected in any samples during exposure or depuration (DL 0.045 µg/kg to 118 µg/kg for PFHxA and PFPeA, and DL 0.067 µg/kg to 177 µg/kg for 6:2 FTS). Perfluorooctanoic acid (PFOA) was only detected in two samples from the "high" treatment after one week of exposure (13.0 µg/kg and 10.8 µg/kg, DL 0.045 µg/kg to 118 µg/kg). Three PFASs were detected, which were not in the nominal mixture of compounds. Perfluorobutanoic acid (PFBA) was detected in a total of 16 fish sampled before, during and after exposure with a maximum concentration of 1.10 µg/kg (DL 0.050 µg/kg to 118 µg/kg). Perfluorononanoic acid (PFNA) was detected in two samples from ten weeks after ended exposure (0.104 µg/kg and 0.084 µg/kg, DL 0.050 µg/kg to 118 µg/kg). Perfluorodecanoic acid (PFDA) was detected in a total of four samples, one sample from week one of exposure (0.057 µg/kg), and three trout collected ten weeks after ended exposure (0.055 µg/kg, 0.131 µg/kg and 0.120 µg/kg, DL 0.050 µg/kg to 118 µg/kg).

3.1.2 Bioconcentration

There were significant increases in PFOS concentrations in trout exposed to the "high" PFASs concentration from week zero to week three, and from week one to week three of exposure (Tukey's HSD, CI $3.46*10^4$ to $9.32*10^4$, $p<0.001$, and CI $1.48*10^4$ to $7.34*10^4$, $p=0.004$, respectively), but there was no significant difference when comparing week zero to week one (Tukey's HSD, CI $-9.53*10^3$ to $4.91*10^4$, $p=0.2$) (Figure 3). No significant differences were detected over time in trout from the "low" treatment (one-way ANOVA, $F_{df\ 2,12}=3.25$, $p=0.07$). There was a significantly higher concentration in fish from the "high" treatment compared to the "control" at week one of exposure (Tukey's HSD on log-transformed values, CI 1.30 to 12.6, $p=0.02$), but no differences between the other treatments were detected (Tukey's HSD on log-transformed values, CI -2.56 to 8.74, $p=0.3$ between the "control" and "low" treatments, and CI -9.51 to 1.79, $p=0.2$ between the "low" and "high" treatments). After three weeks of exposure there were significant increases in the PFOS concentrations from the "control" to the "high" treatment, and from the "low" to the "high" treatment (Tukey's HSD, CI $3.74*10^4$ to $9.01*10^4$, and CI $-8.78*10^4$ to $-3.81*10^4$, $p<0.001$), while no difference was detected between trout from the "control" and "low" treatments (Tukey's HSD, CI $-2.55*10^4$ to $2.71*10^4$, $p=1$).

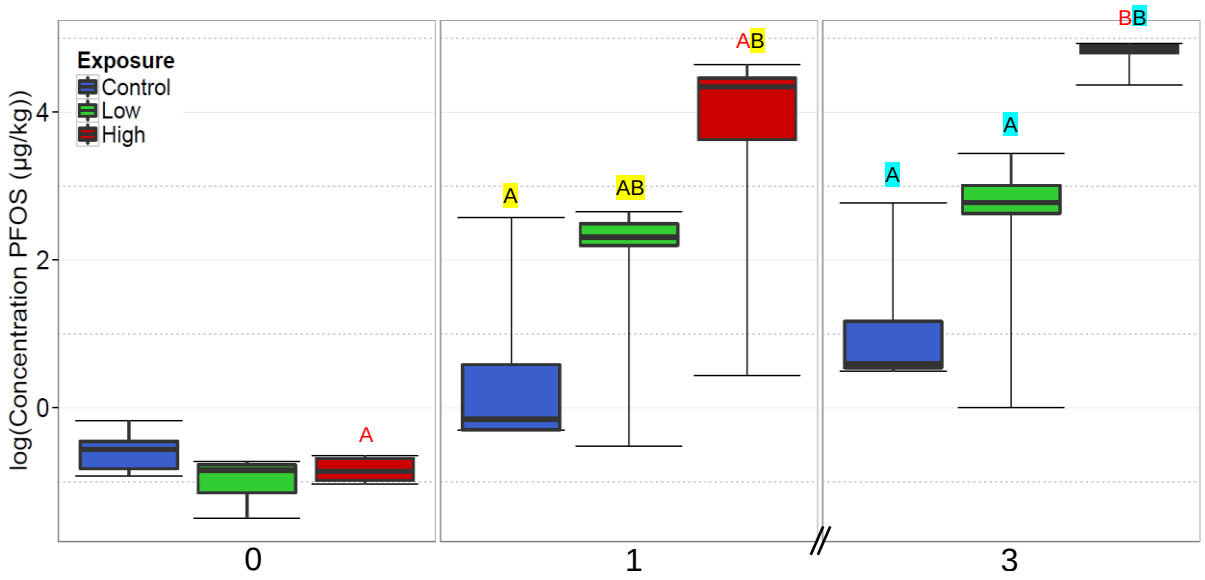


Figure 3. Log-transformed concentrations of perfluorooctane sulfonate (PFOS) in trout from "control" (blue), "low" (green), and "high" (red) treatments at zero (reference), one and three weeks of exposure to PFASs. Median and quartiles are presented (n=5, except "control" at three weeks where n=4). Letters shown in identical colours originate from the same significance test. Unequal letters indicate significant difference ($p<0.05$). The only non-detected sample (week zero, "low" treatment) was set to half DL (DL=0.064 µg/kg).

There was a significant accumulation of PFHxS in trout exposed to the "high" concentration of PFASs from week zero to week one and week three of exposure with p=0.001 and <0.001, respectively (Tukey's HSD on log-transformed values, CI 3.37 to 11.4, and CI 5.61 to 13.7) (Figure 4). The difference was not significant in fish collected at week one compared to week three (Tukey's HSD on log-transformed values, CI -1.78 to 6.26, p=0.3). In the "low" treatment, there was a significant difference in PFHxS concentration over time from week zero to week three of exposure (Tukey's HSD, CI 0.108 to 25.5, p=0.05); however, there were no differences between samples collected at week zero and week one, and week one and week three (CI -6.15 to 19.2, and CI -6.44 to 19.0, p-values=0.4). A significant increase in concentration between trout from the "control" and "high" treatments was present after one week of exposure (Tukey's HSD on log-transformed values, CI 1.10 to 11.2, p=0.02), while there were no differences between the "control" and "low" treatments, and the "low" and "high" treatments (CI -2.26 to 7.89, p=0.3, and CI -8.43 to 1.72, p=0.2). In fish sampled at week three of exposure, there was a significantly higher concentration in the "high" treatment compared to the "control" and "low" treatments (Tukey's HSD, CI 644 to 767, and CI -754 to -638, p-values<0.001), but there was no difference between the "control" and "low" treatments (CI -52.6 to 71.2, p=0.9).

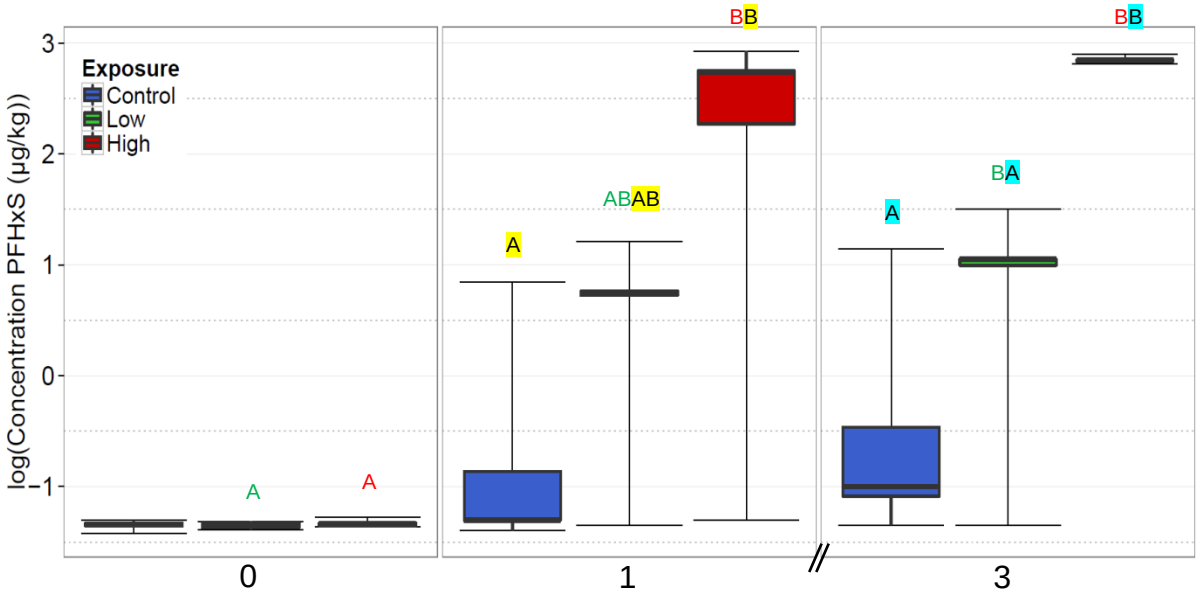


Figure 4. Log-transformed concentrations of perfluorohexane sulfonate (PFHxS) in trout from "control" (blue), "low" (green), and "high" (red) treatments at zero (reference), one and three weeks during exposure to PFASs. Median and quartiles are presented (n=5, except "control" at three weeks where n=4). Letters shown in identical colours originate from the same significance test. Unequal letters indicate significant differences (p<0.05). Concentrations under DL (from 0.065 µg/kg to 90.1 µg/kg) were set to half the DL for the given sample.

Samples from the "control" treatment showed no significant differences during the exposure period, both for PFOS (one-way ANOVA, $F_{df\ 4,18}$=0.92, p=0.5) and PFHxS (one-way ANOVA, $F_{df\ 4,18}$=0.98, p=0.4). Nor were there any differences between the reference samples collected from the aquaria set to receive the "control", "low" and "high" treatments (one-way ANOVA, $F_{df\ 2,12}$=2.80, p=0.1 for PFOS, and $F_{df\ 2,12}$=0.45, p=0.7 for PFHxS).

3.1.3 Bioconcentration factors of PFOS and PFHxS

The concentrations of PFOS and PFHxS in trout from the "high" and "low" treatments were divided by their respective water concentrations when estimating the bioconcentration factors (BCFs) (Table 6). The median BCFs in the "high" treatment were 244 L/kg (175-632 L/kg, n=5) and 6.34 L/kg (5.49-12.0 L/kg, n=5) of PFOS and PFHxS, respectively. For trout from the "low" treatment, PFOS had a BCF of 185 L/kg (0.303-846 L/kg, n=5) and PFHxS had a BCF of 11.2 L/kg (0.025-19.8 L/kg, n=5).

Table 6. Measured concentrations in water and calculated bioconcentration factors (BCFs) of perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) in the "high" and "low" treatments.

Concentration in water (µg/L)				BCF (L/kg)			
PFOS		PFHxS		PFOS		PFHxS	
High	Low	High	Low	High	Low	High	Low
38.1	3.30	54.2	1.80	632	184	6.47	15.2
284	14.8	125	2.10	614	352	12.0	11.2
362	0.500	126	0.500	244	846	5.49	19.8
133	2.90	109	1.00	175	124	6.34	8.62
413	4.80	122	1.30	192	0.303	5.78	0.025

3.1.4 Elimination and half-life of PFOS and PFHxS

There were no significant decreases in PFOS concentrations over time after ended exposure in trout from the "high" (one-way ANOVA, $F_{df\ 2,10}$=3.13, p=0.09) or "low" treatments (one-way ANOVA, $F_{df\ 2,12}$=2.77, p=0.1) (Figure 5). There were, however, significant differences in concentrations between fish from the "control", "low" and "high" treatments after one week of depuration (Tukey's HSD on log-transformed values) where both the "low" and "high" treatments showed higher concentrations than the "control" (CI 5.53 to 9.05, and CI 8.60 to

12.1, p-values<0.001). Furthermore, the PFOS levels in trout from the "high" treatment was significantly higher than the concentrations in fish from the "low" treatment (CI -4.82 to -1.30, p=0.002). The same differences were shown in trout sampled at week ten of depuration (Tukey's HSD on log-transformed values, CI ("control" and "high") 4.53 to 5.18, CI ("control" and "low") 2.11 to 2.68, CI ("low" and "high") -2.77 to -2.15, p-values<0.001).

There were significant decreases in PFHxS concentrations over time in trout exposed to the "high" concentration of PFASs from week zero and week one to week ten after termination of the exposure (Tukey's HSD, CI $-1.12*10^3$ to $-0.224*10^3$, p=0.005, and CI $-1.02*10^3$ to $-0.122*10^3$, p=0.01, respectively) (Figure 6). No decrease was detected from week zero to week one (CI -491 to 286, p=0.8). Trout from the "low" treatment did not show any significant differences in concentrations over time during the depuration period (one-way ANOVA, $F_{df\ 2,12}$=1.88, p=0.2). In fish collected one week after ended exposure, there were significantly higher concentrations of PFHxS in trout from the "high" treatment compared to the "control" (Tukey's HSD, CI 259 to 954, p=0.001) and "low" treatments (CI -906 to 212, p=0.003), but there were no differences between the "low" and "control" treatments (CI -300 to 394, p=0.9). The same results were shown after ten weeks of depuration, with p-values <0.001 between the "high" and "control", and "high" and "low" treatments (Tukey's HSD, CI 34.5 to 37.6, and CI -37.1 to -34.1), and a non-significant p=0.7 when comparing the "low" and "control" treatments (CI -0.960 to 1.82).

The one-way ANOVA did not show any significant differences over time for the concentrations of PFOS or PFHxS in trout from the "control" treatment (F-values$_{df\ 2,10}$=1.18, p-values=0.3).

Figure 5 and 6 show the linear regressions using log-transformed concentrations of perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) in trout exposed to the different concentrations of PFASs from where the half-life ($t_{1/2}$) can be estimated. For PFOS, the linear regression using data from fish exposed to the "high" treatment (Figure 5A) had a positive slope resulting in an increasing (∞) half-life estimate (CI 56.5 to ∞ weeks), while estimation using the "low" treatment (Figure 5B) showed $t_{1/2}$=13 weeks (CI 2.13 to ∞ weeks). The linear regression in Figure 6A resulted in $t_{1/2}$=2.3 weeks (CI 2.03 to 2.70 weeks) for PFHxS in trout from the "high" treatment, and the same half-life estimate in fish exposed to the "low" treatment ($t_{1/2}$=2.3 weeks, CI 1.29 to 8.63 weeks, Figure 6B).

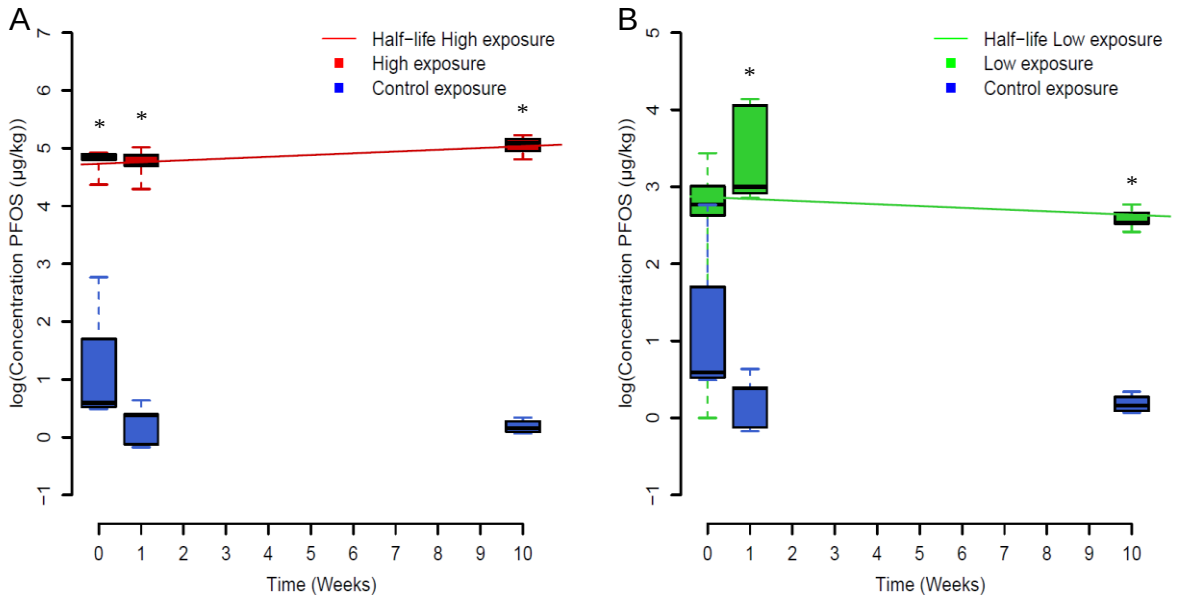


Figure 5. Log-transformed concentrations of perfluorooctane sulfonate (PFOS) in trout from A) "high" (red) and B) "low" (green) treatments at zero through ten weeks after ended exposure to PFASs. Blue boxplots are concentrations in the "control" treatment. Lines represent half-life of PFOS (on log-scale). Median and quartiles are presented (n=5, except ten weeks "control" and "high" where n=4 and n=3, respectively). The asterisk indicate significant difference from control ($p<0.01$).

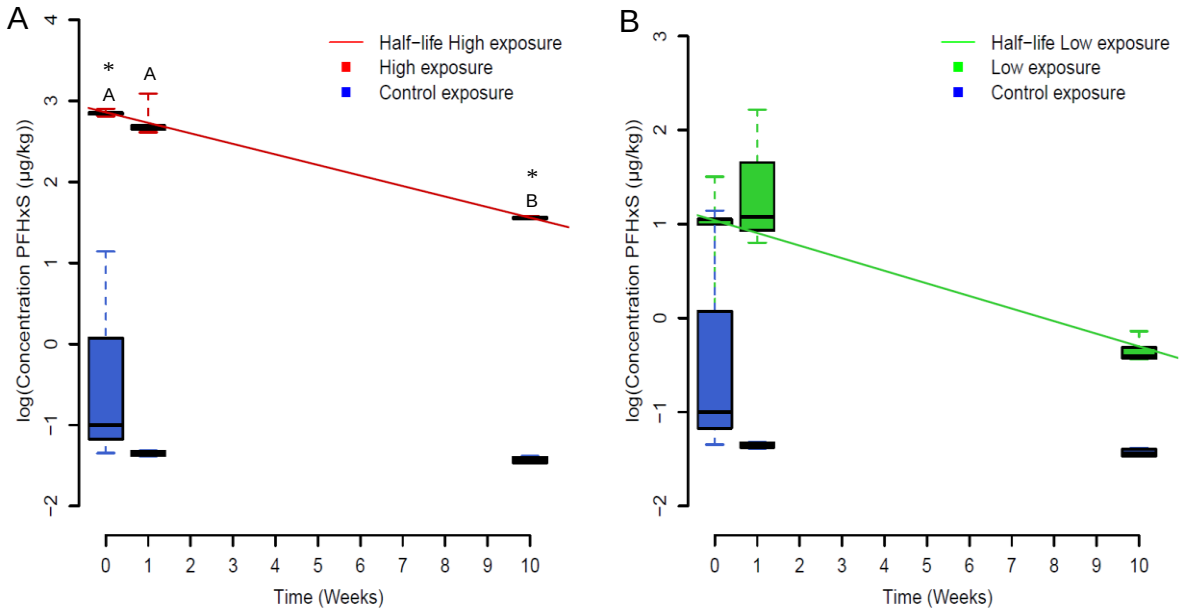


Figure 6. Log-transformed concentrations of perfluorohexane sulfonate (PFHxS) in trout from A) "high" (red) and B) "low" (green) treatments at zero through ten weeks after ended exposure to PFASs. Blue boxplots are concentrations in the "control" treatment. Lines represent half-life of PFHxS (on log-scale). Median and quartiles are presented (n=5, except in ten weeks "control" and "high" where n=4 and n=3, respectively). Unequal letters indicate significant differences ($p<0.05$). The asterisks indicate significant difference from control ($p<0.01$).

3.2 Effects of PFASs

3.2.1 Ethoxyresorufin *O*-deethylase activity in gills

The analysis of ethoxyresorufin *O*-deethylase (EROD) activity was performed to investigate the effect of PFASs exposure on the activity of this metabolic enzyme in the gills of brown trout. Significantly increased EROD activity were found in fish from the "high" treatment after three weeks of exposure (compared to samples from week zero and week one) (Tukey's HSD, CI 0.129 to 0.770, p=0.007, and CI 0.141 to 0.783, p=0.006) (Figure 7). The same observation was made in trout from the "control" treatment (Tukey's HSD, CI 0.067 to 0.535, p=0.01, and CI 0.028 to 0.496, p=0.03 when comparing week three with week zero and week one, respectively). There was no significant difference in the EROD activity of trout from the "low" treatment during exposure (one-way ANOVA, $F_{df\ 2,12}$=0.522, p=0.6). There was, however, an increased activity in fish collected at ten weeks of depuration (week 13), compared to the third week of exposure in trout exposed to the "low" concentration of PFASs (two sample t-test, $t_{df\ 8}$=-2.62, p=0.03). No differences were detected in fish from the "control" and "high" treatments between week three of exposure and week ten after ended exposure, p=0.7 and p=0.2, respectively (two sample t-test, $t_{df\ 8}$=-0.436 and $t_{df\ 8}$=-1.37). Within sampling weeks, there were no differences between trout from various treatments at week one (one-way ANOVA, $F_{df\ 2,12}$= 2.05, p=0.2) and week ten after terminated exposure (one-way ANOVA, $F_{df\ 2,12}$=2.30, p=0.2). For samples collected at week three of exposure, there was a significant increase in EROD activity in the gills of fish from the "high" treatment compared to the "low" treatment (Tukey's HSD, CI -0.748 to -0.073, p=0.02), but trout from neither "high" nor "low" treatments were found to be significantly different from the "control" (Tukey's HSD, CI -0.156 to 0.520, p= 0.4, and CI -0.566 to 0.109, p=0.2). No differences in the activity of EROD were detected between treatments in trout from the reference group (week zero, one-way ANOVA, $F_{df\ 2,12}$=1.30, p=0.3).

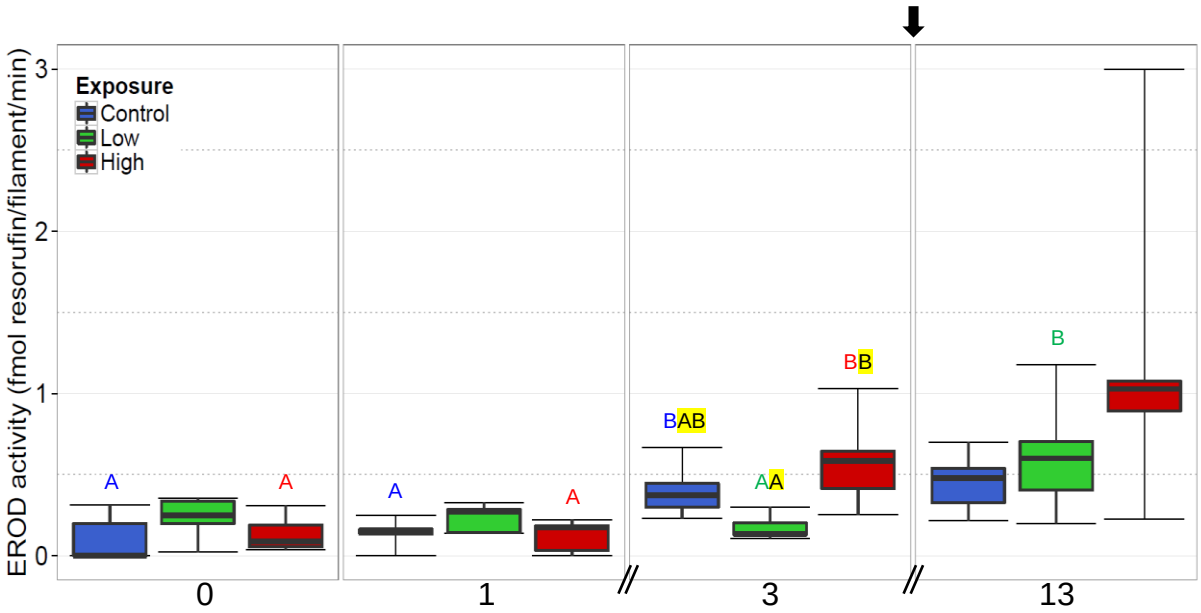


Figure 7. The activity of ethoxyresorufin *O*-deethylase (EROD) in gills of trout from "control" (blue), "low" (green), and "high" (red) treatments at zero, one and three weeks during exposure to PFASs, and at ten weeks after ended exposure (week 13, ended exposure is denoted by the black arrow). Median and quartiles are presented (n=5). Negative EROD values were set to zero (two samples from week zero, and one sample from week one "high" treatment). Letters shown in identical colours originate from the same significance test. Unequal letters indicate significant difference ($p<0.05$).

3.2.2 Thiobarbituric acid reactive substances in gills

The determination of lipid peroxides through the thiobarbituric acid reactive substances (TBARS) method was conducted to evaluate possible oxidative damage to unsaturated fatty acids in gills of trout by the exposure to PFASs. No significant differences were detected in the levels of TBARS in fish sampled from the "low" and "high" treatments during exposure, with p=0.7 and p=0.1, respectively (one-way ANOVA, $F_{df\ 2,12}=0.446$ and $F_{df\ 2,12}=2.57$) (Figure 8). There were no differences between samples from week three of exposure and week ten of depuration (week 13) for the "low" and "high" treatments (two sample t-test, $t_{df\ 8}=1.94$, p=0.09, and $t_{df\ 8}=0.811$, p=0.4, respectively). The one-way ANOVA analysis between treatments did not yield any significant differences when investigating trout collected at each time of sampling (week one, $F_{df\ 2,12}=0.145$, p=0.9; week three, $F_{df\ 2,12}=1.23$, p=0.3; week 13, $F_{df\ 2,12}=0.163$, p=0.9). No differences were detected between treatments in trout from the reference group (week zero, one-way ANOVA, $F_{df\ 2,12}=0.446$, p=0.7), nor over time in fish exposed to the "control" treatment (one-way ANOVA, $F_{df\ 3,16}=1.77$, p=0.2).

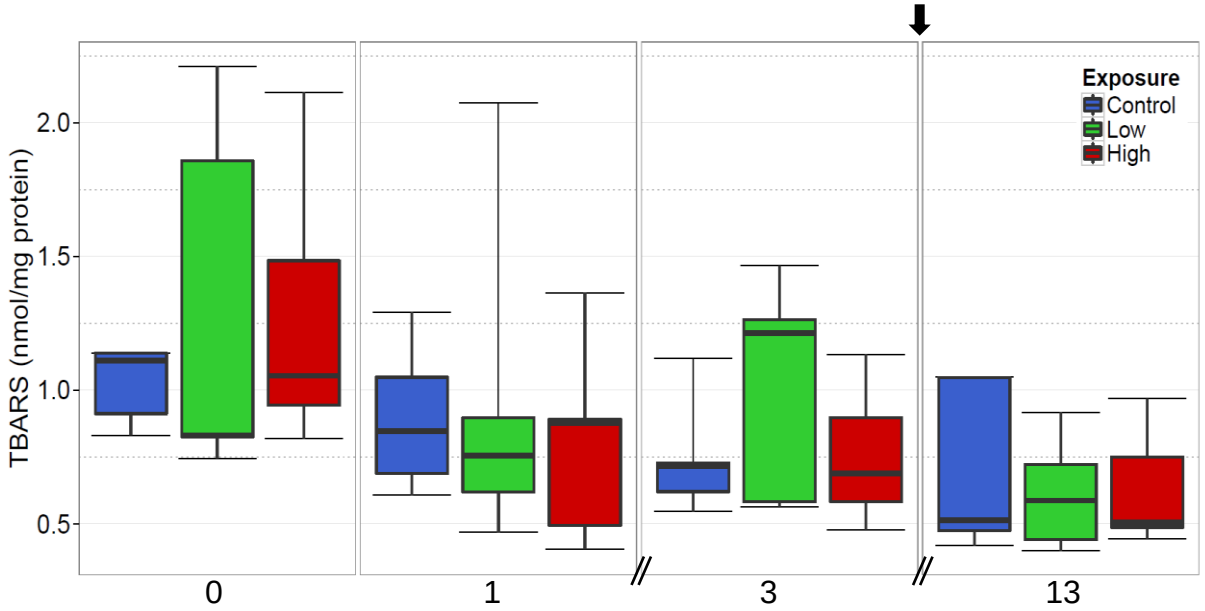


Figure 8. Thiobarbituric acid reactive substances (TBARS) in gills of trout from "control" (blue), "low" (green), and "high" (red) treatments at zero, one and three weeks of exposure to PFASs, and at ten weeks after ended exposure (week 13, ended exposure is denoted by the black arrow). Median and quartiles are presented (n=5).

3.2.3 Thiobarbituric acid reactive substances in liver

Measurement of the hepatic TBARS levels was conducted to investigate if exposure to PFASs possibly causes lipid peroxidation in the liver tissue. There were no significant differences in the hepatic TBARS levels in the trout from the "low" and "high" treatments during exposure to PFASs (one-way ANOVA, $F_{df\ 2,12}$=0.540, p=0.6, and $F_{df\ 2,12}$=1.67, p=0.2), nor were there any significant differences in the "low" or "high" treatments when comparing week three of exposure and week ten of depuration (week 13, two sample t-test, $t_{df\ 8}$=2.18, p=0.06, and $t_{df\ 8}$=-0.569, p=0.6) (Figure 9). The trout from all treatments collected at week one, week three and week 13 showed no significant differences in TBARS (one-way ANOVA, $F_{df\ 2,12}$=1.17, p=0.3, $F_{df\ 2,12}$=2.19, p=0.2, and $F_{df\ 2,12}$=2.93, p=0.09, respectively). There were no differences between samples from the "control", "low" and "high" treatments collected at week zero of exposure (one-way ANOVA, $F_{df\ 2,12}$=0.101, p=0.9), nor over time in trout exposed to the "control" treatment (one-way ANOVA, $F_{df\ 3,16}$=1.76, p=0.2).

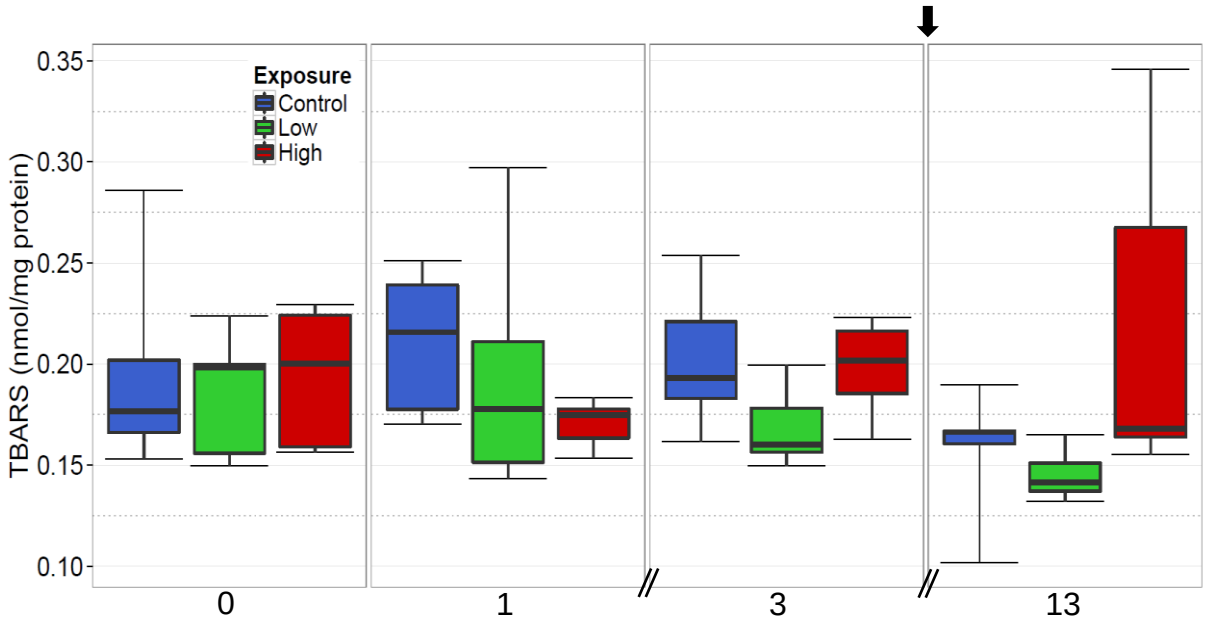


Figure 9. Thiobarbituric acid reactive substances (TBARS) in liver of trout from "control" (blue), "low" (green), and "high" (red) treatments at zero, one and three weeks of exposure to PFASs, and at ten weeks after ended exposure (week 13, ended exposure is denoted by the black arrow). Median and quartiles are presented (n=5).

3.2.4 Peroxisomal acyl-CoA oxidase activity in liver

Analysis of the peroxisomal acyl-CoA oxidase (AOX) activity was performed to investigate if PFASs act as peroxisome proliferators and possibly cause oxidative damage to the liver tissue. No significant differences were found in the hepatic AOX activity in trout from the "low" and "high" treatments during exposure (one-way ANOVA, $F_{df\ 2,12}$=1.65, p=0.2, and Welch *U* test, $F_{df\ 2.0,6.7}$=1.16, p=0.4, respectively) (Figure 10). There was no difference when comparing samples collected at week three of exposure with week ten of depuration (week 13) in fish from the "low" treatment (two sample t-test, $t_{df\ 8}$=-0.0779, p=0.9), nor in trout exposed to the "high" treatment (two sample t-test, $t_{df\ 8}$=2.17, p=0.06). No differences in the activity of AOX were detected between samples from the "control", "low" and "high" treatments at week one (one-way ANOVA, $F_{df\ 2,12}$=0.534, p=0.6) and week three of exposure (one-way ANOVA, $F_{df\ 2,12}$=0.615, p=0.6), or at ten weeks after terminated exposure (week 13, one-way ANOVA, $F_{df\ 2,12}$=1.52, p=0.3). There were no differences between all treatments in samples collected from the reference group (week zero), nor any changes over time occurring in trout exposed to the "control" treatment (one-way ANOVA, $F_{df\ 2,12}$=0.404, p=0.7, and $F_{df\ 3,16}$=0.148, p=0.9, respectively).

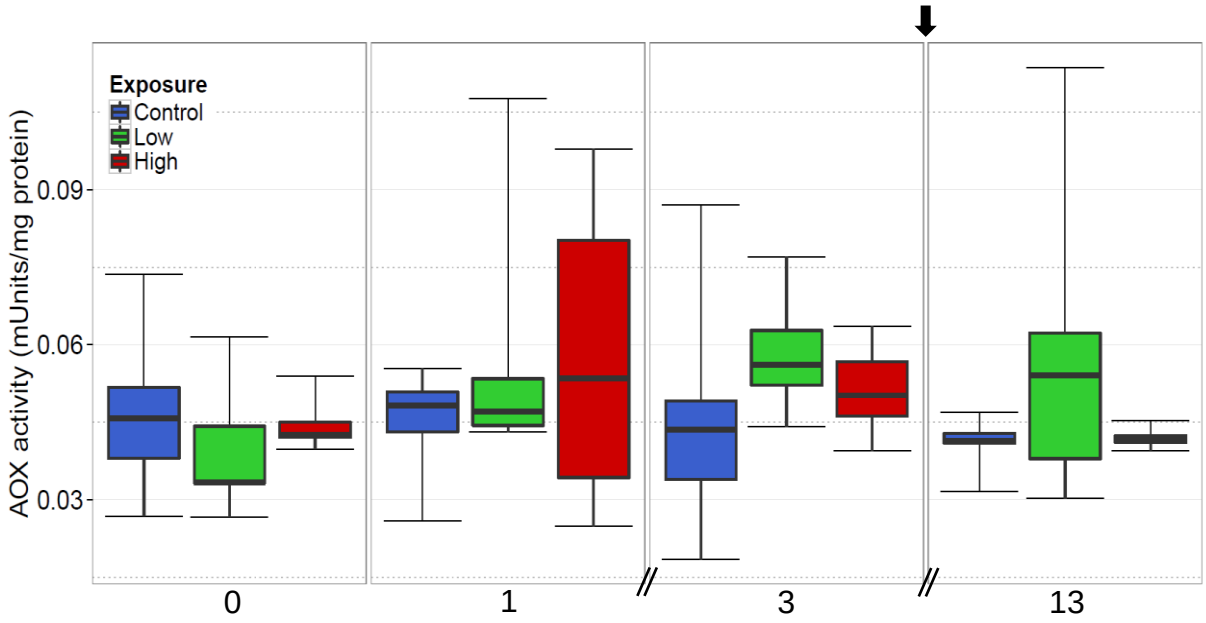


Figure 10. The activity of peroxisomal acyl-CoA oxidase (AOX) in liver of trout from "control" (blue), "low" (green), and "high" (red) treatments at zero, one and three weeks during exposure to PFASs, and at ten weeks after ended exposure (week 13, ended exposure is denoted by the black arrow). Median and quartiles are presented (n=5).

3.2.5 Alanine aminotransferase activity in plasma

The analysis described by Bergmeyer *et al.* (1986) was modified for the detection of alanine aminotransferase (ALAT) activity in smaller volumes of plasma, and further applied to estimate the damage to liver cells of trout after exposure to the PFASs mixtures. Tests during the method modification showed that the pre-incubation period should last for approximately 15 minutes to ensure complete saturation of the alanine aminotransferase and completion of the reactions between NADH and endogenous substances in plasma. During this period, all slopes were slightly decreasing with levels ranging from $-3.07*10^{-5}$ to $-1.12*10^{-5}$ and from $-2.82*10^{-5}$ to $3.04*10^{-6}$ for Tris/L-alanine (overall reaction) and Tris/D-alanine (individual sample blank), respectively.

Almost all test samples showed a lag phase of approximately 300 seconds after the addition of Tris/α-ketoglutaric acid. However, continuous measurements after this period resulted in a monotonously decreasing $\Delta OD/\Delta t$ in samples for the overall reaction. This decrease was significantly higher than in the samples for individual sample blanks (Kruskal-Wallis, $\chi^2_{df\ 1}= 21.5$, $p<0.001$).

Volumes of 5.0, 10 and 20 µL, and 20, 30 and 40 µL from the same plasma sample were tested using two different 96-well plates. The calculated enzyme activities of the first plate (5.0, 10 and 20 µL sample volumes) were slightly lower than the activities of the second plate (20, 30 and 40 µL), with median values of 47.0 Units/L (34.9 to 62.3 Units/L) and 53.5 Units/L (53.1 to 63.0 Units/L), respectively. The ALAT activity calculated using the samples with 5.0 µL plasma had the highest variability (34.9 Units/L to 62.3 Units/L) and was evaluated to not give sufficiently accurate estimates of the enzymatic activity. All other sample volumes yielded relatively identical activity estimates, and a volume of 15 µL for each well in the 96-well plate was chosen to be used in the analysis of ALAT activity in plasma of brown trout exposed to PFASs.

There were no significant differences in the activity of ALAT in plasma during exposure to PFASs in trout from the "low" (one-way ANOVA, $F_{df\ 2,11}$=0.217, p=0.8) or "high" treatments (one-way ANOVA, $F_{df\ 2,12}$=1.57, p=0.2) (Figure 11). Furthermore, there was no significant difference when comparing samples collected at week three of exposure and week ten of depuration (week 13, two sample t-test, $t_{df\ 8}$=1.27, p=0.2, and $t_{df\ 8}$=-1.30, p=0.2 for the "low" and "high" treatments, respectively). The one-way ANOVA did not show any significant changes in the ALAT activities between trout exposed to the "control", "low" and "high" treatments at week one (one-way ANOVA, $F_{df\ 2,10}$=0.232, p=0.8) and week three of exposure (one-way ANOVA, $F_{df\ 2,12}$=1.01, p=0.4), nor at week ten after termination of the exposure (week 13, one-way ANOVA, $F_{df\ 2,12}$=1.45, p=0.3). No differences were found between the treatments in the reference group (week zero, one-way ANOVA on log-transformed data, $F_{df\ 2,11}$=0.829, p=0.5), nor over time in trout exposed to the "control" treatment (one-way ANOVA, $F_{df\ 3,14}$=0.114, p=1).

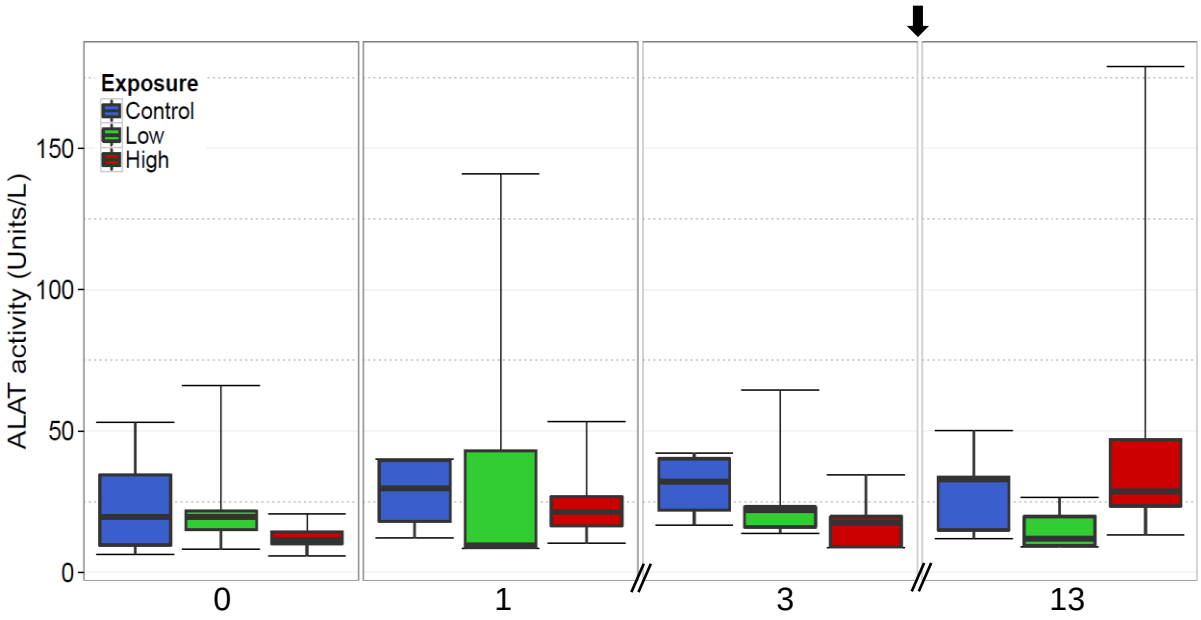


Figure 11. Alanine aminotransferase (ALAT) activity in plasma of trout from "control" (blue), "low" (green), and "high" (red) treatments at zero, one and three weeks during exposure to PFASs, and at ten weeks after ended exposure (week 13, ended exposure is denoted by the black arrow). Median and quartiles are presented (n=5, except "control" at time zero and one where n=4).

3.2.6 Combined analysis of biomarker responses

The biplots from the principal component analyses (PCA) using standardized and centred biomarker responses in brown trout after zero, one, and three weeks of exposure, and ten weeks of depuration to PFASs are shown in Figure 12. Among the reference samples (week zero, Figure 12A), the variation in the responses can be 33% and 28% explained by the first and second principal components (PCs), respectively. The highest proportions of the variance explained by the PCs (75% in total) are in samples from week one of exposure (Figure 12B), where PC1 is 54% and PC2 is (21%). After three weeks of exposure (Figure 12C), PC1 and PC2 explain 40% and 22% of the total variation, respectively. For the last sampling week, at ten weeks of depuration (Figure 12D), PC1 and PC2 explain 39% and 26%, respectively.

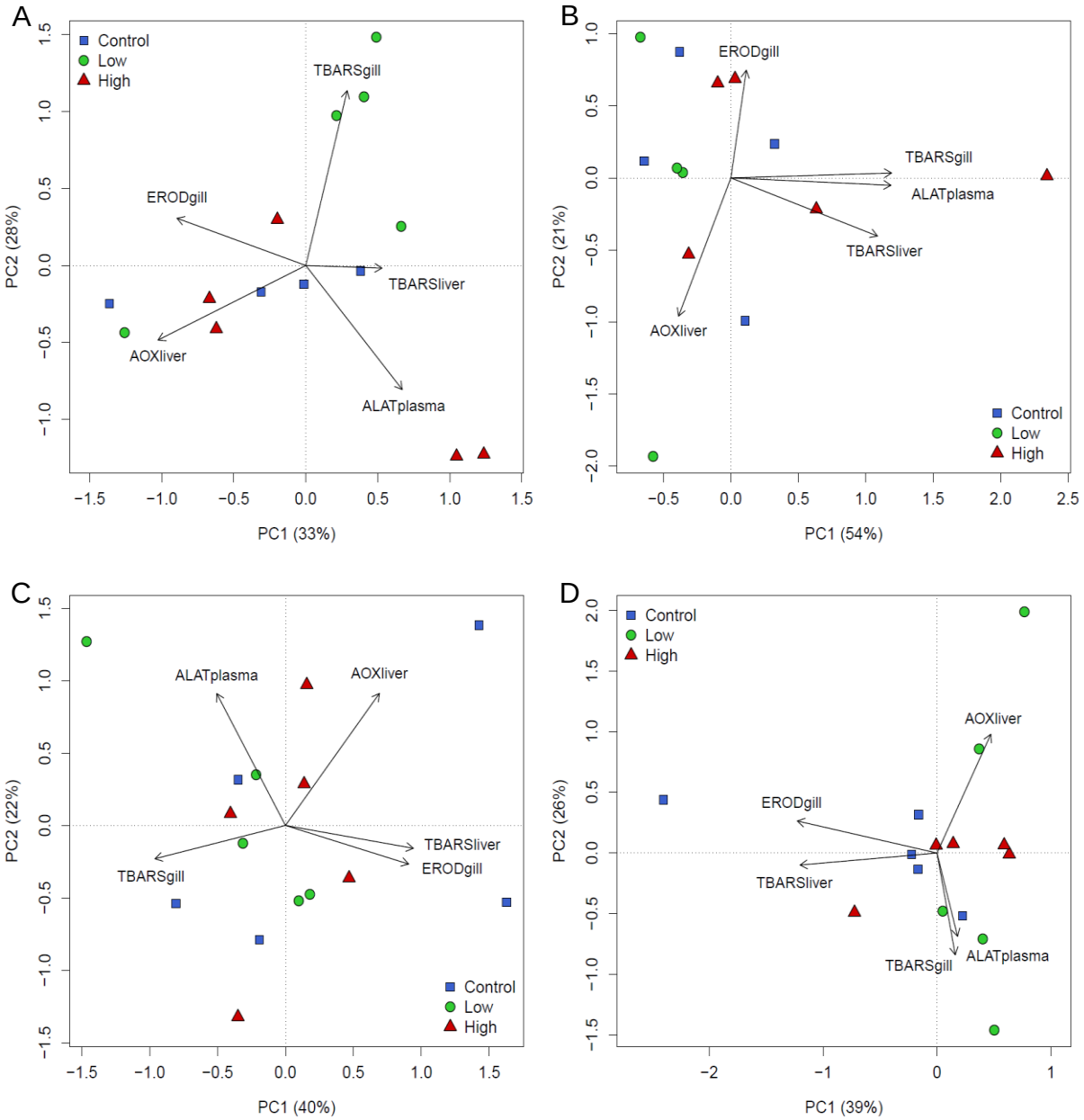


Figure 12. Biplots of standardized and centred biomarker responses in trout from different treatments on their principal components extracted from principal component analyses (PCA) at A) week zero (reference), B) week one, and C) week three of exposure to PFASs, and D) ten weeks after ended exposure. Biomarkers are ethoxyresorufin *O*-deethylase (EROD) activity in gills, thiobarbituric acid reactive substances (TBARS) in gills and liver, peroxisomal acyl-CoA oxidase (AOX) activity in liver, and the activity of alanine aminotransferase (ALAT) in plasma. Arrows represent the loadings of different biomarkers. Percentages of the total variation explained by the PC's are in brackets. (n=5, except A "control" where n=4, and B "control" and "low" where n=4).

The PCA biplots in Figure 13 shows the distributions of the standardized and centred biomarker responses in brown trout exposed to the "high" and "low" concentrations of PFASs sampled at week zero (reference), week one and week three during exposure, and ten weeks after ended exposure (week 13). The PC axes in the biplot of responses in trout exposed to the "high" concentration (Figure 13A) explain 41 and 22% of the total variation, while the biplot for fish from the "low" treatment (Figure 13B) shows a PC1 explaining 43% and a PC2 explaining 21% of the variation.

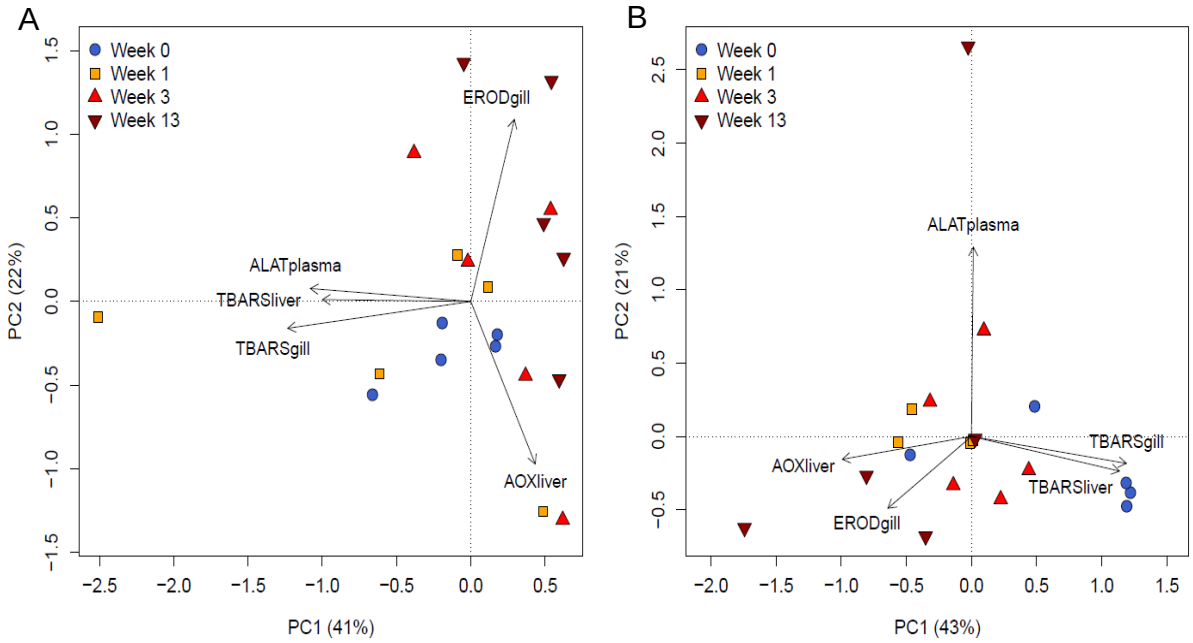


Figure 13. Biplots of the standardized and centred biomarker responses in trout from week zero (reference), one, and three weeks of exposure, and ten weeks after ended exposure to PFASs (week 13) on their principal components extracted from principal component analyses (PCA) in A) "high" and B) "low" treatments. Biomarkers are ethoxyresorufin *O*-deethylase (EROD) activity in gills, thiobarbituric acid reactive substances (TBARS) in gills and liver, peroxisomal acyl-CoA oxidase (AOX) activity in liver, and the activity of alanine aminotransferase (ALAT) in plasma. Arrows represent the loadings of different biomarkers. Percentages of the total variation explained by the PC's are in brackets. (n=5, except B week one where n=4).

4. Discussion

Harstad/Narvik Airport Evenes is arguably one of the most PFAS-polluted airports in Norway (Sweco 2012). This study exposed juvenile brown trout to a mixture of fire foam-related PFASs chosen to resemble the contamination in Lavangsvatnet close to Evenes Airport. The aim was to investigate the bioconcentration, elimination and effects on biomarkers of oxidative stress (lipid peroxidation levels and acyl-CoA oxidase activity), induction of metabolic enzymes (ethoxyresorufin *O*-deethylase activity) and liver damage (alanine aminotransferase activity). In addition, the biomarker results have been briefly discussed in relation to the results from a parallel study investigating the effects on gene expression of selected genes in the same trout exposed to PFASs (Hellstad 2015).

4.1 Bioconcentration and elimination of PFASs in trout

4.1.1 Exposure conditions and detection of PFASs

PFASs were present above the analytical detection limit in fish from both the "high" and "low" treatments. As expected, PFOS had the highest concentrations in trout during the exposure and depuration periods. PFHxS had the second highest concentration and was detected in 59% of the samples taken during and after terminated exposure (not including reference samples). This is in accordance with the PFASs found in fish collected from Lavangsvatnet, where PFOS had the highest concentration followed by PFHxS (Norconsult and Sweco 2015). 6:2 FTS, PFPeA and PFHxA were below the limit of detection in all trout samples, i.e. there were no detectable bioconcentration of these PFASs following three weeks exposure of fish to concentrations at 108, 57.2 and 37.8 µg/L (median levels from the "high" treatment) in water, respectively. Although PFOA had the lowest nominal concentration (in water) of the selected PFASs, this compound was detected in two of the five trout collected from the "high" treatment following one week of exposure. The analyses had relatively low sensitivity and subsequently high limits of detection (maximum DL=177 µg/kg) for the barely and undetected PFASs compared to the preferred DL of 1 µg/kg. Less background noise in the analyses would possibly have resulted in the detection of more PFASs present in the nominal mixture in trout. On the other hand, a higher level of analytical uncertainty follows concentrations close to the detection limits. Due to the low or non-detected concentrations of

6:2 FTS, PFPeA, PFHxA and PFOA, the bioconcentration factors and half-lives could not be calculated for these compounds.

PFOS, PFBA, PFNA and PFDA were detected in trout sampled before initiation of the exposure (reference samples) and in the fish not exposed to PFASs ("control" treatment). This indicates contamination, possibly by food (pellets) given before acclimatization or by cross-contamination from the "high" and "low" treatment aquaria. PFBS and PFHpA were detected in water from the "high" and "low" treatments during exposure indicating that the selected compounds were not of preferred purity and thereby causing the detection of other than nominal PFASs in water. These compounds (PFBS and PFHpA) did, however, not seem to bioconcentrate in trout at the observed concentrations in this study.

The water concentrations (median) of PFOS reached 62% and 57% of the nominal concentration during the three weeks of exposure in trout from the "low" and "high" treatments, respectively. For PFHxS, the concentrations (median) reached 98% and 81% of the nominal concentration. Martin *et al.* (2003a) observed that the concentration of PFOS detected in water were lower than nominal (and of the other PFAS concentrations with fewer C-F bonds) particularly early in their experiment and despite the constant flow-through conditions. This was explained by a higher biomass in the aquaria close to initiation and due to the more lipophilic properties of PFOS, compared to other PFASs with shorter fluoroalkyl chains, which resulted in higher uptake rates and consequently less dissolved compound in the water. In the present study, the other PFASs in the mixture were present at concentrations equal to or slightly higher than nominal concentrations during the exposure period. A different study described a relatively low water solubility for PFOS (910 mg/L), suggesting a greater surface activity for this compound compared to PFOA (3300 mg/L) (Inoue *et al.* 2012), which also supports the results presented here and by Martin and co-authors (2003a).

4.1.2 Bioconcentration

The concentrations of PFOS increased significantly in trout exposed to the "high" concentration from the reference group (week zero) to the samples collected three weeks after initiation of the exposure. The levels were, however, not significantly different after only one week of exposure. In fish exposed to the "low" concentration, PFOS did not increase significantly during the exposure period and individual variations were observed. The PFHxS

concentrations in fish were significantly higher in both treatments after three weeks of exposure, as well as significantly increased after only one week in trout exposed to the "high" concentration. Between the various treatments sampled at week one and week three of the exposure period, only the "high" treatment showed significantly higher concentrations (of both PFOS and PFHxS) compared to trout from both the "low" and "control" treatments.

In accordance with the results from the study presented here, Martin *et al.* (2003a) showed that PFOS and PFHxS bioconcentrated significantly in juvenile rainbow trout (*Oncorhynchus mykiss*) during 12 days of waterborne exposure. The same compounds were also found to accumulate in juvenile and adult rainbow trout after dietary exposure (Martin *et al.* 2003b, Goeritz *et al.* 2013, Falk *et al.* 2015). In the current study, PFASs with a sulfonate group reached higher concentrations in brown trout than the carboxylated compounds. This is consistent with existing knowledge (Martin *et al.* 2003a, Labadie and Chevreuil 2011, Inoue *et al.* 2012, Goeritz *et al.* 2013, Falk *et al.* 2015). The observation is, however, partly biased as the sulfonated PFASs (PFOS and PFHxS) had the highest nominal concentrations in the PFAS mixture. 6:2 FTS was also expected to be detected in the exposed fish because of its third highest concentration and sulfonate functional group. This compound and its alcohol precursor (6:2 FTOH) have, however, previously been found to biodegrade in experiments with bacteria and aerobic soil (Key *et al.* 1998, Liu *et al.* 2010). Short carboxylated perfluoroalkyl substances (seven or less fluorinated carbons) have not been considered to accumulate in organisms (Conder *et al.* 2008), which could explain why PFPeA, PFHxA and PFOA (with the exception of two samples from the "high" treatment) were not detected in trout exposed in this experiment. A higher than preferred detection limit (118 µg/kg compared to 1 µg/kg) made it, however, impossible to detect a low bioconcentration of these compounds had it been present.

Tissues of trout were not analysed separately in this study. Other studies have shown that the rate of uptake, bioconcentration and measured concentrations of PFASs vary with tissues analysed and are generally higher in organs rich in blood (e.g. liver and kidneys) compared to lipid and muscle tissues (Martin *et al.* 2003a, Labadie and Chevreuil 2011, Falk *et al.* 2015). In this study, differences in temperature during the experimental periods were minimal (8.8°C ± 0.8°C); however, higher metabolic rates (and consequently increased bioconcentration of toxicants through active transport or increased ventilation rates) have been reported in fish with increasing temperature (Cairns *et al.* 1975). In the study presented here, both PFOS and

PFHxS bioconcentrated significantly in trout during the three weeks of exposure to the selected PFASs, and thus disproved the first null hypothesis (1.) for these two compounds.

4.1.3 Bioconcentration factors of PFOS and PFHxS

The bioconcentration factors (BCFs) were calculated for two sulfonated PFASs. The BCF of PFOS (244 and 185 L/kg) was 38 and 17 times higher than the BCF of PFHxS (6.34 and 11.2 L/kg) in the "high" and "low" treatments, respectively. This is in accordance with previous knowledge where the BCFs of PFASs have been reported to be positively correlated with the carbon chain length, until a point where it is limited by the compound's molecular size (Conder *et al.* 2008). In addition, BCFs have been shown to be higher for compounds with sulfonate functional groups compared to carboxylated PFASs of the same chain length (Martin *et al.* 2003a), which can be explained by their stronger binding affinities to proteins (Ng and Hungerbühler 2013).

Martin *et al.* (2003a) calculated that PFOS had the highest BCFs (1100 L/kg in carcass) after 12 days of exposure in a flow-through system with juvenile rainbow trout and a mixture of PFASs. A different study exposed juvenile common carp (*Cyprinus carpio*) to PFASs in individual flow-through tanks for 58 days and calculated the BCF of PFOS (whole fish) ranging between 720 and 1300 L/kg (Inoue *et al.* 2012). In adult marbled flounder (*Pseudopleuronectes yokohamae*), the BCF of PFOS was calculated to 920 L/kg after 28 days of exposure (Sakurai *et al.* 2013). These estimates are all in the same range and three to seven-folds higher than the BCF of PFOS shown in this present study. On the other hand, the BCF of PFOS have been calculated to 95 L/kg in northern leopard frog (*Rana pipiens*) (Ankley *et al.* 2004), which is the only estimate of bioconcentration that is lower than those found in the present study. Bioconcentration factors of PFHxS are mostly absent from literature. However, Martin *et al.* (2003a) estimated a considerably lower BCF of PFHxS (9.6 L/kg in carcass) than of PFOS, and this value is in the same range as the BCF of PFHxS estimated in this study.

The lipophilic and hydrophilic properties are usually taken into consideration when calculating the BCF of a compound (Barron 1990). However, PFASs do not partition evenly in lipid or water. Consequently, prudence should be exercised when estimating the BCFs as the current methods may not adequately characterize the bioconcentration of these compounds

(Arnot and Gobas 2006). A steady-state between the uptake and the elimination is assumed when estimating the BCFs (Barron 1990). This was not the case for PFHxS and PFOS in market-size rainbow trout after 28 days dietary exposure to PFASs (Goeritz *et al.* 2013), or after 12 days of waterborne exposure in juvenile rainbow trout (Martin *et al.* 2003a). The time to reach steady state have been predicted to approximately 43 and 30 days for PFOS and PFHxS, respectively (Martin *et al.* 2003a, Martin *et al.* 2003b). In this present study, it cannot be proven that a steady-state was attained, which might have been the cause for the relatively low BCF of PFOS compared to other studies. However, the concentrations of especially PFHxS, but also of PFOS, in water seemed to be stabilizing towards the end of the 20 days of exposure.

4.1.4 Elimination and half-life of PFOS and PFHxS

The concentrations of PFOS in trout from the "high" and "low" treatments did not decrease during the ten weeks of depuration. A significant elimination would, however, likely have occurred with a longer time of sampling after terminated exposure. Two previous studies have estimated the half-life of PFOS in juvenile rainbow trout (Martin *et al.* 2003a) and in juvenile common carp (Inoue *et al.* 2012) after waterborne exposure to be 15 days (carcass) and 45 to 52 days (whole fish). After dietary exposure, the half-life has been calculated to 11, 13 and 16 days in carcass (Martin *et al.* 2003b, Falk *et al.* 2015) and whole body (Goeritz *et al.* 2013) of juvenile and adult rainbow trout. Despite the differences between these estimates, they are all lower than the non-measurable whole body half-life of PFOS in brown trout presented here. Furthermore, the half-life of PFOS in this study was more consistent with the half-lives calculated in chickens (125 and 231 days), monkeys (approximately four months), and humans (4.8 years) as these estimates are longer than previously reported in fish (Olsen *et al.* 2007, Yeung *et al.* 2009, Chang *et al.* 2012, Tarazona *et al.* 2015).

The PFHxS concentrations were found to be significantly reduced in trout from the "high" treatment collected ten weeks after ended exposure. No significant decrease could be detected in fish exposed to the "low" concentration due to individual variation. The half-life of PFHxS in trout (whole body) was estimated to 2.3 weeks (16.1 days) for both the "high" and "low" concentrations. This is approximately five days longer than previously reported for carcass of juvenile rainbow trout after waterborne exposure (Martin *et al.* 2003a), and seven to nine days longer than from carcass and whole body of rainbow trout after exposure through food

(Martin *et al.* 2003b, Goeritz *et al.* 2013, Falk *et al.* 2015). Still, the half-life presented in this study is shorter than the estimates reported in rodents (one month), monkeys (four months) and humans (7.8 years) (Olsen *et al.* 2007, Sundström *et al.* 2012).

Of the PFHxS concentrations measured at week one and week ten of depuration, trout exposed to the "high" concentration showed significantly higher levels of PFHxS compared to the "low" and "control" treatments, but the "low" treatment fish did not differ from the "control". This was, however, not the case for PFOS where the concentrations were significantly different in trout from all treatments sampled at week one and week ten after termination of the exposure, and this corresponds to what was expected from the exposure regime.

A positive relationship between the chain length and the estimated half-life has previously been shown for carboxylated PFASs, but not for sulfonated PFASs (Martin *et al.* 2003a, Martin *et al.* 2003b, Lau *et al.* 2007). In the study presented here, PFHxS was eliminated faster than PFOS, suggesting a possible positive correlation between the fluorinated chain length and the estimated half-life of sulfonated PFASs, as shown for the PFASs with carboxylate functional groups. Several studies have also shown that the sulfonated PFASs (e.g. PFOS and PFHxS) have longer half-lives than the carboxylated PFASs of the same carbon chain length (Martin *et al.* 2003a, Martin *et al.* 2003b, Falk *et al.* 2015). This could, however, not be determined in the present study because only a few of the trout had detectable concentrations of one of the carboxylated PFASs (PFOA) in the nominal mixture.

Estimation of the half-life was assumed to fit a one-compartment model and first-order exponential decay. These assumptions could, however, not be confirmed due to the limited number of sampling weeks after the exposure had been terminated. A longer depuration period and more frequent sampling could have produced different half-life estimates, especially for PFOS. For both PFOS and PFHxS in fish from the "low" treatment, the fact that no decreases in concentrations were detected in trout collected one week after terminated exposure was probably caused by individual and analytical variations. This lag in elimination could, however, also indicate that the half-life estimation of PFASs fit a two or multi-compartment model instead of a one-compartment model (Newman and Clements 2008 p. 119, Timbrell 2009 p. 64). The simple one-compartment model has also been questioned when estimating the pharmacokinetics for PFOS (Tarazona *et al.* 2015), and the half-lives of PFOS and PFHxS have been shown to vary with tissues analysed (Martin *et al.* 2003a, Martin

et al. 2003b, Falk *et al.* 2015). In the present study, PFOS was not eliminated from trout during the depuration period (ten weeks), which supports the second null hypothesis (2.) for this compound. On the other hand, the concentration of PFHxS decreased significantly in trout after terminated exposure, which resulted in a half-life estimate of 2.3 weeks, and disproved the second null hypothesis for this compound.

4.2 Effects of PFASs

4.2.1 Ethoxyresorufin *O*-deethylase activity in gills

No significant increase in the activity of EROD in gills was detected in trout exposed to the "low" PFAS concentration during the three weeks of exposure. Fish from both the "control" and "high" treatments showed significant increases in the EROD activity from week zero to week one and week three of exposure to PFASs. As the same pattern of significance was found in fish from the "control" treatment the elevated activity detected in the "high" treatment probably originated from stress and unknown influences. Handling stress during laboratory experiments did, however, not significantly affect the EROD activity of Arctic charr (*Salvelinus alpinus*) (Jørgensen *et al.* 2001).

In the "low" treatment, EROD activity was elevated in trout collected at ten weeks of depuration, which indicates an induction of the CYP1A enzymes by the selected PFASs. No significant changes were, however, detected in trout exposed to the "high" treatment. Less variation was shown in the activity of EROD between trout from the "control" treatment, compared to the "low" and "high" treatments, and no significant differences were detected between week three of exposure and week ten of depuration. At the last day of exposure (week three), the EROD activity was increased in trout from the "high" treatment and decreased in the "low" treatment compared to the "control". Similar results have not been reported by other studies and this pattern was not detected within any other sampling weeks.

To the author's knowledge, no studies on the induction of EROD in gills of fish by a mixture of fire foam-related PFASs have yet been published. PFOS has been shown not to cause a significant increase in the EROD activity in gills of green mussel (*Perna viridis*) after a seven-day exposure period (Liu *et al.* 2014b). A significant decrease in the activity was, however, observed in mussel exposed to the highest concentration (1.0 mg/L PFOS). The authors suggest that the tested PFASs are not biotransformed by the CYP1A enzymes or that

the EROD enzyme could be inhibited by PFOS. This inhibition has been shown in human hepatocytes, however the CYP1A enzymes showed less inhibition by PFOS than the other CYP-enzymes analysed (Narimatsu *et al.* 2011).

Studies by Kim *et al.* (2010) and Bilbao *et al.* (2010) showed no induction of the EROD enzyme in liver when exposing juvenile common carp and thicklip grey mullet (*Chelon labrosus*) to 50 and 2.0 mg/L PFOS through water (four and 16 days of exposure, respectively). In contrast, the activity of EROD in liver was elevated in freshwater tilapias (*Oreochromis niloticus*) after three days of dietary exposure (10, 20 and 30 mg/L PFOS) (Han *et al.* 2012). Hu *et al.* (2003) showed that PFOS alone did not induce CYP1A1 (EROD activity) in the hepatocytes of desert topminnow (*Poeciliopsis lucida*) and rat hepatoma cells. However, the authors reported an increased activity of as much as 40% when exposing cells to a combination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PFOS, compared to when the cells that were only exposed to TCDD. Hu and co-authors (2003) suggested that PFOS increased the permeability of cells to hydrophobic substances (such as TCDD) by affecting the cell membrane, rather than having specific effects directly on the CYP1A enzymes. It is, however, difficult to extrapolate the results from *in vitro* on to *in vivo* experiments and effects. Hu *et al.* (2003) also investigated whether PFHxS had an effect on the activity of EROD in fish hepatocytes; however, no changes were detected within the range of the tested concentrations. Watanabe *et al.* (2009) found that an exposure to PFOS alone did not change the EROD activity in chicken embryo hepatocytes (0.1, 1.0 and 10 µM exposure after 24 hours incubation). The authors suggested that PFOS is such a weak EROD inducer that if a more prominent inducer was present (e.g. TCDD), PFOS would have no effect on the already induced EROD activity.

From the studies described above it can be concluded that PFOS and PFHxS do not induce EROD activity when administered separately. In this study, the significant induction of this metabolic enzyme detected in gills of trout exposed to the "low" concentration of fire foam-related PFASs (collected at ten weeks of depuration) disproved the third null hypothesis (3.), and the result should be further investigated.

4.2.2 Thiobarbituric acid reactive substances in gills

The levels of lipid peroxides (TBARS) in gills did not change over time in trout from the "control", "low" or "high" treatments during exposure to PFASs, nor were there any differences after ten weeks of depuration. No differences were detected in TBARS when comparing the treatments collected at each sampling week. However, the results during exposure, depuration and between treatments might have been statistically significant if less individual variation had been present.

Not many studies have analysed the levels of lipid peroxidation in gills after exposure to PFASs, and to date there are no published articles on changes in the levels of TBARS in gills of fish caused by the exposure to PFHxS. Munyemera (2014) exposed juvenile Atlantic cod (*Gadus morhua*) to 100 and 200 µg/L PFOS. None of the results were significantly different from the control, but the lipid peroxidation in the gills of cod did slightly increase after three and nine days of exposure to the "low" concentration. After nine days of exposure in cod from the "high" treatment, TBARS were stated as lower compared to the "control" and "low" treatments (Munyemera 2014). In comparison, the study presented here had concentration of PFOS in water (from the "high" treatment) that was 1.4 times higher than the highest concentration yielding no significant changes in the study by Munyemera (2014); in addition to a two-fold longer time of exposure.

Another study detected significant increases in the levels of TBARS in green mussel when exposed to 1.0 and 10 mg/L of PFOS for seven days (Liu *et al.* 2014a). The authors concluded that the induction of TBARS was a result of the ROS production exceeding the antioxidant capacity leading to oxidative damage to lipids. However, caution should be taken when comparing results on toxicity between vertebrates and invertebrates. Consequently, from an increased level of TBARS in the green mussel (after exposure to PFOS) it cannot be concluded that the same result would occur in fish.

The TBARS test should be used with prudence (when body fluids and tissue extracts are analysed) due to the detection of other aldehydes in addition to MDA or that the peroxide decomposition produces radicals that amplifies the responses given by the analysis (Halliwell and Chirico 1993). In the present study, the exposure to PFASs did not cause lipid peroxidation in the gills of trout and the fourth null hypothesis (4.) was supported. More studies should, however, be conducted to examine possible changes in the TBARS levels in gills after exposure to single substances and mixtures of PFASs.

4.2.3 Thiobarbituric acid reactive substances in liver

The levels of lipid peroxidation (TBARS) in the liver of trout did not change during the exposure period in the "low" and "high" PFASs treatments. Although not statistically significant, there were decreases in the hepatic TBARS levels ten weeks after ended exposure in trout exposed to the "low" concentration when compared to the levels detected at week three of exposure. This decrease was, however, also present in the control treatment and was possibly caused by individual variation. No differences were detected in the levels of hepatic lipid peroxidation when comparing trout from the "control", "low" and "high" treatments collected at each time of sampling.

Oakes et al. (2005) investigated the effects of PFOS on different species and life-stages of fish. Male fathead minnow (*Pimephales promelas*), juvenile rainbow trout, adult creek chub (*Semotilus atromaculatus*) (both genders) and female spottail shiner (*Notropis hudsonius*) showed elevated levels of hepatic lipid peroxidation after exposure to 3.0 mg/L PFOS for 12, 14 or 28 days. Female rainbow trout also showed elevated TBARS in the liver after 14 days with 1.0 mg/L exposure, and female fathead minnow had significantly more lipid peroxidation after exposure to the "low" treatment (0.3 mg/L PFOS) for 28 days. Adult white sucker (Catostomus commersoni) of both genders was the only species that did not show changed TBARS levels in the liver after exposure to PFOS for 14 and 21 days (3.0 mg/L) (Oakes *et al.* 2005).

Juvenile goldfish (*Carassius auratus*) showed increased lipid peroxidation levels in the liver after exposure to 0.5 mg/L PFOS for four days (Feng *et al.* 2015). TBARS was also significantly elevated compared to the control in combined exposures with PFOS and copper (0.5 mg/L PFOS + 50 µg/L Cu and 5.0 mg/L PFOS + 200 µg/L Cu) (Feng *et al.* 2015). Juvenile Atlantic salmon (*Salmo salar*) exposed to a diet containing PFOS (0.2 mg/kg) showed significantly elevated hepatic levels of lipid peroxidation after two days and significantly reduced levels after eight days of exposure. An increase was again detected seven days after ended exposure (Arukwe and Mortensen 2011).

PFOS have also been shown to increase the TBARS levels in zebrafish (*Danio rerio*) embryos after 92 hours exposure to 1.0 mg/L PFOS (from four to 96 hpf) (Shi and Zhou 2010), and in *in vitro* studies with human neurotypic cell lines and exposure to 5.0, 12.5 and 25 mg/L PFOS (Slotkin *et al.* 2008, Chen *et al.* 2014). Another study exposed primary cultured hepatocytes of freshwater tilapia to PFOS (0, 1.0, 5.0, 15 and 30 mg/L) for 24 hours (Liu *et al.* 2007), and

found that levels of TBARS were unchanged for all concentrations. This was an unexpected result due to the notable increase of ROS observed in the cells after exposure to 15 mg/L.

The effects on hepatic TBARS have been investigated after an airplane fire and the following application of approximately 48000L aqueous film-forming foam (AFFF) in Toronto, Canada in 2005 (Oakes *et al.* 2010). After the spill, PFOS and 6:2 FTS was two of the detected PFASs in liver of blacknose dace (*Rhinichthys atratulus*) nine days after the AFFF application. Hepatic TBARS of dace were significantly lowered downstream of the application site, while an increase was observed in creek chub. 120 days after the application, only creek chub sampled around the application site had elevated TBARS levels possibly reflecting their diet. The authors concluded that the accidental application of AFFF did not have a significant impact on the fish community. A different field study on wood mice (*Apodemus sylvaticus*), sampled in close approximation to a fluorochemical plant in Antwerp, Belgium in 2002, showed a significant relationship between the hepatic TBARS and the PFOS liver concentrations (0.47 mg/kg to 179 mg/kg) (Hoff *et al.* 2004). This indicated oxidative damage to the hepatic tissues in this heavily polluted population of wood mice.

In comparison with the levels of hepatic TBARS presented here, there is no clear indication that the concentrations of PFASs used in this study should cause significant lipid peroxidation in the exposed trout. Only female fathead minnow (and possibly also juvenile goldfish) showed increased lipid peroxidation at relevant PFOS concentrations, and species and gender differences in the sensitivity of TBARS are evident. Oakes *et al.* (2010) found effects on TBARS when only 219 to 350 µg/kg PFOS was detected in fish liver from field. These concentrations are much lower than PFOS detected in the whole body of trout in this study, but the effects on TBARS found by Oakes and co-authors (2010) were probably influenced by environmental factors and other xenobiotics causing lipid peroxidation. In the study presented here, a mixture of the selected PFASs did not cause lipid peroxidation in the liver of trout and hence supported the fourth null hypothesis (4.).

4.2.4 Peroxisomal acyl-CoA oxidase activity in liver

No significant differences were detected in the activity of hepatic AOX in trout from all the treatments during and after ended exposure to PFASs. There was, however, a slight decrease in the activity from week three of exposure to ten weeks of depuration in fish exposed to the

"high" concentration. No differences were detected in the AOX activity between trout exposed to the "control", "low" and "high" treatments within each time of sampling.

Oakes *et al.* (2005) (in addition to investigating the effect of PFOS on hepatic TBARS) analysed for changes in the AOX activity in liver of different fish species. Juvenile rainbow trout, female rainbow trout, adult creek chub and female spottail shiner showed elevated AOX levels after exposure to 1.0 and 3.0 mg/L PFOS for 12, 14 or 21 days. Adult fathead minnow and adult white sucker (both genders) did, however, not show significant changes in the activities after exposure to 3.0 mg/L PFOS. The authors concluded that PFOS is a peroxisome proliferator in a number of fish species, although species differences are notable. Another study on thicklip grey mullet showed induced AOX activity after two days of exposure to only the "high" concentration of PFOS (2.0 mg/L) (Bilbao *et al.* 2010), and it was concluded that PFOS does not seem to act as a typical peroxisome proliferator in this species of fish.

After the application of AFFF during an airplane fire in Toronto, Canada, the AOX activity in liver of blacknose dace was significantly reduced directly downstream of the application site at day nine, but significantly increased 120 days after application (Oakes *et al.* 2010). A different species of fish (creek chub) showed decreased activity immediately below the application site only at day 120. The effect of PFOS on the hepatic AOX in juvenile common carp after a single intraperitoneal injection was investigated by Hoff *et al.* (2003a). No significant changes were detected for the measured concentrations of 16, 270 and 864 ng/g PFOS in liver five days after injection, but the authors speculated that PFOS could be a weak peroxisome proliferator in carp.

Studies on the activity of AOX in liver of male rats have shown significant increases after single intraperitoneal injections or seven days of dietary exposure to 100 mg/kg PFOS (Berthiaume and Wallace 2002, Elcombe *et al.* 2012). The AOX activity was also increased in male rats after four weeks of exposure to 20 mg/kg PFOS, but this increase was not persistent in samples taken ten weeks later (Seacat *et al.* 2003). The authors concluded that PFOS did not cause peroxisome proliferation in rats at the tested concentrations (20 mg/kg as the highest treatment concentration), which was also supported by a different study using rats showing similar results in the hepatic AOX activity after seven days of dietary exposure to 20 mg/kg PFOS (Elcombe *et al.* 2012). In cynomolgus monkeys (*Macaca fasicularis*), a significant increase in the AOX activity was shown after exposure to 0.75 mg/kg/day of PFOS (Seacat *et al.* 2002). However, the increase was not above the criteria for biological

significance (two-fold higher than the control levels) and the authors concluded that PFOS did not cause peroxisomal proliferation in monkeys.

Differences in the effect of PFOS on the hepatic AOX activity are evident between various species in the studies mentioned above, and little information is available for the effect of PFHxS. In comparison with the study presented here, only higher concentrations have been shown to cause effects on the hepatic AOX and it can be concluded that no peroxisome proliferation was expected from the chosen PFAS concentrations in this study. Some studies have shown that the activities normalized to grams of tissue are more sensitive indicators for the levels of AOX and oxidative damage (Oakes *et al.* 2004, Oakes *et al.* 2005). In the present study, the AOX activity was, however, only reported as values normalized to protein concentrations. No significant peroxisome proliferation was detected in the liver of trout exposed to the mixture of PFASs, and the fourth null hypothesis (4.) was supported. More studies are needed to evaluate the effects of lower concentrations of PFASs, both after singular and combined exposure.

4.2.5 Alanine aminotransferase activity in plasma

After adapting the method for measuring ALAT activity in small plasma samples from trout, the analysis was used to investigate the liver tissue damage during and after terminated exposure to the mixture of PFASs. No differences in ALAT activity were detected in trout during the exposure and depuration periods. Neither were there any differences between fish exposed to the "control", "low" and "high" treatments within each time of sampling.

A detailed method for detecting the levels of ALAT in smaller volumes of plasma has previously not been published. The plates run as tests showed a more rapid decrease in samples added the overall reaction mixture (Tris/L-alanine) than for those added the mixture for individual sample blank (Tris/D-alanine), and this indicated a functioning analysis for measuring ALAT. A minimal volume of 10 µL plasma should be used to get a more accurate estimate of the activity. Only two test-plates were, however, used to evaluate which volumes yielded sufficient accuracy of the estimated activities. Had more replicates of the smallest plasma volume (5.0 µL) been tested, the results might have showed that this minimalistic volume would be enough to acquire precise and detectable activities.

Hoff *et al.* (2003a) was the first to report *in vivo* effects of PFOS on the activity of ALAT in fish. This study exposed juvenile common carp to PFOS through a single intraperitoneal injection, and after five days showed that the activity had a clear dose-dependent relationship with increasing liver concentrations of PFOS (16.0 to 864 µg/kg). The ALAT activities were significantly elevated in the groups with 561, 670 and 864 µg/kg PFOS in the liver. The researchers concluded that PFOS had deleterious effects on the membrane integrity (however not caused by lipid peroxidation) which might have resulted in necrosis of the liver cells.

Other studies have measured the activity of ALAT in field samples, and Hoff *et al.* (2003b) found that the PFOS concentrations in liver and the ALAT activities in plasma significantly correlated in bib (*Trisopterus luscus*), however not in plaice (*Pleuronectes platessa*). The authors speculate that the link between PFOS concentrations and ALAT activities in bib could have been influenced by environmental factors varying along the Western Scheldt. Possible species differences was also shown by Hoff *et al.* (2005) who found that the ALAT activity was significantly positively correlated with the PFOS concentration in liver of common carp and eel (*Anguilla anguilla*), but not in gibel carp (*Carassius auratua gibelio*) collected in Flanders, Belgium. Fair *et al.* (2013) also found a positive correlation between the activity of ALAT and both PFOS and sum sulfonated PFASs (including PFOS and PFHxS) concentrations in plasma of a highly exposed population of Atlantic bottlenose dolphins (*Tursiops truncates*). The possibility of PFOS to induce hepatic damage in the field is plausible; however, the relationships observed could have been affected by the exposure to other xenobiotics (e.g. PAH and BKME have been shown to affect the ALAT activity (van der Oost *et al.* 2003)).

In wood mice collected near a fluorochemical plant in Antwerp, Belgium in 2002, the activity of ALAT showed a negative correlation to the liver PFOS concentration (ranging from 0.47 to 179 µg/g) (Hoff *et al.* 2004). This contradicts what have been shown in male rats exposed to 20 mg/kg PFOS through the diet for 14 weeks (which increased the ALAT activity) (Seacat *et al.* 2003), and in plasma of approximately 47000 adult humans of the C8 Health Project (a significantly positive relationship between the ALAT activity and the PFOS concentration in plasma) (Gallo *et al.* 2012). Cynomolgus monkeys exposed to PFOS (0.03, 0.15, or 0.75 mg/kg/day) for at least 26 weeks did, however, not show any significant increases in the activity of ALAT (Seacat *et al.* 2002). Neither did male rats exposed to 20 or 100 mg/kg PFOS for seven days in a different experiment by Elcombe *et al.* (2012), and it was suggested that PFOS did not cause hepatotoxicity in these species.

Little information on the toxicity of PFHxS has been documented. Butenhoff *et al.* (2009) exposed rats to different concentrations of PFHxS (0.3, 1.0, 3.0 and 10 mg/kg) in a study investigating the reproductive and developmental toxicity of this substance. After 42 days of exposure, no significant differences were detected in the activity of ALAT.

There are evident species-dependent differences in the sensitivity of ALAT when exposed to PFASs. In studies detecting correlations between PFOS and ALAT from field samples it is plausible that these organisms were exposed to additional compounds possibly confounding the observed relationships. From the results presented in this study, it seemed that PFOS (at median water concentrations of 284 and 3.30 µg/L) in a mixture with other PFASs did not cause liver damage in brown trout, and thus supported the fifth null hypothesis (5.). More research is needed to fully characterize the hepatotoxicity of PFASs in fish (singular or in combination), especially conducted in controlled laboratory studies with constant exposure regimes.

4.2.6 Combined analysis of biomarker responses

By conducting a principal component analysis (PCA) it is possible to visualize patterns of observations and variables in a dataset (Bro and Smilde 2014). When producing biplots of the samples within each week, and for trout from the "low" and "high" treatments, no obvious patterns and clustering were observed in the score plots of trout exposed to a mixture of PFASs. Some samples had relatively high values (possible outliers) even after standardization and centration. These observations were not removed from the PCA and are presumably not incorrect, but rather assumed to be extremes due to individual variation (Bro and Smilde 2014).

When investigating the loading plot of the reference samples (week zero), no obvious correlations between the variables were present. After one week of exposure, the levels of TBARS in gills and the ALAT activity in plasma seemed to be positively correlated and largely explained by the first principal component. This pattern was not present after three weeks of exposure, but again visible in the samples collected after ten weeks of depuration. To the author's knowledge, an increased ALAT activity does not have any reasonable connection to the level of lipid peroxidation in gills. However, the correlation could indicate that the trout with low TBARS levels in gills also had low ALAT levels in plasma indicating

low or non-detectable effects of the PFASs. Biomarker variables in the biplots of trout collected at week three of exposure and week ten of depuration both showed a possible positive correlation between the TBARS in liver and the EROD activity in gills. These variables have no likely connections and the correlation was probably due to randomness and individual variation between the trout exposed to PFASs.

The levels of TBARS in gills and liver of trout seemed to be positively correlated in the biplot of fish exposed to the "high" concentration of PFASs. This relationship was also visible in the biplot of trout from the "low" treatment and could be explained by the PFASs causing similar oxidative damage effect patterns to lipids in the liver (due to the high metabolic activity and capacity for elimination of this organ) as to lipids in the gills (as the organ of uptake and possible elimination). In the biplot of biomarker responses in trout from the "high" treatment of PFASs, the activity of ALAT in plasma seem to be positively correlated with both TBARS in the liver and in the gills. This relationship was to be expected as an increased concentration of TBARS indicates higher levels of lipid peroxidation. The cell membranes largely consist of lipids (Newman and Clements 2008 p. 95) and an increase in the hepatic TBARS could result in increased damage to membranes and following leakage of ALAT out from the liver cells and into the extracellular fluid. This relationship between TBARS and ALAT was, however, not visible in trout exposed to the "low" concentration of PFASs. The hepatic TBARS levels were also expected to correlate with the AOX activity in the liver, but this was not observed in any of the biplots.

In conclusion, no obvious patterns were observed in the score plots created by the principal component analysis. The levels of lipid peroxidation in the gills and the liver showed a possible positive correlation in the loading plots of trout exposed to the "high" and "low" PFAS concentrations. None other consistent relationships were observed between the biomarker responses presented in the biplots.

4.3 Relationship of biomarker responses to gene expression

In a parallel study (in addition to the biomarker analyses presented in this study) liver and gills of the same brown trout were analysed for the effects on gene expression of selected genes (Hellstad 2015).

Expression of the CYP1A gene would be expected to be related to the activity of EROD in a sample (Abrahamson 2007, Bilbao *et al.* 2010). The CYP1A expression in gills did not show any significant differences in trout from the "control", "low" and "high" treatments during exposure to PFASs. A non-significant increase in the expression was, however, observed in trout exposed to the "low" concentration from week three of exposure to week ten of depuration. This corresponds to the significantly increased EROD activity in trout from the "low" treatment observed in the present study, and supports the conclusion that the "low" PFAS concentration induces EROD after ended exposure.

The genes PPARα, PPARγ and AOX are involved in lipid metabolism and peroxisome proliferation (Corton *et al.* 2000), and expected to be related to the detection of TBARS and the activity of AOX. Expression of the PPARα and PPARγ genes were not significantly changed in gills of trout following exposure or depuration to PFASs, which coincides with the levels of lipid peroxidation (TBARS) in gills observed in this study (no significant differences). The PPARα gene in liver had a significantly lower expression when comparing week three of exposure to week ten of depuration in trout from the "control" treatment. This trend, however not significant, was also observed in the levels of hepatic TBARS in this study and could be caused by individual variation. The expression of PPARγ in liver increased significantly from week zero to week three of exposure in trout from the "low" treatment, but this was not observed in the measured levels of hepatic TBARS in this study. Gene expression of the AOX enzyme in liver increased significantly in trout from the "low" treatment when comparing samples collected at week zero and week one of exposure to PFASs. The same increase, although not significant, was detected in the hepatic AOX activity presented in this study. No other significant changes in expression of the AOX gene was observed between the other sampling weeks.

Hellstad (2015) also investigated the effect on the ABCb1 (P-glycoprotein) gene, which would be expected to be related to the expression of the ATP-binding cassette transport proteins (Luckenbach *et al.* 2014), and the results showed a significantly elevated expression in the liver of trout exposed to the "high" concentration of PFASs when comparing week three of exposure and week ten of depuration. The hepatic expression of vitellogenin (the egg yolk precursor protein normally only present in oviparous female vertebrates (Mommsen and Walsh 1988)) was significantly increased in fish from the "high" treatment collected at three weeks of exposure. In addition, trout exposed to the "high" concentration of PFASs had elevated expression of vitellogenin compared to fish from the "control" treatment sampled at

week three of exposure and at week ten of depuration. Genes involved in oxidative stress responses (metallothionein, γ-glutamyl synthetase and thioredoxin) were also investigated in the liver and gills of the trout and the results showed no significant induction during the exposure and depuration periods.

5. Conclusions

The bioconcentration, elimination and effects of a fire foam-related PFAS mixture, simulating contamination in Lavangsvatnet near Evenes Airport, were investigated in juvenile brown trout. Of the six PFASs in the nominal exposure, only PFOS and PFHxS were detected in more than two trout sampled during the three weeks of exposure. These compounds were shown to bioconcentrate significantly during the exposure period with BCFs of 244 and 185 L/kg, and 6.34 and 11.2 L/kg for PFOS and PFHxS, respectively. Only the concentrations of PFHxS increased significantly in trout exposed to the "low" levels of PFASs (made to resemble the concentrations detected in Lavangsvatnet).

PFOS was not significantly eliminated from trout during the ten weeks of depuration. The concentrations of PFHxS in trout exposed to the "high" treatment were significantly decreased during the depuration period and the whole body half-life was estimated to 2.3 weeks in both the "high" and "low" treatments. No significant decreases could be detected in fish exposed to the "low" concentration due to individual variation.

The induction of the metabolic CYP1A enzyme (by analysis of the EROD activity) in gills of trout was significantly increased during the exposure period in trout exposed to the "control" and "high" concentrations of PFASs. During depuration, the EROD activity was significantly induced in trout from the "low" treatment (PFAS concentrations resembling contamination in Lavangsvatnet) sampled ten weeks after ended exposure. There were no significant effects on the oxidative damage responses (lipid peroxidation levels and the activity of AOX) or the liver damage (ALAT activity) in trout exposed to the selected fire foam-related PFASs.

Based on the results of this study, the trout population in Lavangsvatnet would be expected to bioconcentrate PFOS and PFHxS, but not the other PFASs likely to originate from the contamination by AFFFs (6:2 FTS, PFPeA, PFHxA and PFOA). In addition, PFOS would be expected to reside in the trout and induce the activity of CYP1A in gills a while after terminated exposure. The concentrations of PFASs found in Lavangsvatnet do, however, not cause oxidative damage or liver damage in the trout when exposed for a period of three weeks.

6. Future recommendations

From the results presented in this study it can be concluded that the contamination of aquatic environments due to the use of AFFFs containing PFASs should be assessed as a hazard in risk assessment, especially in terms of the possible bioconcentration of PFASs in biota. More research is needed to better determine the toxicokinetics of PFASs in fish. Although differences in the sensitivity to PFASs have been documented between various species, little information is available on the lipid peroxidation and the induction of the CYP1A enzyme in gills of fish following exposure to PFASs (both singular and in combination). More controlled laboratory studies are needed to investigate the oxidative damage, induction of metabolic enzymes and possible liver damage in fish exposed to mixtures of PFASs. Biomarkers of endocrine, developmental and immunotoxic responses should also be further examined to get a more holistic understanding of the biological effects in fish from exposure to PFASs.

7. References

Abrahamson, A. (2007). *Gill EROD Activity in Fish. A Biomarker for Waterborne Ah-Receptor Agonists*. Ph.D thesis. Uppsala University.

Ankley, G. T., Kuehl, D. W., Kahl, M. D., Jensen, K. M., Butterworth, B. C. and Nichols, J. W. (2004). Partial Life-Cycle Toxicity and Bioconcentration Modeling of Perfluorooctanesulfonate in the Northern Leopard Frog (*Rana pipiens*). *Environmental Toxicology and Chemistry*, **23**: 2745-2755.

Arnot, J. A. and Gobas, F. A. (2006). A Review of Bioconcentration Factor (BCF) and Bioaccumulation Factor (BAF) Assessments for Organic Chemicals in Aquatic Organisms. *Environmental Reviews*, **14**: 257-297.

Arukwe, A. and Mortensen, A. S. (2011). Lipid Peroxidation and Oxidative Stress Responses of Salmon Fed a Diet Containing Perfluorooctane Sulfonic- or Perfluorooctane Carboxylic Acids. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **154**: 288-295.

Barron, M. G. (1990). Bioconcentration. Will water-borne organic chemicals accumulate in aquatic animals? *Environmental Science & Technology*, **24**: 1612-1618.

Benninghoff, A. D., Bisson, W. H., Koch, D. C., Ehresman, D. J., Kolluri, S. K. and Williams, D. E. (2011). Estrogen-Like Activity of Perfluoroalkyl Acids *In Vivo* and Interaction with Human and Rainbow Trout Estrogen Receptors *In Vitro*. *Toxicological Sciences*, **120**: 42-58.

Benninghoff, A. D., Orner, G. A., Buchner, C. H., Hendricks, J. D., Duffy, A. M. and Williams, D. E. (2012). Promotion of Hepatocarcinogenesis by Perfluoroalkyl Acids in Rainbow Trout. *Toxicological Sciences*, **125**: 69-78.

Bergmeyer, H. U., Hørder, M. and Rej, R. (1986). IFCC Methods for the Measurement of Catalytic Concentration of Enzymes. Part 3. IFCC Method for Alanine Aminotransferase. *Clinical Chemistry*, **24**: 481-495.

Berthiaume, J. and Wallace, K. B. (2002). Perfluorooctanoate, Perflourooctanesulfonate, and N-Ethyl Perfluorooctanesulfonamido Ethanol; Peroxisome Proliferation and Mitochondrial Biogenesis. *Toxicology Letters*, **129**: 23-32.

Bilbao, E., Raingeard, D., de Cerio, O. D., Ortiz-Zarragoitia, M., Ruiz, P., Izagirre, U., Orbea, A., Marigómez, I., Cajaraville, M. P. and Cancio, I. (2010). Effects of Exposure to Prestige-Like Heavy Fuel Oil and to Perfluorooctane Sulfonate on Conventional Biomarkers and Target Gene Transcription in the Thicklip Grey Mullet *Chelon labrosus*. *Aquatic Toxicology*, **98**: 282-296.

Blum, A., Balan, S. A., Scheringer, M., Trier, X., Goldenman, G., Cousins, I. T., Diamond, M., Fletcher, T., Higgins, C., Lindeman, A. E., Peaslee, G., de Voogt, P., Wang, Z. and Weber, R. (2015). The Madrid Statement on Poly- and Perfluoroalkyl Substances (PFASs). *Environmental Health Perspectives*, **123**: A107-A111.

Botsoglou, N. A., Fletouris, D. J., Papageorgiou, G. E., Vassilopoulos, V. N., Mantis, A. J. and Trakatellis, A. G. (1994). Rapid, Sensitive, and Specific Thiobarbituric Acid Method for Measuring Lipid Peroxidation in Animal Tissue, Food, and Feedstuff Samples. *Journal of Agricultural and Food Chemistry*, **42**: 1931-1937.

Bro, R. and Smilde, A. K. (2014). Principal Component Analysis. *Analytical Methods*, **6**: 2812-2831.

Bucher, F. and Hofer, R. (1990). Effects of Domestic Wastewater on Serum Enzyme Activities of Brown Trout (*Salmo trutta*). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, **97**: 381-385.

Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., de Voogt, P., Jensen, A. A., Kannan, K., Mabury, S. A. and van Leeuwen, S. P. (2011). Perfluoroalkyl and Polyfluoroalkyl Substances in the Environment: Terminology, Classification, and Origins. *Integrated Environmental Assessment and Management*, **7**: 513-541.

Buck, R. C., Murphy, P. M. and Padon, M. (2012). Chemistry, Properties, and Uses of Commercial Fluorinated Surfactants. *In:* Knepper, T. and Lange, F. *Polyfluorinated chemicals and transformation products*. Berlin, Germany: Springer.

Butenhoff, J. L., Chang, S.-C., Ehresman, D. J. and York, R. G. (2009). Evaluation of Potential Reproductive and Developmental Toxicity of Potassium Perfluorohexanesulfonate in Sprague Dawley Rats. *Reproductive Toxicology*, **27**: 331-341.

Cairns, J. J., Heath, A. G. and Parker, B. C. (1975). The Effects of Temperature Upon the Toxicity of Chemicals to Aquatic Organisms. *Hydrobiologia*, **47**: 135-171.

Cajaraville, M. P., Cancio, I., Ibabe, A. and Orbea, A. (2003). Peroxisome Proliferation as a Biomarker in Environmental Pollution Assessment. *Microscopy Research and Technique*, **61**: 191-202.

Cancio, I. and Cajaraville, M. P. (2000). Cell Biology of Peroxisomes and Their Characteristics in Aquatic Organisms. *International Review of Cytology*, **199**: 201-293.

Chang, S.-C., Noker, P. E., Gorman, G. S., Gibson, S. J., Hart, J. A., Ehresman, D. J. and Butenhoff, J. L. (2012). Comparative Pharmacokinetics of Perfluorooctanesulfonate (PFOS) in Rats, Mice, and Monkeys. *Reproductive Toxicology*, **33**: 428-440.

Chen, N., Li, J., Li, D., Yang, Y. and He, D. (2014). Chronic Exposure to Perfluorooctane Sulfonate Induces Behavior Defects and Neurotoxicity through Oxidative Damages, *In Vivo* and *In Vitro*. *PLoS ONE*, **9**: e113453.

Conder, J. M., Hoke, R. A., de Wolf, W., Russell, M. H. and Buck, R. C. (2008). Are PFCAs Bioaccumulative? A Critical Review and Comparison with Regulatory Criteria and Persistent Lipophilic Compounds. *Environmental Science & Technology*, **42**: 995-1003.

Corton, J. C., Anderson, S. P. and Stauber, A. (2000). Central Role of Peroxisome Proliferator–Activated Receptors in the Actions of Peroxisome Proliferators. *Annual Review of Pharmacology and Toxicology*, **40**: 491-518.

Cowi and Sweco (2012). *Miljøprosjektet DP 2, Miljøtekniske Grunnundersøkelser Harstad/Narvik lufthavn - Evenes.* Avinor AS. Report nr: 168180-280-1. [In Norwegian].

Dalton, T. P., Puga, A. and Shertzer, H. G. (2002). Induction of Cellular Oxidative Stress by Aryl Hydrocarbon Receptor Activation. *Chemico-Biological Interactions*, **141**: 77-95.

Dawn-Linsley, M., Ekinci, F. J., Ortiz, D., Rogers, E. and Shea, T. B. (2005). Monitoring Thiobarbituric Acid-Reactive Substances (TBARs) as an Assay for Oxidative Damage in

Neuronal Cultures and Central Nervous System. *Journal of Neuroscience Methods*, **141**: 219-222.

De Silva, A. O., Tseng, P. J. and Mabury, S. A. (2009). Toxicokinetics of Perfluorocarboxylate Isomers in Rainbow Trout. *Environmental Toxicology and Chemistry*, **28**: 330-337.

DeWitt, J. C., Peden-Adams, M. M., Keller, J. M. and Germolec, D. R. (2012). Immunotoxicity of Perfluorinated Compounds: Recent Developments. *Toxicologic Pathology*, **40**: 300-311.

Ding, G. and Peijnenburg, W. J. (2013). Physicochemical Properties and Aquatic Toxicity of Poly- and Perfluorinated Compounds. *Critical Reviews in Environmental Science and Technology*, **43**: 598-678.

Du, Y., Shi, X., Liu, C., Yu, K. and Zhou, B. (2009). Chronic Effects of Water-Borne PFOS Exposure on Growth, Survival and Hepatotoxicity in Zebrafish: A Partial Life-Cycle Test. *Chemosphere*, **74**: 723-729.

Elcombe, C. R., Elcombe, B. M., Foster, J. R., Chang, S.-C., Ehresman, D. J., Noker, P. E. and Butenhoff, J. L. (2012). Evaluation of Hepatic and Thyroid Responses in Male Sprague Dawley Rats for up to Eighty-Four Days Following Seven Days of Dietary Exposure to Potassium Perfluorooctanesulfonate. *Toxicology*, **293**: 30-40.

Fair, P. A., Romano, T., Schaefer, A. M., Reif, J. S., Bossart, G. D., Houde, M., Muir, D., Adams, J., Rice, C., Hulsey, T. C. and Peden-Adams, M. (2013). Associations Between Perfluoroalkyl Compounds and Immune and Clinical Chemistry Parameters in Highly Exposed Bottlenose Dolphins (*Tursiops truncatus*). *Environmental Toxicology and Chemistry*, **32**: 736-746.

Falk, S., Failing, K., Georgii, S., Brunn, H. and Stahl, T. (2015). Tissue Specific Uptake and Elimination of Perfluoroalkyl Acids (PFAAs) in Adult Rainbow Trout (*Oncorhynchus mykiss*) After Dietary Exposure. *Chemosphere*, **129**: 150-156.

Feng, M., He, Q., Meng, L., Zhang, X., Sun, P. and Wang, Z. (2015). Evaluation of Single and Joint Toxicity of Perfluorooctane Sulfonate, Perfluorooctanoic Acid, and Copper to *Carassius auratus* Using Oxidative Stress Biomarkers. *Aquatic Toxicology*, **161**: 108-116.

Filipe, A. F., Markovic, D., Pletterbauer, F., Tisseuil, C., De Wever, A., Schmutz, S., Bonada, N. and Freyhof, J. (2013). Forecasting Fish Distribution Along Stream Networks: Brown Trout (*Salmo trutta*) in Europe. *Diversity and Distributions*, **19**: 1059-1071.

Frost, W. E. and Brown, M. E. (1967). *The Trout*. London, UK: Collins.

Gallo, V., Leonardi, G., Genser, B., Lopez-Espinosa, M.-J., Frisbee, S. J., Karlsson, L., Ducatman, A. M. and Fletcher, T. (2012). Serum Perfluorooctanoate (PFOA) and Perfluorooctane Sulfonate (PFOS) Concentrations and Liver Function Biomarkers in a Population with Elevated PFOA Exposure. *Environmental Health Perspectives*, **120**: 655-660.

Giesy, J. P. and Kannan, K. (2001). Global Distribution of Perfluorooctane Sulfonate in Wildlife. *Environmental Science & Technology*, **35**: 1339-1342.

Giesy, J. P. and Kannan, K. (2002). Peer Reviewed: Perfluorochemical Surfactants in the Environment. *Environmental Science & Technology*, **36**: 146A-152A.

Goeritz, I., Falk, S., Stahl, T., Schäfers, C. and Schlechtriem, C. (2013). Biomagnification and Tissue Distribution of Perfluoroalkyl Substances (PFASs) in Market-Size Rainbow Trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, **32**: 2078-2088.

Goksøyr, A. and Förlin, L. (1992). The Cytochrome P-450 System in Fish, Aquatic Toxicology and Environmental Monitoring. *Aquatic Toxicology*, **22**: 287-311.

Hagenaars, A., Meyer, I. J., Herzke, D., Pardo, B. G., Martinez, P., Pabon, M., De Coen, W. and Knapen, D. (2011). The Search for Alternative Aqueous Film Forming Foams (AFFF) With a Low Environmental Impact: Physiological and Transcriptomic Effects of Two Forafac® Fluorosurfactants in Turbot. *Aquatic Toxicology*, **104**: 168-176.

Halliwell, B. and Chirico, S. (1993). Lipid Peroxidation: Its Mechanism, Measurement, and Significance. *The American Journal of Clinical Nutrition*, **57**: 715S-724S.

Han, X., Snow, T. A., Kemper, R. A. and Jepson, G. W. (2003). Binding of Perfluorooctanoic Acid to Rat and Human Plasma Proteins. *Chemical Research in Toxicology*, **16**: 775-781.

Han, Z.-X., Zhang, M. and Lv, C.-X. (2011). Toxicokinetic Behaviors and Modes of Perfluorooctane Sulfonate (PFOS) and Perfluorooctane Acid (PFOA) on Tilapia (*Oreochromis niloticus*). *African Journal of Biotechnology*, **10**: 12943-12950.

Han, Z., Liu, Y., Wu, D., Zhu, Z. and Lü, C. (2012). Immunotoxicity and Hepatotoxicity of PFOS and PFOA in Tilapia (*Oreochromis niloticus*). *Chinese Journal of Geochemistry*, **31**: 424-430.

Hellstad, S. (2015). *Bioconcentration and Transcriptional Effects of Fire Foam-Related PFAS on Brown Trout (Salmo Trutta)*. M.Sc thesis. University of Oslo.

Hoff, P. T., Scheirs, J., Van de Vijver, K., Van Dongen, W., Esmans, E. L., Blust, R. and De Coen, W. (2004). Biochemical Effect Evaluation of Perfluorooctane Sulfonic Acid-Contaminated Wood Mice (*Apodemus sylvaticus*). *Environmental Health Perspectives*, **112**: 681-686.

Hoff, P. T., Van Campenhout, K., Van de Vijver, K., Covaci, A., Bervoets, L., Moens, L., Huyskens, G., Goemans, G., Belpaire, C., Blust, R. and De Coen, W. (2005). Perfluorooctane Sulfonic Acid and Organohalogen Pollutants in Liver of Three Freshwater Fish Species in Flanders (Belgium): Relationships with Biochemical and Organismal Effects. *Environmental Pollution*, **137**: 324-333.

Hoff, P. T., Van de Vijver, K., Van Dongen, W., Esmans, E. L., Blust, R. and De Coen, W. M. (2003b). Perfluorooctane Sulfonic Acid in Bib (*Trisopterus luscus*) and Plaice (*Pleuronectes platessa*) From the Western Scheldt and the Belgian North Sea: Distribution and Biochemical Effects. *Environmental Toxicology and Chemistry*, **22**: 608-614.

Hoff, P. T., Van Dongen, W., Esmans, E. L., Blust, R. and De Coen, W. M. (2003a). Evaluation of the Toxicological Effects of Perfluorooctane Sulfonic Acid in the Common Carp (*Cyprinus carpio*). *Aquatic Toxicology*, **62**: 349-359.

Hu, W. Y., Jones, P. D., De Coen, W., King, L., Fraker, P., Newsted, J. and Giesy, J. P. (2003). Alterations in Cell Membrane Properties Caused by Perfluorinated Compounds. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **135**: 77-88.

Inoue, Y., Hashizume, N., Yakata, N., Murakami, H., Suzuki, Y., Kikushima, E. and Otsuka, M. (2012). Unique Physicochemical Properties of Perfluorinated Compounds and Their Bioconcentration in Common Carp *Cyprinus carpio* L. *Archives of Environmental Contamination and Toxicology*, **62**: 672-680.

Jensen, A. A. and Leffers, H. (2008). Emerging Endocrine Disrupters: Perfluoroalkylated Substances. *International Journal of Andrology*, **31**: 161-169.

Jones, K. C. and de Voogt, P. (1999). Persistent Organic Pollutants (POPs): State of the Science. *Environmental Pollution*, **100**: 209-221.

Jones, P. D., Hu, W., De Coen, W., Newsted, J. L. and Giesy, J. P. (2003). Binding of Perfluorinated Fatty Acids to Serum Proteins. *Environmental Toxicology and Chemistry*, **22**: 2639-2649.

Jönsson, E. M., Brandt, I. and Brunström, B. (2002). Gill Filament-Based EROD Assay for Monitoring Waterborne Dioxin-Like Pollutants in Fish. *Environmental Science & Technology*, **36**: 3340-3344.

Jönsson, M., Abrahamson, A., Brunström, B., Brandt, I., Ingebrigtsen, K. and Jørgensen, E. H. (2003). EROD Activity in Gill Filaments of Anadromous and Marine Fish as a Biomarker of Dioxin-Like Pollutants. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **136**: 235-243.

Jørgensen, E. H., Celander, M., Goksøyr, A. and Iwata, M. (2001). The Effect of Stress on Toxicant-Dependent Cytochrome P450 Enzyme Responses in the Arctic Charr (*Salvelinus alpinus*). *Environmental Toxicology and Chemistry*, **20**: 2523-2529.

Kannan, K., Corsolini, S., Falandysz, J., Fillmann, G., Kumar, K. S., Loganathan, B. G., Mohd, M. A., Olivero, J., Wouwe, N. V., Yang, J. H. and Aldous, K. M. (2004). Perfluorooctanesulfonate and Related Fluorochemicals in Human Blood from Several Countries. *Environmental Science & Technology*, **38**: 4489-4495.

Key, B. D., Howell, R. D. and Criddle, C. S. (1998). Defluorination of Organofluorine Sulfur Compounds by *Pseudomonas Sp.* Strain D2. *Environmental Science & Technology*, **32**: 2283-2287.

Kim, W.-K., Lee, S.-K. and Jung, J. (2010). Integrated Assessment of Biomarker Responses in Common Carp (*Cyprinus carpio*) Exposed to Perfluorinated Organic Compounds. *Journal of Hazardous Materials*, **180**: 395-400.

Kim, W. R., Flamm, S. L., Di Bisceglie, A. M. and Bodenheimer, H. C. (2008). Serum Activity of Alanine Aminotransferase (ALT) as an Indicator of Health and Disease. *Hepatology*, **47**: 1363-1370.

Kishi, T. and Arai, M. (2008). Study on the Generation of Perfluorooctane Sulfonate from the Aqueous Film-Forming Foam. *Journal of Hazardous Materials*, **159**: 81-86.

Kissa, E. (2001). *Fluorinated Surfactants and Repellents.* Second Edition. New York, USA: Marcel Dekker Inc.

Klaunig, J. E., Hocevar, B. A. and Kamendulis, L. M. (2012). Mode of Action Analysis of Perfluorooctanoic Acid (PFOA) Tumorigenicity and Human Relevance. *Reproductive Toxicology*, **33**: 410-418.

Kottelat, M. and Freyhof, J. (2007). *Handbook of European Freshwater Fishes*. Cornol, Switzerland: Publications Kottelat.

Kruskal, W. H. and Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*, **47**: 583-621.

Labadie, P. and Chevreuil, M. (2011). Partitioning Behaviour of Perfluorinated Alkyl Contaminants Between Water, Sediment and Fish in the Orge River (Nearby Paris, France). *Environmental Pollution*, **159**: 391-397.

Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A. and Seed, J. (2007). Perfluoroalkyl Acids: A Review of Monitoring and Toxicological Findings. *Toxicological Sciences*, **99**: 366-394.

Lau, C., Butenhoff, J. L. and Rogers, J. M. (2004). The Developmental Toxicity of Perfluoroalkyl Acids and Their Derivatives. *Toxicology and Applied Pharmacology*, **198**: 231-241.

Levene, H. (1960). A Robust Approximate Confidence Interval for Components of Variance. *Annals of Mathematical Statistics*, **31**: 534-535.

Liu, C., Chang, V. W. and Gin, K. Y. (2014a). Oxidative Toxicity of Perfluorinated Chemicals in Green Mussel and Bioaccumulation Factor Dependent Quantitative Structure–Activity Relationship. *Environmental Toxicology and Chemistry*, **33**: 2323-2332.

Liu, C., Gin, K. Y. and Chang, V. W. (2014b). Multi-Biomarker Responses in Green Mussels Exposed to PFCs: Effects at Molecular, Cellular, and Physiological Levels. *Environmental Science and Pollution Research International*, **21**: 2785-2794.

Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K., Wu, R. S. and Zhou, B. (2007). Induction of Oxidative Stress and Apoptosis by PFOS and PFOA in Primary Cultured Hepatocytes of Freshwater Tilapia (*Oreochromis niloticus*). *Aquatic Toxicology*, **82**: 135-143.

Liu, J., Wang, N., Szostek, B., Buck, R. C., Panciroli, P. K., Folsom, P. W., Sulecki, L. M. and Bellin, C. A. (2010). 6-2 Fluorotelomer Alcohol Aerobic Biodegradation in Soil and Mixed Bacterial Culture. *Chemosphere*, **78**: 437-444.

Lock, E. A., Mitchell, A. M. and Elcombe, C. R. (1989). Biochemical Mechanisms of Induction of Hepatic Peroxisome Proliferation. *Annual Review of Pharmacology and Toxicology*, **29**: 145-163.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein Measurement With the Folin Phenol Reagent. *Journal of Biological Chemistry*, **193**: 265-275.

Luckenbach, T., Fischer, S. and Sturm, A. (2014). Current Advances on ABC Drug Transporters in Fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **165**: 28-52.

Luebker, D. J., Hansen, K. J., Bass, N. M., Butenhoff, J. L. and Seacat, A. M. (2002). Interactions of Flurochemicals with Rat Liver Fatty Acid-Binding Protein. *Toxicology*, **176**: 175-185.

Løland, B. (2014). *Fate and Transport of PFCs in a Peat Bog Environment*. M.Sc thesis. University of Oslo.

Martin, J. W., Mabury, S. A., Solomon, K. R. and Muir, D. C. (2003a). Bioconcentration and Tissue Distribution of Perfluorinated Acids in Rainbow Trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, **22**: 196-204.

Martin, J. W., Mabury, S. A., Solomon, K. R. and Muir, D. C. (2003b). Dietary Accumulation of Perfluorinated Acids in Juvenile Rainbow Trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, **22**: 189-195.

McComb, R. B., Bond, L. W., Burnett, R. W., Keech, R. C. and Bowers, G. N. (1976). Determination of the Molar Absorptivity of NADH. *Clinical Chemistry*, **22**: 141-150.

Mommsen, T. P. and Walsh, P. J. (1988). Vitellogenesis and Oocyte Assembly. *Fish Physiology*, **11A**: 347-406.

Moody, C. A. and Field, J. A. (2000). Perfluorinated Surfactants and the Environmental Implications of Their Use in Fire-Fighting Foams. *Environmental Science & Technology*, **34**: 3864-3870.

Moody, C. A., Hebert, G. N., Strauss, S. H. and Field, J. A. (2003). Occurrence and Persistence of Perfluorooctanesulfonate and Other Perfluorinated Surfactants in Groundwater at a Fire-Training Area at Wurtsmith Air Force Base, Mickigan, USA. *Journal of Environmental Monitoring*, **5**: 341-345.

Moody, C. A., Martin, J. W., Kwan, W. C., Muir, D. C. and Mabury, S. A. (2002). Monitoring Perfluorinated Surfactants in Biota and Surface Water Samples Following an Accidental Release of Fire-Fighting Foam into Etobicoke Creek. *Environmental Science & Technology*, **36**: 545-551.

Muir, D. C. and de Wit, C. A. (2010). Trends of Legacy and New Persistent Organic Pollutants in the Circumpolar Arctic: Overview, Conclusions, and Recommendations. *Science of The Total Environment*, **408**: 3044-3051.

Munyemera, I. (2014). *Combined Effects of Perfluorooctane Sulfonic Acid (PFOS) and Carbon Dioxide (CO2) on Oxidative Stress Responses in the Gill of Atlantic Cod (Gadus morhua)*. M.Sc thesis. Norwegian University of Science and Technology (NTNU).

Narimatsu, S., Nakanishi, R., Hanioka, N., Saito, K. and Kataoka, H. (2011). Characterization of Inhibitory Effects of Perfluorooctane Sulfonate on Human Hepatic Cytochrome P450 Isoenzymes: Focusing on CYP2A6. *Chemico-Biological Interactions*, **194**: 120-126.

Newman, M. C. and Clements, W. H. (2008). *Ecotoxicology, A Comprehensive Treatment*. California, USA: CRC Press.

Ng, C. A. and Hungerbühler, K. (2013). Bioconcentration of Perfluorinated Alkyl Acids: How Important Is Specific Binding? *Environmental Science & Technology*, **47**: 7214-7223.

Norconsult and Sweco (2015). *Harstad/Narvik Lufthavn Evenes, Undersøkelser av PFAS i Jord, Vann og Biota med Risikovurdering*. [Online]. Avinor AS. [12.05.2015]. Available from: https://avinor.no/globalassets/_konsern/miljo-lokal/miljorapporter/pfos-rapport-harstad-narvik-lufthavn-april-2015.pdf. [In Norwegian].

Oakes, K. D., Benskin, J. P., Martin, J. W., Ings, J. S., Heinrichs, J. Y., Dixon, D. G. and Servos, M. R. (2010). Biomonitoring of Perfluorochemicals and Toxicity to the Downstream Fish Community of Etobicoke Creek Following Deployment of Aqueous Film-Forming Foam. *Aquatic Toxicology*, **98**: 120-129.

Oakes, K. D., Sibley, P. K., Martin, J. W., Maclean, D. D., Solomon, K. R., Mabury, S. A. and Van Der Kraak, G. J. (2005). Short-Term Exposures of Fish to Perfluorooctane Sulfonate: Acute Effects on Fatty Acyl-CoA Oxidase Activity, Oxidative Stress, and Circulating Sex Steroids. *Environmental Toxicology and Chemistry*, **24**: 1172-1181.

Oakes, K. D., Sibley, P. K., Solomon, K. R., Mabury, S. A. and Van Der Kraak, G. J. (2004). Impact of Perfluorooctanoic Acid on Fathead Minnow (*Pimephales promelas*) Fatty Acyl-Coa Oxidase Activity, Circulating Steroids, and Reproduction in Outdoor Microcosms. *Environmental Toxicology and Chemistry*, **23**: 1912-1919.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction. *Analytical Biochemistry*, **95**: 351-358.

Olsen, G. W., Burris, J. M., Ehresman, D. J., Froehlich, J. W., Seacat, A. M., Butenhoff, J. L. and Zobel, L. R. (2007). Half-Life of Serum Elimination of Perfluorooctanesulfonate, Perfluorohexanesulfonate, and Perfluorooctanoate in Retired Fluorochemical Production Workers. *Environmental Health Perspectives*, **115**: 1298-1305.

Pabon, M. and Corpart, J. M. (2002). Fluorinated Surfactants: Synthesis, Properties, Effluent Treatment. *Journal of Fluorine Chemistry*, **114**: 149-156.

Paul, A. G., Jones, K. C. and Sweetman, A. J. (2009). A First Global Production, Emission, and Environmental Inventory for Perfluorooctane Sulfonate. *Environmental Science & Technology*, **43**: 386-392.

Peakall, D. B. and Walker, C. H. (1994). The Role of Biomarkers in Environmental Assessment (3), Vertebrates. *Ecotoxicology*, **3**: 173-179.

Prevedouros, K., Cousins, I. T., Buck, R. C. and Korzeniowski, S. H. (2006). Sources, Fate and Transport of Perfluorocarboxylates. *Environmental Science & Technology*, **40**: 32-44.

Qin, P., Liu, R., Pan, X., Fang, X. and Mou, Y. (2010). Impact of Carbon Chain Length on Binding of Perfluoroalkyl Acids to Bovine Serum Albumin Determined by Spectroscopic Methods. *Journal of Agricultural and Food Chemistry*, **58**: 5561-5567.

Reichling, J. and Kaplan, M. (1988). Clinical Use of Serum Enzymes in Liver Disease. *Digestive Diseases and Sciences*, **33**: 1601-1614.

Remde, A. and Debus, R. (1996). Biodegradability of Fluorinated Surfactants Under Aerobic and Anaerobic Conditions. *Chemosphere*, **32**: 1563-1574.

Renner, R. (2001). Growing Concern Over Perfluorinated Chemicals. *Environmental Science & Technology*, **35**: 154-160.

Sakurai, T., Kobayashi, J., Kinoshita, K., Ito, N., Serizawa, S., Shiraishi, H., Lee, J.-H., Horiguchi, T., Maki, H., Mizukawa, K., Imaizumi, Y., Kawai, T. and Suzuki, N. (2013). Transfer Kinetics of Perfluorooctane Sulfonate from Water and Sediment to a Marine Benthic Fish, the Marbled Flounder (*Pseudopleuronectes yokohamae*). *Environmental Toxicology and Chemistry*, **32**: 2009-2017.

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.

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. *Toxicological Sciences*, **68**: 249-264.

Shi, X., Du, Y., Lam, P. K., Wu, R. S. and Zhou, B. (2008). Developmental Toxicity and Alteration of Gene Expression in Zebrafish Embryos Exposed to PFOS. *Toxicology and Applied Pharmacology*, **230**: 23-32.

Shi, X. and Zhou, B. (2010). The Role of Nrf2 and MAPK Pathways in PFOS-Induced Oxidative Stress in Zebrafish Embryos. *Toxicological Sciences*, **115**: 391-400.

Slotkin, T. A., MacKillop, E. A., Melnick, R. L., Thayer, K. A. and Seidler, F. J. (2008). Developmental Neurotoxicity of Perfluorinated Chemicals Modeled *in Vitro*. *Environmental Health Perspectives*, **116**: 716-722.

Small, G. M., Burdett, K. and Connock, M. J. (1985). A Sensitive Spectrophotometric Assay for Peroxisomal Acyl-CoA Oxidase. *Biochemical Journal*, **227**: 205-210.

Sparks, T. H., Scott, W. A. and Clarke, R. T. (1999). Traditional Multivariate Techniques: Potential for Use in Ecotoxicology. *Environmental Toxicology and Chemistry*, **18**: 128-137.

Sundström, M., Chang, S.-C., Noker, P. E., Gorman, G. S., Hart, J. A., Ehresman, D. J., Bergman, Å. and Butenhoff, J. L. (2012). Comparative Pharmacokinetics of Perfluorohexanesulfonate (PFHxS) in Rats, Mice, and Monkeys. *Reproductive Toxicology*, **33**: 441-451.

Sweco (2012). *Miljøprosjektet - DP 2, Miljøtekniske Grunnundersøkelser ved Avinors Lufthavner.* Avinor AS. Report nr: 168180-1. [In Norwegian].

Tarazona, J. V., Rodríguez, C., Alonso, E., Sáez, M., González, F., San Andrés, M. D., Jiménez, B. and San Andrés, M. I. (2015). Toxicokinetics of Perfluorooctane Sulfonate in Birds under Environmentally Realistic Exposure Conditions and Development of a Kinetic Predictive Model. *Toxicology Letters*, **232**: 363-368.

Tukey, J. W. (1949). Comparing Individual Means in the Analysis of Variance. *Biometrics*, **5**: 99-114.

UNEP (2009). *Report of the Conference of the Parties of the Stockholm Convention on Persistent Organic Pollutants on the Work of Its Fourth Meeting.* [Online]. United Nations Environment Programme. Stockholm Convention on Persistent Organic Pollutants. [16.04.2015]. Available from: http://www.chem.unep.ch/ddt/documents/SC_COP4_report_full.pdf.

Valenzuela, A. (1991). The Biological Significance of Malondialdehyde Determination in the Assessment of Tissue Oxidative Stress. *Life Sciences*, **48**: 301-309.

van der Oost, R., Beyer, J. and Vermeulen, N. P. (2003). Fish Bioaccumulation and Biomarkers in Environmental Risk Assessment: a Review. *Environmental Toxicology and Pharmacology*, **13**: 57-149.

Watanabe, M. X., Jones, S. P., Iwata, H., Kim, E.-Y. and Kennedy, S. W. (2009). Effects of Co-Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin and Perfluorooctane Sulfonate or Perfluorooctanoic Acid on Expression of Cytochrome P450 Isoforms in Chicken (*Gallus gallus*) Embryo Hepatocyte Cultures. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **149**: 605-612.

Welch, B. L. (1938). The Significance of the Difference Between Two Means When the Population Variances are Unequal. *Biometrika*, **29**: 350-362.

Whitlock, M. C. and Schluter, D. (2009). *The Analysis of Biological Data*. Colorado, USA: Roberts and Company Publishers.

Whyte, J. J., Jung, R. E., Schmitt, C. J. and Tillitt, D. E. (2000). Ethoxyresorufin-*O*-Deethylase (EROD) Activity in Fish as a Biomarker of Chemical Exposure. *Critical Reviews in Toxicology*, **30**: 347-570.

Yeung, L. Y., Loi, E. H., Wong, V. Y., Guruge, K., Yamanaka, N., Tanimura, N., Hasegawa, J., Yamashita, N., Miyazaki, S. and Lam, P. S. (2009). Biochemical Responses and Accumulation Properties of Long-Chain Perfluorinated Compounds (PFOS/PFDA/PFOA) in Juvenile Chickens (*Gallus gallus*). *Archives of Environmental Contamination and Toxicology*, **57**: 377-386.

Ziegenhorn, J., Senn, M. and Bücher, T. (1976). Molar Absorptivities of Beta-NADH and Beta-NADPH. *Clinical Chemistry*, **22**: 151-160.

8. Appendix

Appendix 1: PFASs in trout

Appendix 2: PFASs in water

Appendix 3: Oxygen, temperature and nitrite

Appendix 4: Biomarker responses

Appendix 5: Buffers and solutions

Appendix 6: Chemicals and lab equipment

Appendix 1: PFASs in trout

Table A1. Range (min-max) and median concentrations (µg/kg) of perfluorooctane sulfonate (PFOS) detected in brown trout from different treatments ("control", "low" and "high") collected at week zero (reference), one and three during exposure, and week one (4) and ten (13) after ended exposure to PFASs. (n=5 if not otherwise stated).

Week	Control		Low		High	
	Range	Median	Range	Median	Range	Median
0	0.120 - 0.671	0.350	0.0702 - 0.185	0.156 (n=4*)	0.0925 - 0.226	0.138
1	0.500 - 376	0.700	0.300 - 445	202	2.70 - 43900	21900
3	3.10 - 585	3.95 (n=4)	1.00 - 2730	594	23400 - 84000	69400
4	0.674 - 4.34	2.43	714 - 13700	1000	19800 - 104000	51700
13	1.17 - 2.19	1.455 (n=4)	260 - 588	343	64200 - 168000	123000 (n=3)

*The concentrations of one fish were measured to be below the detection limit (0.0643 µg/kg).

Table A2. Range (min-max) and median detection limits (µg/kg) of Perfluoropentanoic acid (PFPeA), Perfluorohexanoic acid (PFHxA) and Perfluorooctanoic acid (PFOA) in brown trout from different treatments ("control", "low" and "high") collected at week zero (reference), one and three during exposure, and week one (4) and ten (13) after ended exposure to PFASs. (n=5 if not otherwise stated).

Week	Control		Low		High	
	Range	Median	Range	Median	Range	Median
0	0.0507 - 0.0661	0.0602	0.0545 - 0.0643	0.0601	0.0582 - 0.0702	0.0626
1	0.050 - 0.600	0.060	0.060 - 6.80	0.60	0.07 - 62.2	11.7
3	0.060 - 0.600	0.060 (n=4)	0.060 - 6.70	5.70	50.5 - 118	105
4	0.0547 - 0.0641	0.0593	0.541 - 61.7	1.14	46.3 - 66.9	63.7
13	0.0449 - 0.0552	0.0489 (n=4)	0.238 - 0.525	0.487	46.8 - 50.5	47.0 (n=3)

Table A3. Range (min-max) and median detection limits (µg/kg) of 6:2 fluorotelomer sulfonate (FTS) in brown trout from different treatments ("control", "low" and "high") collected at week zero (reference), one and three during exposure, and week one (4) and ten (13) after ended exposure to PFASs. (n=5 if not otherwise stated).

Week	Control		Low		High	
	Range	Median	Range	Median	Range	Median
0	0.0761 - 0.0991	0.094	0.0818 - 0.0965	0.0901	0.0873 - 0.105	0.0918
1	0.08 - 1.0	0.1	0.09 - 10.2	0.9	0.1 - 93.3	17.5
3	0.09 - 0.9	0.09 (n=4)	0.09 - 10.1	8.5	75.7 - 177	157
4	0.082 - 0.0961	0.0889	0.811 - 90.1	1.710	69.4 - 100	95.5
13	0.0674 - 0.0828	0.0734 (n=4)	0.357 - 0.788	0.731	70.2 - 75.8	70.6 (n=3)

Table A4. Concentration (µg/kg) of Perfluorohexane sulfonate (PFHxS) detected in brown trout from different treatments ("control", "low" and "high") collected at week zero (reference), one and three during exposure, and week one (4) and ten (13) after ended exposure to PFASs. Samples marked with < are not detected. Concentrations after < are detection limits (varying with sample volume and sensitivity of analysis). (NA = not available).

Week	Control					Low					High				
0	<0,090	<0,088	<0,076	<0,094	<0,099	<0,085	<0,094	<0,097	<0,082	<0,090	<0,087	<0,088	<0,094	<0,105	<0,092
1	0,137	<0,080	<0,100	<0,100	7,00	16,2	5,30	5,80	<0,09	5,60	<0,10	187	848	557	549
3	NA	0,10	0,10	<0,090	13,9	31,9	11,2	9,90	11,2	<0,09	705	650	686	799	705
4	<0,089	<0,082	<0,084	<0,095	<0,096	165	6,33	120	<90,1	8,55	408	446	453	1230	496
13	NA	<0,078	<0,083	<0,069	<0,067	<0,731	<0,746	0,486	<0,788	0,725	<70,6	<75,8	NA	NA	<70,2

Appendix 2: PFASs in water

Table A5. 6:2 fluorotelomer sulfonate (FTS), Perfluoropentanoic acid (PFPeA), Perfluorohexane sulfonate (PFHxS), Perfluorooctane sulfonate (PFOS), Perfluorohexanoic acid (PFHxA) and Perfluorooctanoic acid (PFOA) in water sampled at different times during and after exposure to PFASs (exposure ended at day 20). Samples were collected from one randomly chosen aquarium of every treatment at each day. The aquaria sampled denoted day 27 were the only aquaria sampled twice. Samples marked with < are not detected. Concentrations after < are detection limits (varying with sample volume and sensitivity of analysis).

Time (Days)	Treatment	6:2 FTS (µg/L)	PFPeA (µg/L)	PFHxS (µg/L)	PFOS (µg/L)	PFHxA (µg/L)	PFOA (µg/L)
3	High	30.4	36.4	54.2	38.1	23.1	8.40
3	Low	1.50	1.20	1.80	3.30	0.70	0.400
3	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
6	High	108	57.2	125	284	37.8	27.5
6	Low	2.00	1.00	2.10	14.8	0.600	0.500
6	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
10	High	114	57.6	126	362	38.7	28.3
10	Low	0.400	0.70	0.500	0.500	0.400	0.0900
10	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
15	High	72.2	107	109	133	67.4	18.5
15	Low	1.00	0.500	1.00	2.90	0.300	0.20
15	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
20	High	121	55.4	122	413	37.0	28.8
20	Low	1.40	0.500	1.30	4.80	0.400	0.300
20	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
27	High	< 0.800	< 0.500	< 0.800	< 0.500	< 0.500	< 0.500
27	Low	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100
27	Control	< 0.0200	< 0.0100	< 0.0200	< 0.0100	< 0.0100	< 0.0100

Table A6. Perfluorobutane sulfonate (PFBS) and Perfluoroheptanoic acid (PFHpA) in water sampled at different times during and after exposure to PFASs (exposure ended at day 20). Samples were collected from one randomly chosen aquarium of every treatment at each day. The aquaria denoted day 27 were the only aquaria sampled twice. Samples marked with < are not detected. Concentrations after < are detection limits (varying with sample volume and sensitivity of analysis).

Time (Days)	Treatment	PFBS (µg/L)	PFHpA (µg/L)
3	High	< 0.8	0.9
3	Low	< 0.02	0.03
3	Control	< 0.02	< 0.01
6	High	< 0.8	1.6
6	Low	< 0.02	0.02
6	Control	< 0.02	< 0.01
10	High	< 0.8	1.6
10	Low	< 0.02	0.01
10	Control	< 0.02	< 0.01
15	High	0.9	2.0
15	Low	< 0.02	0.01
15	Control	< 0.02	< 0.01
20	High	< 0.8	1.5
20	Low	0.02	0.01
20	Control	< 0.02	< 0.01
27	High	< 0.8	< 0.5
27	Low	< 0.02	< 0.01
27	Control	< 0.02	< 0.01

Appendix 3: Oxygen, temperature and nitrite

Table A7. Oxygen saturation (%) in water from all experiment aquaria ("control", "low" and "high" treatments) measured at different days after started exposure to PFASs. (Exposure ended at day 20).

Day	Control exposure		Low exposure		High exposure	
	Range	Median	Range	Median	Range	Median
3	89.4 - 95.1	92.8	91.4 - 96.2	94.1	84.3 - 95.2	93.3
7	90.8 - 97.0	95.1	93.3 - 95.8	95.0	91.2 - 95.2	93.9
10	82.8 - 95.7	90.6	93.4 - 96.1	95.8	89.6 - 97.7	96.4
14	89.9 - 96.0	93.4	95.4 - 97.3	95.8	92.8 - 95.8	95.6
17	93.0 - 96.0	95.0	92.7 - 95.7	94.4	93.0 - 97.1	95.0
21	89.1 - 98.0	95.1	96.6 - 97.4	97.0	93.0 - 97.5	95.5
24	90.9 - 96.7	95.1	93.5 - 96.0	95.8	92.8 - 95.5	94.2
28	93.0 - 99.1	98.0	96.0 - 98.0	97.3	95.5 - 97.8	97.4
31	94.5 - 97.6	96.4	95.9 - 96.6	96.1	95.2 - 97.2	96.4
35	92.6 - 97.6	94.8	94.1 - 96.8	94.6	94.6 - 96.9	96.2
38	93.7 - 96.2	95.9	93.9 - 95.4	95.2	94.2 - 94.7	94.3
42	95.4 - 98.9	97.3	94.8 - 99.1	95.1	95.8 - 98.2	96.9
45	93.2 - 96.6	95.0	95.1 -96.4	95.8	93.9 - 96.2	95.4
49	93.8 - 96.0	95.5	94.8 - 95.9	95.2	94.1 - 95.7	94.9
52	95.5 - 98.3	97.5	96.2 - 98.8	97.2	96.9 - 99.5	97.5
56	95.4 - 98.4	97.4	95.9 - 98.1	97.9	96.7 - 97.6	97.3
59	95.7 - 97.5	97.0	94.8 - 97.8	97.2	96.8 - 97.9	97.2
63	93.7 - 99.3	96.7	96.6 - 98.2	97.3	95.1 - 98.9	98.0
66	93.0 - 98.4	97.4	95.0 - 97.8	97.2	95.8 - 97.9	97.6
70	92.7 - 97.7	95.6	94.3 - 96.7	96.2	94.4 - 97.0	95.9
73	94.4 - 96.6	95.9	94.4 -97.9	96.2	94.4 - 97.1	96.6
77	95.8 - 98.4	96.3	95.9 - 97.9	96.3	96.5 - 97.8	97.1
80	94.9 - 96.9	95.3	95.5 - 97.4	97.1	95.5 - 97.8	96.6
84	94.8 - 100	97.2	97.0 - 98.7	98.3	95.8 - 99.4	98.1
87	88.1 - 97.8	95.3	93.2 - 96.4	95.2	89.3 - 96.8	95.2

Table A8. Temperature (°C) in water from all experiment aquaria ("control", "low" and "high" treatments) measured at different days after started exposure to PFASs. (Exposure ended at day 20).

Day	Control exposure		Low exposure		High exposure	
	Range	Median	Range	Median	Range	Median
0	8.1 - 9.7	8.8	7.8 - 9.3	8.1	8.1 - 9.7	8.2
3	8.1 - 9.6	8.7	7.8 - 9.3	8.2	7.8 - 9.5	8.2
7	8.4 - 10	8.9	8.3 - 9.5	8.3	8.2 - 9.9	8.8
10	8.2 - 10	9.1	8.1 - 9.8	8.6	8.1 - 9.9	8.9
14	8.2 - 9.9	8.8	8.1 - 9.3	8.2	8.2 - 9.7	8.6
17	7.8 - 9.6	8.6	7.7 - 9.1	8.0	7.8 - 9.3	8.2
21	7.7 - 9.5	8.7	7.6 - 8.8	8.4	7.6 - 9.3	8.6
24	7.5 - 8.6	8.1	7.4 - 8.2	7.5	7.5 - 8.6	7.8
28	8.6 - 10	8.7	8.4 -9.7	8.7	8.5 - 10	8.7
31	8.1 - 9.5	8.6	8.0 - 9.1	8.1	8.1 - 9.4	8.2
35	8.3 - 9.6	8.9	8.1 - 9.5	8.3	8.2 - 9.7	8.6
38	7.7 - 9.2	8.7	7.6 - 9.1	8.3	7.9 - 9.3	8.5
42	8.0 - 9.1	8.7	7.7 - 8.9	8.1	8.1 - 9.2	8.3
45	7.8 - 9.0	8.6	7.7 - 8.8	8.1	7.8 - 9.0	8.1
49	7.6 - 8.8	8.3	7.5 - 8.7	7.8	7.7 - 9.0	7.9
52	7.8 - 9.2	8.6	7.7 - 8.8	7.9	7.8 - 9.2	8.2
56	7.7 - 9.1	8.5	7.6 - 8.6	8.0	7.8 - 9.1	8.2
59	7.7 - 9.3	8.7	7.8 - 8.8	8.2	8.1 - 9.2	8.3
63	8.1 - 9.7	8.8	8.1 - 9.0	8.3	8.3 - 9.7	8.6
66	8.1 - 9.8	9.0	8.1 - 9.1	8.5	8.2 - 9.7	8.8
70	8.8 - 10	9.6	8.7 - 9.8	9.0	8.9 - 10	9.5
73	9.0 - 11	9.6	8.8 - 9.9	9.2	9.1 - 11	9.5
77	8.9 - 11	9.6	8.6 - 9.2	8.8	9.1 - 10	9.4
80	9.3 - 11	10	9.1 - 10	9.6	9.2 - 11	9.8
84	9.6 - 11	10	9.3 - 10	9.7	9.6 - 11	10
87	9.7 - 12	10	9.4 - 11	9.9	9.7 - 11	10

Table A9. Nitrite levels (mg/L) in water from all experiment aquaria ("control", "low" and "high" treatments) measured at different times after started exposure to PFASs. (Exposure ended at day 20).

Day	Control exposure					Low exposure					High exposure				
-1	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
15	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
27	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
91	<0.3	0.3	0.3	0.3	<0.3	<0.3	<0.3	0.3	<0.3	<0.3	<0.3	0.3	<0.3	0.3	<0.3

Appendix 4: Biomarker responses

Table A10. Ethoxyresorufin *O*-deethylase activity in gills (EROD, fmol resorufin/filament/minute), thiobarbituric acid reactive substances in gills and liver (TBARS, nmol/mg protein), peroxisomal acyl-CoA oxidase activity in liver (AOX, mUnits/mg protein) and alanine aminotransferase activity in plasma (ALAT, Units/L) in trout from the "control", "low" and "high" treatments measured at week zero (reference), one and three of exposure, and week ten (marked 13) of depuration to a mixture of PFASs. EROD activities set to zero were originally -0.01879, -0.01052 and -0.02156, respectively. (NA: not available).

Time	Treatment	EROD gills	TBARS gills	TBARS liver	AOX liver	ALAT plasma
0	Control	0.00260885	1.13851113	0.20194223	0.03802873	NA
0	Control	0.00000000	0.83060020	0.28602793	0.04577241	52.9600144
0	Control	0.00000000	1.13741842	0.16616235	0.02683826	10.6899937
0	Control	0.31309896	0.91132764	0.17673278	0.05175291	28.3787687
0	Control	0.19805142	1.10976604	0.15312374	0.07358372	6.35961880
0	Low	0.02245230	0.82540592	0.14989764	0.03334642	66.0238481
0	Low	0.19797630	0.74392726	0.19974322	0.04422010	8.14509624
0	Low	0.33532298	1.85689201	0.22397965	0.03334648	21.6796991
0	Low	0.35276633	0.83122466	0.15595951	0.06152993	19.6705661
0	Low	0.24832119	2.21097705	0.19850651	0.02664844	15.1109812
0	High	0.03999504	2.11341962	0.20036263	0.04255040	5.75380565
0	High	0.08688480	1.05332144	0.22415705	0.03983613	14.3004766
0	High	0.05501449	0.94290619	0.15912421	0.04207595	10.1988930
0	High	0.30900628	0.81778287	0.22940776	0.05388709	11.4283701
0	High	0.18996034	1.48314215	0.15631727	0.04501129	20.5794819
1	Control	0.25033283	0.60674981	0.25105204	0.04313131	NA
1	Control	0.16307248	1.29202100	0.23907976	0.04823239	39.3784559
1	Control	0.15813418	0.68812935	0.17759052	0.05087089	12.1593899
1	Control	0.13508466	0.84656337	0.17047954	0.02591106	40.0247882
1	Control	0.00231710	1.04805981	0.21573067	0.05539412	19.8800850
1	Low	0.26987917	2.07510473	0.29728526	0.04307637	140.829061
1	Low	0.32753924	0.89608529	0.15140925	0.05344361	8.97205544
1	Low	0.14017014	0.61912165	0.17773404	0.04708033	10.3845983
1	Low	0.28440598	0.46775390	0.14347594	0.04437794	8.41563313
1	Low	0.14388856	0.75371694	0.21100204	0.10766497	NA
1	High	0.03433559	0.49320847	0.18353679	0.09792152	26.7152124
1	High	0.17720089	0.40479256	0.16343225	0.05347794	10.2933434
1	High	0.00000000	1.36393406	0.15354941	0.02481166	53.2761526
1	High	0.18515225	0.87752062	0.17475211	0.03428549	21.3211798
1	High	0.22182964	0.89138665	0.17770672	0.08024332	16.4422963
3	Control	0.30026344	0.71492428	0.16151516	0.04908315	32.0290060
3	Control	0.23044336	0.72724856	0.19298565	0.03397260	40.1783566
3	Control	0.44574243	0.61988741	0.18297248	0.04352503	16.7328636
3	Control	0.37274497	1.11745257	0.22104684	0.01842558	21.9339167
3	Control	0.66906652	0.54636268	0.25385367	0.08704870	42.1787062

3	Low	0.10824293	0.58244668	0.17822035	0.07701986	23.1485253
3	Low	0.30144058	1.21292124	0.14962283	0.04411113	16.0036640
3	Low	0.13090254	1.26362948	0.19959934	0.06276030	13.7955581
3	Low	0.20221758	1.46536677	0.15644860	0.05217358	64.3427723
3	Low	0.13314134	0.56361736	0.16007343	0.05607534	22.0321827
3	High	0.41436920	0.89711237	0.20180533	0.04615647	17.4958773
3	High	0.58577610	1.13291162	0.18540479	0.03946956	19.7635796
3	High	1.03159183	0.47716961	0.22290043	0.06363887	8.98843065
3	High	0.25259194	0.68807430	0.21631316	0.05669787	8.69518765
3	High	0.64405197	0.58207753	0.16288410	0.05020222	34.5926289
13	Control	0.53931440	1.04928058	0.16639285	0.04690759	12.0209709
13	Control	0.32825067	0.47452228	0.10174258	0.04097429	50.1893832
13	Control	0.21653895	1.04559859	0.16048578	0.04138652	14.9909608
13	Control	0.47937797	0.51327968	0.16689612	0.04285402	32.7593845
13	Control	0.70067771	0.41835795	0.18967931	0.03156717	33.6573004
13	Low	0.19994901	0.72212924	0.13715339	0.05407215	19.7706449
13	Low	1.17988209	0.58688793	0.14149760	0.03027108	26.4400937
13	Low	0.70343349	0.91626081	0.16499103	0.03793846	11.9875519
13	Low	0.60043631	0.44039053	0.13205152	0.11370235	8.90968574
13	Low	0.40423920	0.39862303	0.15104121	0.06225979	9.49213999
13	High	0.22591420	0.74981780	0.16795854	0.04106019	178.980317
13	High	2.99999789	0.44430519	0.34594636	0.03949995	28.5902206
13	High	1.07755247	0.50530025	0.16387666	0.0453046	23.4935222
13	High	0.89239931	0.48652728	0.15521159	0.04147473	46.9059446
13	High	1.02860044	0.96740499	0.26752743	0.04239122	13.3442976

Appendix 5: Buffers and solutions

EROD		
Hepes-Cortland buffer, pH 7.7 (adjust pH if necessary).	KCl	0.38 g/L
	NaCl	7.74 g/L
	$MgSO_4*7H_2O$	0.23 g/L
	$CaCl_2$	0.17 g/L
	$NaH_2PO_4*H_2O$	0.33 g/L
	Hepes	1.43 g/L
	Glucose	1.00 g/L
Reaction buffer	Hepes-Cortland buffer	35 mL
	Dicumarol (10 mM)	35 µL
	7-ethoxyresorufin (2.59 mM) dissolved in DMSO	13.7 µL

TBARS		
K-PO_4 buffer (0.1 M), pH 7.4 (adjust pH if necessary).	KH_2PO_4	2.59 g/L
	$K_2HPO_4*3H_2O$	18.48 g/L
Homogenization buffer, pH 7.4, in each cryo-tube.	K-PO_4 buffer 0.1 M, pH 7.4	200 µL
	Triton X-100	0.1%
	Butylated hydroxytoluene dissolved in methanol	18.15 nM
Trichloroacetic acid (0.73 M)	Trichloroacetic acid	12 g
	Distilled H_2O	100 mL
Thiobarbituric acid (0.051 M) (use ultra-sound, heating and mixing by magnets to solve).	Thiobarbituric acid	0.73 g
	Distilled H_2O	100 mL
Tris buffer (60 mM), pH 7.4, containing 0.1 mM diethylenetriaminepentaacetic acid	Trizma HCl	7.932 g/L
	Trizma base	1.164 g/L
	diethylenetriaminepentaacetic acid	0.0393 g/L

AOX		
TVBE homogenization buffer, pH 7.6	NaHCO3	84 mg/L
	Na-EDTA	10 mL/L
	Ethanol	1 mL/L
	10% Trition X-100	1 mL/L
2'7'-dichlorofluorescein diacetate (2.6 mM)	2'7'-dichlorofluorescein diacetate	50 mg
	dimetylformamide	3.846 mL
	NaOH (0.01 M)	34.62 mL
Reaction medium (for 100 mL)	K-PO4 buffer (0.5 M), pH 7.4	2 mL
	2'7'-dichlorofluorescein diacetate (2.6 mM)	2 mL
	Horseradish peroxidase (1200 Units/mL)	1 mL
	Sodium azide (4 M)	1 mL
	10% Trition X-100	200 µL
	Distilled H_2O	93.8 mL
Palmitoyl-CoA (3 mM)	Palmitoyl-CoA	30.2 mg
	Distilled H_2O	10 mL

Protein		
Tris buffer (0.1 M), pH 8.0 at 4 °C (adjust pH if necessary).	Trizma HCl	13.22 g/L
	Trizma base	1.94 g/L

ALAT		
Tris buffer (0.11 M), pH 7.8 (adjust pH if necessary).	Trizma HCl	11.704 g/L
	Trizma base	4.334 g/L
Tris/L-alanine (1260 mM), pH 7.8 (adjust pH with 1 M HCl).	Trizma base	1.33 g
	L-alanine	11.225 g
	Distilled H_2O	100 mL
Tris/D-alanine (1260 mM), pH 7.8 (adjust pH with 1 M HCl).	Trizma base	1.33 g
	D-alanine	11.225 g
	Distilled H_2O	100 mL
Pyridoxal 5'-phosphate solution (0.63 mM)	Pyridoxal 5'-phosphate hydrate	16.7 mg
	Tris buffer (0.11 M), pH 7.8	100 mL
β-NADH solution (6.12 mM)	β-NADH	108.6 mg
	Tris buffer (0.11 M), pH 7.8	25 mL
Lactic dehydrogenase solution (225 Units/L)	Lactic dehydrogenase in glycerol (2250 Units/L)	60 µL
	Tris buffer (0.11 M), pH 7.8	540 µL
Tris/α-ketoglutaric acid (180 mM), pH 7.8 (adjust pH with 12 M and 5 M HCl).	Trizma base	492 mg
	α-ketoglutaric acid disodium salt dihydrate	1052 mg
	Distilled H_2O	40 mL
NaCl (154 mM)	NaCl	0.9 g
	Distilled H_2O	100 mL
Reaction mixture (50 mL)	Tris/L-alanine or Tris/D-alanine solution (1260 mM), pH 7.8	19.71 mL
	Pyridoxal 5'-phosphate solution (0.63 mM)	7.93 mL
	β-NADH solution (6.12 mM)	5.675 mL
	Lactic dehydrogenase solution (225 Units/L)	0.267 mL
	Tris buffer (0.11 M), pH 7.8	16.42 mL

Appendix 6: Chemicals and lab equipment

Chemical name	CAS number	Producer
6:2 Fluorotelomer sulfonate (6:2FTS)	27619-97-2	Chiron, Trondheim, Norway
Acetone	67-64-1	VWR, Oslo, Norway
Alkaline copper tartrate solution (Reagent A)	500-0113*	Bio-Rad, Oslo, Norway
Bovine serum albumin protein standard	9048-46-8	Sigma-Aldrich, Oslo, Norway
Butylated hydroxytoluene	128-37-0	Sigma-Aldrich, Oslo, Norway
Calcium chloride ($CaCl_2$)	10043-52-4	Sigma-Aldrich, Oslo, Norway
D-alanine	338-69-2	Sigma-Aldrich, Oslo, Norway
Dicumarol	66-76-2	Sigma-Aldrich, Oslo, Norway
Diethylenetriaminepentaacetic acid (DTPA)	67-43-6	Sigma-Aldrich, Oslo, Norway
Dimethyl sulfoxide (DMSO)	67-68-5	Sigma-Aldrich, Oslo, Norway
Ethanol	64-17-5	Sigma-Aldrich, Oslo, Norway
Ethoxyresorufin	5725-91-7	Sigma-Aldrich, Oslo, Norway
Ethylenediaminetetraacetic acid disodium salt dihydrate (Na-EDTA)	6381-92-6	Sigma-Aldrich, Oslo, Norway
Folin reagent (Reagent B)	500-0114*	Bio-Rad, Oslo, Norway
Glucose	50-99-7	Sigma-Aldrich, Oslo, Norway
Glycerol	56-81-5	Sigma-Aldrich, Oslo, Norway
Heparin	9041-08-1	Sigma-Aldrich, Oslo, Norway
Hepes	7365-45-9	Applichem, Darmstadt, Germany
Hydrogen chloride (HCl)	7647-01-0	Sigma-Aldrich, Oslo, Norway
Hydrogen peroxide (H_2O_2)	7722-84-1	Sigma-Aldrich, Oslo, Norway
Lactic dehydrogenase	9001-60-9	Sigma-Aldrich, Oslo, Norway
L-alanine	56-41-7	Sigma-Aldrich, Oslo, Norway
Magnesium sulfate heptahydrate ($MgSO_4*7H_2O$)	10034-99-8	Merck, Drammen, Norway
Malondialdehyde (MDA) tetrabutylammonium salt	1000683-54-3	Sigma-Aldrich, Oslo, Norway
Methanol	67-56-1	Sigma-Aldrich, Oslo, Norway
n-Perfluoropentanoic acid (PFPeA)	2706-90-3	Chiron, Trondheim, Norway
Palmitoyl-CoA	188174-64-3	Sigma-Aldrich, Oslo, Norway
Perfluorohexane sulfonate (PFHxS)	3871-99-6	Chiron, Trondheim, Norway
Perfluorohexanoic acid (PFHxA)	307-24-4	Chiron, Trondheim, Norway
Perfluorooctane sulfonate (PFOS)	1763-23-1	Chiron, Trondheim, Norway
Perfluorooctanoic acid (PFOA)	335-67-1	Chiron, Trondheim, Norway
Potassium chloride (KCl)	7447-40-7	Merck, Drammen, Norway
Pyridoxal 5'-phosphate hydrate	853645-22-4	Sigma-Aldrich, Oslo, Norway
Resorufin standard	635-78-9	Sigma-Aldrich, Oslo, Norway
Sodium azide (NaH_3)	26628-22-8	Sigma-Aldrich, Oslo, Norway
Sodium bicarbonate ($NaHCO_3$)	144-55-8	Biochrom AG
Sodium chloride (NaCl)	7647-14-5	VWR, Oslo, Norway
Sodium phosphate monobasic monohydrate ($NaH_2PO_4*H_2O$)	231-449-2	Sigma-Aldrich, Oslo, Norway
Thiobarbituric acid (TBA)	504-17-6	Sigma-Aldrich, Oslo, Norway
Trichloroacetic acid (TCA)	76-03-9	Merck

Triton X-100	9002-93-1	Sigma-Aldrich, Oslo, Norway
Trizma base	77-86-1	Sigma-Aldrich, Oslo, Norway
Trizma HCl	1185-53-1	Sigma-Aldrich, Oslo, Norway
α-ketoglutaric acid disodium salt dihydrate	305-72-6	Sigma-Aldrich, Oslo, Norway
β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH)	606-68-8	Sigma-Aldrich, Oslo, Norway

*Product number.

Instrument/Software	Producer
500 series process pumps	Watson Marlow
Centrifuge 5425	Eppendorf
Centrifuge 5702R	Eppendorf
Cryolys	Bertin Technologies
Gen5	BioTek Instruments
Magnetic mixer CS-C10	VWR
Magnetic mixer VMS-A	VWR
PHM 92 LAB pH METER	Radiometer Copenhagen
Precellys ®24	Bertin Technologies
Schego airpump prima	Webzoo.net
Spectrafuge™ Mini Laboratory Centrifuge	Labnet International
Synergy MX Platereader	BioTek Instruments
Termaks T1056 UV Incubator	Termaks AS
Water-bath type 1003	GFL
Whirlimixer Vortex mixer	Fisons