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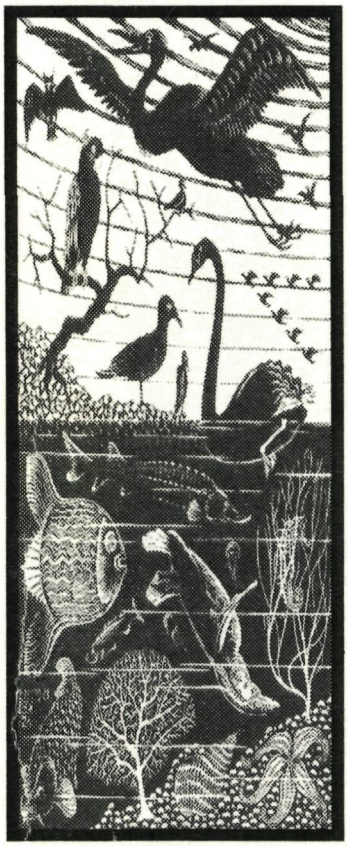

Perfluorooctane sulfonic acid associated risks in aquatic and terrestrial wildlife

Impactevaluatie van perfluorooctaan sulfonzuur op aquatische en terrestrische organismen

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen aan de Universiteit Antwerpen

te verdedigen door Philippe Hoff

Promotor: prof. dr. Wim De Coen **Antwerpen, 2005**

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I always say the chief end of man is to form general propositions
– adding that no general proposition is worth a damn.

Oliver Wendell Holmes (1809-1894)

De auteur dankt de Escher Company voor toelating tot gebruik van het grafisch werk van de kunstenaar ("Animals" © 2005, the M.C. Escher Company, Baarn, Nederland. Alle rechten voorbehouden. www.mcesher.com).

Dankwoord

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Nooit zal ik onze ijskoude, ongewoon ruikende, te zout smakende, zeeziek makende, en letterlijk "adembenemende" (hè, Inneke) vistochten

vergeten. Het was **FANTASTISCH**!!! Trouwens, Karen, ge hebt nog een stuk van 20 frank te goed, hé, en waar is uwe "caoutchoue lap"?

Ik besef eigenlijk nu pas dat deze dankbetuigingen vijgen na Pasen zijn en ik wou dat ik jullie doorheen de jaren meer dankbaarheid, openheid, vrijgevigheid en positieve "vibes" had laten ervaren. Misschien is dit besef ook een positief resultaat van mijn verblijf in het "aquarium van het zevende"? Ik kan er alleen nog maar meer dankbaar om zijn…

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Frequently used abbreviations

ABPL	average DNA basepair length
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
cDNA	complementary deoxyribonucleic acid
CC	*cis*-chlordane
DDE	dichlorodiphenylethane
DDD	dichlorodiphenyldichloroethane
DDT	dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
ES	electrospray
FOC	fluorinated organochemical
EC	effect concentration
FABP	fatty acid binding protein
GJIC	gap junction intercellular communication
GLM	general linear model analysis
HCH	hexachlorocyclohexane
HCB	hexachlorobenzene
Kow	n-octanol/water partition coefficient
LMM	linear mixed model analysis
LOD	limit of detection
LOAEL	lowest observed adverse effect level
LOEL	lowest observed effect level
LOQ	limit of quantitation
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
ND	not determined

N-EtFOSAA	N-ethylperfluorooctanesulfonamidoacetate
N-MeFOSAA	N-methylperfluorooctanesulfonamidoacetate
NOAEL	no observed adverse effect level
NOEC	no observed effect concentration
NOEL	no observed effect level
NS	not significant
OCP	organochlorine pesticide
OxC	oxychlordane
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PBDE	polybrominated biphenyl ether
PFAO	peroxisomal fatty acid oxidation
PFBS	perfluorobutane sulfonic acid
PFDA	perfluorodecanoic acid
PFDOA	perfluorododecanoic acid
PFHS	perfluorohexane sulfonic acid
PFNA	perfluorononanoic acid
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
PFOSA	perfluorooctanesulfonamide
PFOSAA	perfluorooctanesulfonamidoacetate
PFUA	perfluoroundecanoic acid
PLS	partial least square
POSF	perfluorooctanesulfonylfluoride
PPAR	peroxisome proliferator activated receptor
QCB	pentachlorobenzene
RNA	ribonucleic acid
SD	standard deviation
SF	safety factor

SRM	single reactant monitoring
SSH	suppressive subtraction hybridization
TAA	total antioxidant activity
TC	*trans*-chlordane
TN	*trans*-nonachlor
ww	wet weight

Contents

Introduction

In ecotoxicology, "risk" is defined as "the probability of occurrence of adverse effects on man or environment resulting from exposure to a chemical or mixture" (van Leeuwen and Hermens, 1995). Consequently, the risk assessment of a chemical is the integration of both its chemical exposure and hazard assessment. Exposure assessment entails the determination of emission, distribution, transformation and degradation of a chemical in order to determine human and environmental exposure. Hazard assessment, on the other hand, is the inherent capacity of a chemical to cause adverse effects to man or environment.

Historically, two main factors have triggered the need for evaluating risk associated with perfluorooctane sulfonic acid (PFOS) exposure in humans and wildlife. The first factor was the publication of a paper on the global distribution of PFOS (Giesy and Kannan, 2001). This paper showed for the first time that PFOS, an industrially produced chemical, was present as a contaminant in a great diversity of wildlife species and that PFOS could biomagnify through the food chain. Around that time, Hansen al. (2001) also reported on the presence of PFOS in serum of non-occupationally exposed humans and showed that PFOS was present in human serum at higher concentrations than other related perfluorochemicals. The second factor was the scarcity of information on possible adverse effects related to PFOS exposure at the time its worldwide occurrence was reported for the first time, especially for non-rodent species.

At present, it is clear that these two factors have contributed to a growing interest in PFOS-associated risk. This is demonstrated by the recent

accumulation of information dealing with human and environmental PFOS levels and the compounds toxicological effects. An overview of this information is given below taking into account scientific literature until February 2005. The information on the toxicological effects of PFOS is focusing on mammals, fishes and birds.

1. Use and production volumes of PFOS

PFOS is a perfluorooctanesulfonylfluoride (POSF) based chemical that is mainly produced by the 3M Company using electrochemical fluorination of octanesulfonyl fluoride (3M, 2000c). In 2000, the volume of PFOS and its salts that were commercialized as finished products was less than 91 metric tons (3M, 2000a). PFOS is also used as a chemical intermediate for the production of PFOS-based chemicals.

These chemicals can be divided in three main classes: products for surface treatment applications such as textile, leather and carpet production and aftermarket treatment, products for paper protection for food and non-food applications and performance chemicals such as fire fighting foams, insecticides and floor polishes. The estimated global production volume of these PFOS-based chemicals was 4481 metric tons in 2000 (3M, 2000a).

PFOS-based products have been commercialized for over 40 years but information on cumulative production volumes has not been released by 3M so far. Aside from 3M manufacturing units in Decatur (AL, USA) and Antwerp (Belgium) other countries such as Italy and Japan also have production capacity. It is, however, unknown what their contribution to PFOS production is. Another contributing factor might be residual fluorochemicals that are present at concentrations of 1-2 % in final products. These residuals might potentially degrade or metabolize to

PFOS (3M, 2000a). Since 3M has announced phasing out the production of POSF-derived chemicals in May 2000, it is assumed that POSF-production has declined to zero by the end of 2002 (3M, 2000b).

2. Structure and physicochemical properties of PFOS

PFOS is an amphipatic molecule consisting of a hydrophobic perfluorinated carbon tail consisting of eight carbon atoms and a hydrophilic sulfonyl group.

Because PFOS forms three layers in octanol/water due to its amphipatic structure an n-octanol/water partition coefficient (Kow) cannot be determined. PFOS has respective mean solubilities of 519 mg/l and 370 mg/l in pure water and fresh water at 24-25°C, 12.4 mg/l in natural seawater at 22-23°C and 25 mg/l in filtered seawater 22-24°C (3M, 2001a). In octanol, PFOS´ solubility is 56.0 mg/l (3M, 2001b). Therefore, PFOS is presumed to remain in the water phase once it is discharged to a water source although almost no information is at present available on PFOS´ presence in particulate matter or sediment or its binding potential to particulate matter. PFOS´ vapor pressure was determined to be 3.31 x 10^{-4} Pa at 20 °C, corresponding with 3.27 x 10^{-9} atm (3M, 1999).

These vapor pressures and solubilities yield following Henry´s law constants of 3.05 and 4.7 x 10^{-9} atm.m^3/mol in pure and fresh water, respectively. In unfiltered and seawater Henry´s law constants were 1.4 x 10^{-7} and 2.4 x 10^{-8} atm.m^3/mol. This is in agreement with PFOS´ low air/water partition coefficient (< 22 x 10^{-6} Pa.m^3/mol). PFOS is also resistant to photolysis (Hatfield, 2001).

3. Biodegradability and metabolisation

PFOS is not biodegradable (Kurume laboratory, 2002; Key et al., 1998) but seems to be an end metabolite of some perfluorinated compounds. Xu et al. (2004) showed that N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide can undergo N-deethylation to N-(2-hydroxyethyl)perfluorooctanesulfonamide that can be deethylated to perfluorooctanesulfonamide (PFOSA). PFOSA can undergo metabolisation to PFOS in rat liver slices. In rainbow trout (*Oncorynchus mykiss*) microsomes it has been shown that N-ethylperfluorooctanesulfonamide can be converted to PFOS (Tomy et al., 2004). Biodegradation to PFOS as persistent end metabolite has also theoretically been predicted (Dimitrov et al., 2004).

4. Tissue distribution and pharmacodynamics

PFOS is readily absorbed in rats and distributes preferably to liver, less to plasma or serum and the least in kidney, lung, muscle, skin, bone marrow, spleen, eyes, brain and testes. Also in the mouse the liver is the preferential organ for PFOS accumulation compared with serum (Thibodeaux et al., 2003).

In the rat, urinary excretion is the most important route of elimination for PFOS. The serum elimination half-life in rats was found to be 7.5 days (Johnson et al., 1979a, 1979b). It is suggested that PFOS is subjected to enterohepatic circulation (Johnson et al., 1984) and that the compound binds to serum albumin and fatty acid binding protein (Luebker et al., 2002; Jones et al., 2003). Placental PFOS transfer has been demonstrated in rats as fetuses have elevated hepatic PFOS concentrations when dams are exposed to PFOS (Thibodeaux et al., 2003).

In PFOS exposed cynomolgus monkeys, liver-to-serum PFOS ratios ranged between 1:1 to 2:1 with average serum half lifes of 200 days (Seacat et al., 2002).
Also in feral seals, liver PFOS concentrations exceed serum PFOS concentrations about five times, and both concentrations are significantly and positively correlated (Kannan et al., 2002b).

In rainbow trout (*Oncorhynchus mykiss*), PFOS bioaccumulated to approximately the same extent in liver, plasma, gall bladder kidney and less in blood cells, muscle, gills, gonads and adipose tissue days (Martin et al.,2003a). PFOS was also shown to bioconcentrate in rainbow trout, bluegill sunfish (*Lepomis macrochirus*, 3M, 2001b) and common carp (*Cyprinus carpio*, Martin et al., 2003b).

In humans, the mean PFOS liver-to-serum ratios was 1.3:1 (Olsen et al., 2003d) and serum halflife for PFOS elimination of occupationally exposed subjects was 8.67 years (Burris et al., 2002).

5. Environmental exposure

5.1 Abiotic monitoring

Several studies have reported on PFOS concentrations in surface water, ground water and drinking water samples in North-America and East-Asia in order to determine PFOS contamination levels and detect possible PFOS contamination sources. Aside from PFOS, the measurement of some other related perfluorinated compounds was included in some of these studies. Reports on sediment and dust analyses of PFOS concentrations are less abundant than water analyses.

5.1.1 Water

Elevated PFOS concentrations have been shown downstream from the wastewater outlet of fluorochemical production units. Hansen et al. (2002) showed that PFOS could be detected in the Tennessee river at mean concentrations measured of 32 and 114 ng/l upstream and downstream from a fluorochemical manufacturing unit in Decatur (AL, USA), respectively. Next to PFOS, perfluorooctanoic acid (PFOA) was also measured at mean levels of < 25 and 394 ng/l in the upstream and downstream zones, respectively. The PFOS and perfluorooctanoic acid (PFOA) concentrations appeared to be constant within the upstream and downstream sampling zones, suggesting that there was no important degree of volatilization or adsorption and no additional source of PFOS or PFOA over the river stretch under analysis.

Next to releases from fluorochemical production sites, aquatic PFOS contamination can also originate from accidental releases of finished products, such as fire fighting foams. Moody et al. (2002) reported a maximum aquatic PFOS concentration of 2210 µg/l in a creek after an accidental spill of 22000 l of fire fighting foam. The measured perfluorohexane sulfonic acid (PFHS) and PFOA concentrations were at least an order of magnitude lower than the measured PFOS concentrations and aquatic PFOS concentations were suggested to decrease with time.

Fire fighting foams used at air force bases might also be sources of perfluoroalkanesulfonate contamination (Moody et al., 2003). The authors measured PFOS concentrations from 4 to 110 µg/l in groundwater from such a site, next to PFHS (9-120 µg/l), PFOA (< 3-105

μg/l) and perfluorohexanoic acid (< 3-20 μg/l). Perfluorobutane sulfonic acid (PFBS), perfluoropentanesulfonate and perfluoroheptane sulfonic acid concentrations were < 3μg/l. The total concentrations of the perfluoroalkanesulfonates was generally greater than the total perfluorocarboxylate concentration. As these concentrations were measured several years after cessation of fire fighting foam activity, it was suggested that these compounds are persistent in the sub-surface environment. The results of his study also showed migration of these compounds from the presumable contamination site.

In a Japanese study, the PFOS concentration range of 142 river surface water samples was reported to be 0.3-157 ng/l and coastal sea water concentrations ranged between 0.2 and 25.2 ng/l. PFOS concentrations were more elevated at sewer discharge sites (Saito et al., 2003).
In another study by Saito et al. (2004), the reported PFOS concentration ranges in some Japanese rivers were 0.24-37.32 ng/l. Coastal water and tap water PFOS concentration ranges were 0.61-27.69 and 0.1-12.0 ng/l, respectively. A significant correlation was found between surface water PFOA and PFOS concentrations. PFOS concentrations were higher at water discharge sites.
Taniyasu et al. (2003) measured maximum PFOS concentrations in Japanese freshwater and seawater samples of 59 ng/l while PFHS and PFBS concentrations were < 11 and < 60 ng/l, respectively with the highest concentrations measured in industrialized and urbanized areas.

In coastal waters of China and Korea, PFOS and PFOA concentrations are comparable to Japanese coastal seawater (So et al., 2004) and also the possibility of cities and industry being contamination sources is

confirmed in this study. The importance of river discharges into the sea influencing perfluorinated compound contamination was also shown.

Water from the Great Lakes (Canada/USA) has been demonstrated to have PFOS concentrations between 21 and 70 ng/l and to contain the PFOS precursors N-ethylperfluorooctanesulfonamidoacetate (N-EtFOSAA), PFOSA and perfluorooctane sulfinate at maximal concentrations of 17 ng/l (Boulanger et al., 2004).

5.1.2 Sediment

Average PFOS concentrations in sediment from some cities in the USA ranged between < 0.2 and 2740 ng/g dry weight. Maximal estimated PFOS pore water concentrations ranged between < 0.04 and 1792 ng/ml. The most elevated concentrations were measured in sediment from a fluorochemical plant outfall area (3M, 2003).

5.1.3 Dust

Moriwaki et al. (2003) measured PFOS concentrations of 11-2500 ng/g dust in vacuum cleaners. PFOA concentrations were ranging between 69 and 3700 ng/g and correlated significantly with the PFOS concentrations in the same samples suggesting identical sources for both compounds.

5.2 Biomonitoring

PFOS tissue concentrations have been measured in wildlife species and humans and measurements have generally been performed on blood, serum, plasma, cord blood and liver tissue. The wildlife species

investigated are mainly vertebrate species with diverse origins. Occupationally PFOS exposure levels as well as screenings of general human populations in America, Europe and eastern Asia have also been reported. Many of these reports do not only focus on PFOS but also include concominant measurements of other perfluorinated compounds.

5.2.1 Human exposure

Human biomonitoring revealed that PFOS is present in serum from fluorochemical production employees of a POSF production units in Decatur (Alabama, USA) and Antwerp (Belgium) at mean concentrations higher than 1 μg/ml serum (Olsen et al., 1999, 2003a, 2003b). The PFOS concentrations in Antwerp employees were approximately 50 % lower serum compared to the Decatur plant (Olsen et al., 2003a). Chemical plant employees had PFOS concentrations approximately one order of magnitude higher than film-plant employees where fluorochemicals are not produced. (Olsen et al., 2003b). According to Olsen et al. (2003b) females had lower serum PFOS levels than males but also less years of occupational exposure. It is unclear if this difference is due to different exposure patterns or differences in pharmacokinetics. Serum PFOS concentrations declined over the period 1994-2000 (Olsen et al., 2003a).

In addition to PFOS, other perfluorochemicals were detectable in fluorochemical manufacturing employees: N-EtFOSAA, N-methylperfluorooctanesulfonamidoacetate (N-MeFOSAA), perfluorooctanesulfonamidoacetate (PFOSAA), PFOSA, PFOA and PFHS. PFOS and PFOA were the compounds with the highest serum concentrations (Olsen et al., 2003b).

Next to occupational exposure, also non-occupational PFOS exposure has been investigated. Hansen al. (2001) measured PFOS concentrations from 6.7 to 81.5 ng/ml in human serum from biological supply companies. In Canadian and Japanese volunteers, PFOS concentration ranges of 3.7-63.9 ng/ml serum and 3.5-28.1 ng/ml blood were measured, respectively (Kubwabo et al., 2004; Harada et al., 2004), which corroborates PFOS concentrations in blood measured by Taniyasu et al. (2003).

Olsen et al. (2004) measured an average PFOS concentration of 31 ng/ml in elderly subjects from Seattle (WA, USA) with lower PFOS concentrations in the most elderly among subjects with age 65-96. This average PFOS concentration was comparable with younger adult subjects who had an average PFOS concentration of 34.9 ng/ml (Olsen et al., 2003c). Olsen et al. (2003c, 2004) and Taniyasu et al. (2003) did not observe sex differences in blood PFOS concentration but Harada et al. (2004) measured higher PFOS concentrations in males and serum PFOS concentrations in Japanese females were shown to be increased by a factor 3 over the last 25 years. Harada et al. (2004) did not show any age effect but regional differences in PFOS concentrations were shown.

Also in non-occuptionally exposed human blood, serum and plasma, PFOS concentrations were generally higher than concentrations of other measured perfluorinated compounds such as N-EtFOSAA, N-MeFOSAA, PFOSAA, PFOSA, perfluoroheptanoic acid, PFOA, perfluorononanoic acid, perfluorodecanoic acid, perfluorododecanoic acid, perfluoroundecanoic acid, PFBS, PFHS, perfluorodecane sulfonic acid (PFDS) (Harada et al., 2004; Inoue et al., 2004; Kannan et al., 2004; Kubwabo et al., 2004; Olsen et al., 2003c, 2004; Taniyasu et al., 2003). PFOS in serum was shown to strongly correlate with PFOA concentrations (Olsen et al., 2003c, 2004).

Also in human cord blood, PFOS has been shown to be the predominant perfluorinated compound (Inoue et al., 2004). In this study, PFOS concentrations in cord blood were about three times lower than in maternal blood but both concentrations were shown to be well correlated.

A comparative study assessing PFOS concentrations in human blood, serum and plasma samples from different countries (Kannan et al., 2004) showed that PFOS concentrations were the highest in samples from Poland and the USA (> 30 ng/ml), moderate in Korea, Belgium, Malaysia, Brazil, Italy and Colombia (3-29 ng/ml) and lowest in India (< 3 ng/ml). Also in this study, no gender- or age-related differences in PFOS concentration were shown. The association of the PFOS concentration and concentrations of other perfluorinated compounds varied depending on the origin of the samples, suggesting different exposure patterns.

Aside from serum, PFOS has also been measured in donor liver tissue at concentrations between < 4.5 ng/g and 57.0 ng/g with a mean liver to serum ratio of 1.3:1. Liver and serum concentrations of PFOSA, PFOA and PFHS were mostly below the limit of quantitation (ranges 3.4-18.5 ng/g for liver and 1.2-3 ng/ml for serum; Olsen et al., 2003d).

5.2.2 Wildlife exposure

PFOS is present in a great diversity of feral animals all over the world. Tissue PFOS concentrations have been assessed in fishes (Giesy and Kannan, 2001; Taniyasu et al., 2003; Kannan et al., 2002b), birds (Giesy and Kannan, 2001; Kannan et al. 2001a, 2002a, 2002b; Martin et al., 2004; Rattner et al., 2004), oysters (Kannan et al., 2002c), shrimps, crabs,

starfishes (Van de Vijver et al., 2003a), turtles and frogs (Giesy and Kannan, 2001) and terrestrial and marine mammals (Kannan et al. 2001b, 2002b, 2002d; Martin et al., 2004; Van de Vijver et al., 2003b, 2004).

Overall, the measured tissue PFOS concentrations in wildlife are below 1000 ng/g wet weight (ww) or 1000 ng/ml. The highest PFOS concentrations are usually measured in tissues from top predators such as mink liver or bald eagle plasma in which maximal PFOS tissue concentrations of 5140 ng/g ww and 2570 ng/ml have been measured (Giesy and Kannan, 2001, Kannan et al., 2002d). Relatively high PFOS concentrations were also measured in predators such as the bottlenose dolphin liver (1520 ng/g ww), polar bear liver (> 4000 ng/g ww) and cormorant liver (1780 ng/g ww) (Kannan et al., 2001a, 2001b; Martin et al., 2004) suggesting that PFOS can biomagnify through food chains. This is supported by a study by Van de Vijver et al. (2003b) who showed that marine mammals with a higher trophic position have more elevated hepatic PFOS concentrations and by a study of Martin et al. (2004b) on a food web in Lake Ontario.

A general observation in these biomonitoring surveys were the relatively lower PFOS concentrations in widlife tissues from remote locations compared to more populated and industrialized areas (Giesy and Kannan, 2001; Kannan et al., 2001a, 2002d). Also, PFOS concentrations were generally higher than the other measured perfluorinated compounds such as perfluorocarboxylates, perfluorinated sulfonates and PFOSA (Martin et al., 2004; Taniyasu et al., 2003; Kannan et al., 2002a, 2002b, 2002d; Rattner et al., 2004).

Age differences in PFOS tissue concentrations were not observed in birds, minks, river otters and ringed and grey seals (Kannan et al., 2001a,

2001b, 2002a, 2002b, 2002d) although Kannan et al. (2001a) observed that bald eagles nestlings might have relatively lower PFOS concentrations than adult birds. Sex-related differences were also not shown in birds, minks or ringed seals but PFOS concentrations in blood or liver of male grey seals were reported to be significantly higher than in females (Kannan et al., 2001b, 2002a, 2002b, 2002d).

Although data are scarce, liver PFOS concentrations in wildlife could have increased since the seventies as suggested for white-tailed sea eagles (Kannan et al., 2002b).
PFOS concentrations in liver and blood were shown to be significantly related in seals (Kannan et al., 2002b).

5.3 Toxicologic effects in animals

Overall, the investigated toxicologic effects of PFOS can be classified as effects at the biochemical level, effects on organ level and effects on mortality, growth, body weight gain, reproduction and developmental effects. Also behavioural endpoints and visual judgement of anomalies were considered as endpoints for toxicity. Biochemical effects of PFOS exposure are exclusively documented for mammalia while mortality, growth, body weight gain and hatching are the only endpoints investigated in fishes and birds. The overview given below is limited to mammalia, fishes and birds because information on PFOS´ toxicology is almost exclusively available for these classes.

5.3.1 Monkey

In a subchronic PFOS exposure study in cynomolgus monkeys, serum cholesterol decrease was the earliest endpoint to be significantly affected among a set of biological, hematological, serum clinical chemistry and hormonal endpoints (Seacat et al., 2002). As well in males as in females, serum cholesterol concentrations decreased significantly at serum PFOS levels > 100 µg/ml. Other significant changes were a decrease in body weight, increases in liver weight, relative liver weight, decreases in serum high density lipoprotein, triiodithyronine, estradiol and bilirubin levels, increase in serum bile acid concentration and lipid accumulation in the liver. No peroxisomal proliferation in liver, pancreas, testes or hepatic cell proliferation was observed. The endpoints were shown to be reversibly altered. Mortality was observed in the highest dose exposure group. Average no observed effect levels (NOELs) were 82.6 and 66.8 µg/ml serum in males and females, respectively.

Mortality was also observed in rhesus monkey studies by Goldenthal et al. (1978a, 1979) and a cynomolgus monkey study by Thomford et al. (1998, 2000). In these studies, the monkeys showed several clinical signs of toxicity including body weight loss, trembling, weakness, convulsions and reapiratorial and intestinal tract toxicity. At necropsy, congestion, hemorrhage, lipid depletion of the adrenal cortex and atrophy of pancreatic exocrine cells, and hepatocellular hypertrophy and vacuolation were noted. Also in these studies a decrease in serum cholesterol was shown in addition to high density lipoprotein cholesterol, alkaline phosphatase activity and bilirubin concentration reductions and an elevation of bile acid concentration. Also decreases in estradiol values

Tests on ~~PFOS~~ monkeys showed that all monkeys died at exposures of 10 mg/kg bw/day. No ~~PFOS~~ monkeys died at 3,5 mg/kg bw/day

and triiodothyronine were observed. Liver weights, liver-to-body weight ratio and liver-to-brain ratios were ratios were increased.

5.3.2 Rat

A PFOS LC_5 concentration of 5.2 mg/l was determined by Rusch et al. (1979) after exposure to PFOS dust in air. Toxicological effects included emaciation, breathing disturbance, nasal discharge, yellow-stained anogential region and general poor condition. Also discoloration of liver, lungs and discoloration and distention of the small intestine was apparent. The rat oral LD50 value was approximately 250 mg/kg (Dean et al., 1978). This study revealed PFOS-induced hypoactivity, decreased limb tone and ataxia in addition to stomach distention, yellow material around the urogenital region, stomach distention, irritation of glandular mucosa and lung congestion.

A PFOS-mediated effect that has been confirmed by different authors is the induction of hepatic hypertrophy that is independent from cell proliferation (Austin et al., 2003; Berthiaume and Wallace, 2002; Haughom and Spydevold, 1992; Ikeda et al., 1987; Kozuka et al., 1991; Seacat et al., 2003). Austin et al. (2003) and Berthiaume and Wallace (2002) also observed a decrease in body weight while Hu et al. (2002) observed a significant decrease in body weight gain. A decrease in food intake was observed by Austin et al. (2003) which was probably related to a decrease in body fat.

Several studies have investigated the effect of PFOS exposure on the lipid metabolism of the rat. Ikeda et al. (1987) observed significant inductions of several peroxisomal activities of enzymes involved in the fatty acyl-CoA oxidizing system, carnitine acetyl and palmitoyl transferase and

catalase in addition to a co-induction of cytochrome P450 ω-hydroxylation and some drug metabolizing enzyme activities such as aminopyrine demethylase, ethoxycoumarin deethylase and propoxycoumarin depropylase. Kozuka et al. (1991) also observed a significant PFOS-mediated induction of carnitine acetyltransferase activity.

Also Berthiaume and Wallace (2002) and Seacat et al. (2003) reported a PFOS-mediated increase in peroxisomal β-oxidation activity in addition to a significant decrease in serum cholesterol concentration.

Haughom and Spydevold (1992) showed that PFOS exposure can reduce cholesterol synthesis and cholesterol esterification in the liver because of downregulation of hydroxymethyl glutaryl-CoA reductase and acyl-CoA cholesterol acyltransferase. It was also suggested that phospholipid synthesis in the liver was reduced. In the serum, cholesterol and triacylglycerol levels were decreased. The hypothesis of PFOS reducing the release of lipids from the liver was supported by the increase in liver triglyceride levels and the concominant decrease of fatty acid synthesis in the liver.

PFOS did not affect mitochondrial biogenesis in rat liver as suggested by the unaltered cytochrome c activity. Also, cytochrome a, b and c1 and the mitochondrial DNA copy number per cell were not affected by PFOS exposure (Berthiaume and Wallace, 2002). PFOS can affect mitochondrial membrane integrity, however, as shown by a PFOS-mediated increase in proton leakage of the inner mitochondrial membrane *in vitro* (Starkov and Wallace, 2002).

On the rat liver microsomal level, PFOS has been shown to be an inducer of carboxylesterase RL4 activity (Derbel et al., 1996).

A decrease in serum aspartate and an increase in alanine aminotransferase activity and urea nitrogen and decreases in serum glucose were observed in PFOS exposed rats (Seacat et al., 2003). No-observed-adverse effect levels for males and females were 44 and 64 μg/ml serum and corresponded to PFOS concentrations of 358 and 370 μg/g ww in liver, respectively.

PFOS has also been documented to have neuroendocrine effects: estrous cyclicity was affected in female rats after intraperitoneal PFOS injection. Serum corticosterone was shown to be increased and serum leptin concentrations were decreased in addition to a norepinephrine increase in the paraventricular nucleus of the hypothalamus (Austin et al., 2003).

PFOS can reversibly inhibit gap junctional intercellular communication (GJIC) in rat liver cells in a dose-dependent fashion *in vitro* (Hu et al., 2002). Derived NOELs and EC_{50} values were 3.1 and 14.98 μg/ml. *In vivo*, GJIC was observed at a similar extent after three days and three weeks of exposure corresponding with PFOS liver concentrations of 125.6 and 725.5 μg/g, respectively. No differences were observed in response between these exposure periods or between male and female rats.

Chronic PFOS exposure of rats resulted in an increased occurrence of hepatocellular adenoma, combined hepatocellular adenoma and carcinoma and thyroid follicular cell adenoma in male rats. The number of females with an hepatocellular adenoma and the combined incidence of hepatocellular adenoma and carcinoma was also increased after PFOS exposure, as was the combined incidence of thyroid follicular cell

adenoma and carcinoma. Also, the incidence of mammary adenoma and the combination of mammary adenoma and carcinoma increased after PFOS exposure (3M, 2002a).

Maternal weight gain during gestation was found to be suppressed by PFOS exposure as were food and water consumption. Fetal weights were found to be significantly reduced (Grasty et al., 2003; Thibodeaux et al., 2003; Lau et al., 2004).

In pregnant PFOS gavaged rat dams, relative liver weights were significantly increased and serum thyroxine and triiodothyronine levels were reduced. Triglyceride, but not cholesterol levels were also significantly reduced and the relative liver weight was increased. Neonatal thyroxine levels and choline acetyltransferase levels in the prefrontal cortex were significantly reduced.The number of live fetus implantations at term was not altered. Also, an increase in the number of cleft palates, anasarca, ventricular septal defects and enlargements of the right atrium was observed (Thibodeaux et al., 2003). Lau et al. (2003) observed that PFOS exposure of rat dams during gestation also increased pup age at eye opening and reduced neonatal rat survival. Grasty et al. (2003) suggested that PFOS exposure of dams late during gestation was the most susceptible period for induction of neonatal pup mortality and that inhibition of lung maturation might be involved. Reduced pup survival seems to be mainly the result of *in utero* exposure and postnatal exposure via lactation contributes to the reduction of pup survival exposed to PFOS in utero. PFOS exposure via lactation alone was not shown to have an adverse affect on postnatal viability (Christian et al., 1999).

5.3.3 Mouse

In accordance with PFOS rat toxicity, PFOS induces body weight loss, hepatomegaly, mitochondrial and cytosolic catalase activity and peroxisome proliferation in mouse liver (Sohlenius et al., 1993). As in rats, induction of peroxisomal β-oxidation is parallelled by an induction of ω-hydroxylation. Sohlenius et al. (1993) also showed significant increases in mitochondrial and microsomal protein content and increases in cytosolic glutathione transferase, epoxide hydrolase and DT-diaphorase activities.

PFOS has been shown to activate mouse peroxisome proliferator-activated receptor α (PPARα) transcription *in vitro* with respective EC_{50} and EC_{90} values of approximately 6 and 16 μg/ml. The authors also showed that PFOS induces transcription of acyl CoA oxidase, peroxisomal bifunctional enzyme and urate oxidase. PFOS-mediated induction of peroxisomal 3-ketoacyl-CoA thiolase was shown by Western blotting (Shipley et al., 2004).

Effects of PFOS in pregnant mouse dams include: decreases in body weight gain, food and water consumption and serum thyroxine and triglyceride levels. The dam liver weight and relative liver weights were significantly increased. Significant developmental effects were: a decrease in number of live fetuses, increases in pup liver and relative liver weight and an increased incidence of cleft palates, sternal defects, ventricular septal defects and enlargements of the right atrium (Thibodeaux et al., 2003).

5.3.4 Rabbit

PFOS developmental studies revealed body weight losses in PFOS treated pregnant rabbits, reduction in food consumption, increased maternal mortality, reduced fetal weights, increased abortion incidence, decreased litter size and increased resorption. The fetal weight was reduced and reversible delays in ossification were observed (Case et al., 2001). Also eye irritation has been reported (Biesemeyer and Harris, 1974).

5.3.5 Dolphin

Hu et al. (2002) showed that *in vitro* exposure experiment of dolphin kidney cells showed that PFOS could induce gap junctional intercellular communication (GJIC). The derived NOEL and EC_{50} values for GJIC were 3.1 and 12.8 μg/ml.

5.3.6 Birds

Acute PFOS toxicity tests have been carried out with the mallard duck (*Anas platyrhynchos*) and the northern bobwhite quail (*Colinus virginianus*). The most sensitive endpoint in these study in which mortality, body weight, growth and feed consumption were studied, was reduction in body weight for which an 8-day lowest observed adverse effect level (LOAEL) of 29.7 μg PFOS/g liver was derived for the mallard duck and 70.3 μg/g liver for the northern bobwhite quail. No observed adverse effect levels (NOAELs) in these studies were 15.4 and 44.0 μg/g liver for mallard duck and northern bobwhite quail,

respectively (United States Environmental Protection Agency, 2004a, 2004b).

Two chronic PFOS exposure studies considering mortality, body weight, feed consumption, liver weight, histopathology and reproductive endpoints showed a liver NOAEL range of 3.39-10.8 µg/g and a LOAEL of 60.9µg/g in the mallard duck and a liver NOAEL range of 4.9-88.5 µg/g in the northern bobwhite quail (United States Environmental Protection Agency, 2004c, 2004d).

5.3.7 Fishes

In an acute PFOS exposure studie, fathead minnow (*Pimephales promelas*) had a minimal 96 h LC_{50} value of 4.7 mg/l (3M, 1994) which is lower than the minimal 96 h LC_{50} value of 7.8 mg/l for bluegill sunfish (*Lepomis macrochirus;* 3M, 1979) and rainbow trout (*Oncorhynchus mykiss;* 3M, 1985a). In saltwater, a 96 h LC_{50} value of 13.7 mg/l has been determined for rainbow trout (3M, 1985b) and for sheepshead minnow (*Cyprinodon variegatus*) this value was > 15 mg/l (3M, 2002b). 96h no observed effect concentration values (NOECs) for bluegill sunfish and fathead minnow were 4.5 and 3.3 mg/l, respectively (3M, 1979, 2000d).

5-day and 42-day PFOS NOECs for survival, growth and hatching of fathead minnow were ≥ 0.3 mg/l (3M, 2000e). After 30 days of exposure no observed effects were observed at 1 mg PFOS/l (3M, 1978). Chronic PFOS exposure of bluegill sunfish for 62 days was characterized by a NOEC for mortality between 0.086 and 0.87 mg/l (3M, 2001b).

5.4 Toxicological effects in humans

Toxicological effects of PFOS in humans have been investigated *in vivo* by assessing biochemical endpoints in blood and urine of occupationally exposed fluorochemical workers and mortality assessment. The exploration of PFOS´ toxicity related with non-occupational exposure is limited and also *in vitro* studies on the toxicological effects of PFOS are scarce.

5.4.1 *In vitro*

Shipley et al. (2004) showed that PFOS can activate human PPARα in vitro. This activation is known to be involved in the regulation of genes involved in lipid metabolism and homeostasis, peroxisome proliferation and cell growth (Corton et al., 2000). So far, it is not clear whether this effect also occurs *in vivo*.

5.4.2 *In vivo*

Information on the relationships between PFOS exposure and possible biological effects in humans has been investigated in 3M POSF production units in Decatur (Alabama, USA) and Antwerp (Belgium). The available effect assessments included hematological, lipid, hepatic, thyroid and urinary endpoints and mortality.

In a combined cohort of male production and non-production employees from the Antwerp and Decatur production plants with a median serum PFOS concentration of 2.46 µg/ml the serum triglyceride, alkaline phosphatase, alanine aminotransferase and triiodothyronine levels were significantly increased compared with male employees from the cohort

with an average serum PFOS concentrations of 0.29 µg/ml. In female employees, the cohort with a median serum PFOS concentration of 1.34 µg/ml had significantly higher alkaline phosphatase and gamma glutamyl transferase levels than the cohort with a median PFOS concentration of 0.08 µg/ml. It was also shown that in the highest PFOS exposure male cohort a higher percentage of employees had one or more endpoints above the reference range value compared to the lower expoure cohorts. However, when adjusting for possible confounding factors, no substantial or clinically relevant associations were observed between the serum PFOS concentration and the investigated endpoints (Olsen et al., 2003a). Also stratification for sampling year and production plant identity did not reveal consistent substantial associations between PFOS levels and changes in hematological endpoints (Olsen et al., 1999).

Alexander et al. (2003) reported an excess of deaths from bladder cancer in workers who had high exposure jobs at the 3M POSF production facility in Decatur. The major drawbacks of this study, however, such as the limited number of bladder cancers, the estimation of the relative PFOS exposure levels and the lack of information on possible confounding factors warrant a further assessment of this observation.

The only study assessing possible clinical effects in non-occupationaly exposed humans has been conducted on blood of neonati. This study investigated thyroxine and thyroid stimulating hormone levels in blood of neonati and PFOS concentrations in cord blood but no significant correlation between both endpoints was shown, suggesting no PFOS-mediated effects on the investigated endpoints (Inoue et al., 2004).

Objective

The conducted research described in this thesis aimed at providing information enabling an initial assessment of risk associated with PFOS exposure in feral carp, eel, gibel carp, bib, plaice, blue and great tit and wood mouse in Belgium and The Netherlands. This information was obtained from laboratory exposure experiments dealing with the study of biochemical and histological effects of PFOS exposure in the common carp (chapters 1-2) and from field studies in which relationships were investigated between hepatic PFOS concentrations and biochemical and organismal endpoints in feral carp, eel, gibel carp, bib, plaice, blue and great tit and wood mouse (chapters 3-6).

Chapter 1

Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp (*Cyprinus carpio*)

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1.1 Abstract

In the present study we evaluated the toxicological effects of a scarcely documented environmental pollutant, perfluorooctane sulfonic acid (PFOS), on selected biochemical endpoints in the common carp, *Cyprinus carpio*. Juvenile organisms were exposed to PFOS through a single intraperitoneal injection (liver concentrations ranging from 16 ng/g to 864 ng/g after 5 days of exposure) and after 1 and 5 days effects were assessed in liver and serum of the exposed organisms. The investigation of the hepatotoxicity of PFOS included the determination of the peroxisome proliferating potential (peroxisomal palmitoyl CoA oxidase and catalase activity) and the compounds influence on the average DNA basepair length (ABPL) by agarose gel electrophoresis. Total antioxidant activity (TAA), cholesterol and triglyceride levels were monitored in the serum. After 1 day of exposure the ABPL was significantly increased in the 270 and 864 ng/g treatment groups. After 5 days of exposure significant increases relative to the control were observed for the 16, 270 and 864 ng/g treatment groups. Enzyme leakage from the liver was investigated by measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum. At 561, 670 and 864 ng/g PFOS a significant increase in serum ALT activity became apparent after 5 days of exposure with values ranging from 159 % to 407 % relative to the control. For serum AST activity significant increases for the 561 ng/g and 864 ng/g treatment groups were observed with values ranging from 75 % to 112 % relative to the control respectively. Determination of the polymorphonuclear leukocyte migration into liver tissue as assessed through myeloperoxidase (MPO) activity in liver, was used as an indicator for inflammation. It appeared that inflammation was

not involved in the observed membraneous enzyme leakage for the 561, 670 and 864 ng/g PFOS treatment groups.

The results of this study suggest that PFOS induces inflammation-independent enzyme leakage through liver cell membranes that might be related to cell necrosis. Furthermore, results show that PFOS does not significantly affects serum antioxidant levels nor does it clearly induce peroxisome proliferation in carp. This study also points out that PFOS might interfere with homeostasis of the DNA metabolism.

Histology did not reveal tissue damage or peroxisome proliferation.

The results of these biochemical analyses were used to perform an initial hazard assessment study indicating that PFOS levels observed in tissues of wildlife populations could induce a clear rise in serum transaminase levels indicative for disruption of hepatocyte membrane integrity.

1.2 Introduction

Among the halogenated organochemicals the fluorinated organochemicals (FOCs) have been studied less intensively than the chlorinated and brominated organics regarding their ecotoxicological properties. Nonetheless, these compounds are being produced since decades and have a broad application spectrum in industry and household as surfactants, lubricants, adhesives, refrigerants, fire retardants, propellants, agrochemicals and medicines (Key et al., 1997).

One class of FOCs, the perfluorinated sulfonates, are used as catalysts and surfactants. PFOS is one of these compounds with important applications as wetting and foaming agent and as precursor of other surfactants and pesticides (Abe and Nagase, 1982).

Recently it was reported by Giesy and Kannan (2001) and Kannan et al. (2001b) that PFOS is occuring worldwide in wildlife tissues with relatively high concentrations in top predators (e.g. 3680 ng/g wet liver weight in mink). Even in remote areas PFOS is present in detectable concentrations in a great diversity of organisms. Since fish-eating animals have higher PFOS burdens than their prey, it was suggested that PFOS has a tendency to bioaccumulate to higher trophic levels in the food chain. The presence of this compound has also been established in sera of fluorochemical production employees with a maximum observed concentration of 12.83 ng/ml (Olsen et al., 1999). PFOS has also been detected in commercially available non-industrially exposed human sera at concentrations up to 81.5 ng/ml (Hansen et al., 2001).

Notwithstanding its universal presence in the environment and its resistance to biotransformation (Key et al., 1998), available data on the toxicity of PFOS are rather scarce. A limited number of reports suggest that PFOS influences membrane function and structure (Gadelhak, 1992; Hu et al., 2000; Hu et al., 2001). Further effects of PFOS that have been demonstrated are hypolipemia (Haughom and Spydevold, 1992) and induction of microsomal liver carboxylesterase RL4 in rats (Derbel et al., 1996). PFOS is also shown to have potent hepatic peroxisome proliferating capacities in rats and mice (Ikeda et al., 1987; Sohlenius et al., 1992), a phenomenon that has been intimately correlated with hepatocarcinogenesis (Ashby et al., 1994).

Based on this information concerning the toxicology of PFOS, a selection of biochemical endpoints were evaluated for the common carp. Biochemical endpoints were chosen in order to unravel the working mechanism of PFOS and because of their function as early reporters in

comparison with endpoints at higher levels of biological organisation (Peakall, 1992). Because the ecotoxicological data concerning PFOS and fish is thus far limited to the study of PFOS distribution in fish eggs, liver and muscle tissue in fish populations in the USA, the Mediterranean Sea and the Pacific Ocean (Giesy and Kannan, 2001), carp was chosen as test species. Furthermore, carp is an internationally accepted test organism for aquatic effect assessments (OECD, 1993).

Lipid metabolism was investigated by monitoring serum triglyceride and cholesterol levels in addition to cyanide-insensitive palmitoyl CoA oxidation and catalase activities in liver which are classically being used as indicators of peroxisome proliferation. Further endpoints considered in the present study included monitoring of the total antioxidant activity (TAA) of serum and the DNA strand breaks in liver after PFOS exposure. PFOS´ membrane disrupting potential was determined by measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum reflecting hepatocyte leakage of both transaminases. The pro-inflammatory action of PFOS was assessed by determination of myeloperoxidase (MPO) activity in perfused liver tissue.
A preliminary hazard assessment for wildlife populations in general was performed based on the comparison of the experimentally obtained EC_{10}-values in the present study with the available exposure data from Giesy and Kannan (2001).

1.3 Materials and methods

1.3.1 Animals and treatment

Juvenile carps (Wageningen University, the Netherlands) of 6 months old were allowed to acclimatise for 2 weeks in a flow-through system (flow rate 15 l/min) consisting of glass aquaria with fully aerated softened tap water (17 ± 1 °C, pH 7.29 ± 0.14, $CaCO_3$ 86.8 ± 1.0 mg /l) and fed 1 % (w/w) Tetra Pond sticks (Tetra, Germany) once per day. PFOS (Aldrich, USA) was dissolved in physiological saline (9 g NaCl/l) and fish were injected once intraperitoneally with 2000, 25000 and 75000 ng/g PFOS resulting in mean concentrations of 16, 270 and 864 ng/g PFOS in liver after 5 days respectively. In a second experiment, additional treatments of 40000, 50000 and 60000 ng/g PFOS were added to the experimental setup yielding respective mean concentrations of 472, 561 and 670 ng/g PFOS in the liver after 5 days. The control fish only received a single intraperitoneal injection with saline. Before injection, the fish were anesthesized with MS-222 (0.125g/l). After 1 and 5 days, the fish were killed by an overdose of MS-222.

1.3.2 Liver enzymatic assays

All enzymatic assays were performed on the mitochondrial fraction prepared from liver homogenized in sucrose (0.25M) as described by Meijer et al. (1987) for the control group and the 16, 270 and 864 ng/g treatment groups. Catalase activity was measured spectrophotometrically at 240 nm reflecting direct H_2O_2 breakdown (Aebi, 1984). Palmitoyl CoA oxidase activity measurements were based on the peroxidase-linked oxidation of 4-hydroxyphenylacetic acid as electron donor according to

the fluorimetric method of Kvannes and Flatmark (1991) with minor modifications. Spectrophotometric determination of myeloperoxidase activity in livers perfused via ventricular perfusion with ice cold 5 mM Tris buffer containing 150 mM KCl was assessed as described previously (Junge et al., 2001). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad, Germany).

1.3.3 DNA strand integrity

Extraction of DNA from liver was performed with a phenol/chloroform extraction method for the control group and the 16, 270 and 864 ng/g treatment groups. Extracts were subjected to electrophoresis on 0.5 %-agarose gels in TBE-buffer (boric acid 45 mM, EDTA 2.5 mM, Tris base 135 mM, pH 8.0) together with Lambda DNA-*Hind III* digest (Biolabs, USA) as DNA molecular weight marker. Gels were stained with SYBR Green I (Molecular Probes, USA). Pictures from the gels were taken with Polaroid films type 667 (Pharmacia Biotech, USA) and scanned with Adobe Photoshop 5.0 software (Adobe, USA). The average basepair length (ABPL) was calculated as described in Black et al. (1996). Briefly, the ABPL was assessed by summing the percentage of digital light units (DLU) of the different fragment classes as defined by the band pattern of the Lambda-*Hind III* DNA (class I: > 9416 bp, class II: 9416-6557bp, class III: 6557-4361bp, class IV: 4361-2322bp, class V: 2322-2027bp, classVI: < 2027bp) times the mean basepair length for each class. Relative DLUs were determined using Optiquant Image Analysis software (Packard, USA).

1.3.4 Serum endpoints

Blood from carp was collected via caudal puncture. Serum was prepared by centrifugation (4000 rpm, 5 min) and collection of the supernatant. Serum cholesterol and triglycerides were measured spectrophotometrically. The cholesterol content was determined as described previously (Allain et al., 1974) while the triglyceride content was measured according to Spayd et al. (1978). The serum total antioxidant activities (TAAs) were measured using the spectrophotometric method of Arnao et al. (1999) with minor modifications. The TAAs of the sera were expressed as equivalent trolox concentrations eliciting the same antioxidant effect as the samples. All these endpoints were assessed for the control group and the 16, 270 and 864 ng/g treatment groups. Alanine aminotransferase and aspartate aminotransferase activities were determined according to Bergmeyer et al. (1986a) and Bergmeyer et al. (1986b) respectively for the control groups and all the treatment groups.

1.3.5 Histology

Liver coupes for electron microscopy were prepared for three fishes per exposure group. Glutaraldehyde fixation and alkaline 3,3′-diaminobenzidine treatment for staining of peroxisomes and mitochondria and preparation of electron microscopy coupes were done according to Braunbeck et al. (1990).

1.3.6 Determination of PFOS concentrations

After 5 days of exposure, the PFOS concentrations in the livers of the animals of all the treatment groups were measured using combined liquid chromatography-mass spectrometry according to Giesy and Kannan (2001) performed on a CapLC system (Waters, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech 11300, Sercolab, Belgium). The analysis was performed on a Betasil C18 column (50 mm x 1 mm i.d., Keystone Scientific) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH_4OAc (A) / CH_3OH (B). A gradient elution was used starting at 45 % B and going to 90 % B in 3 min. After 5 min initial conditions were resumed. PFOS was measured under (-) electrospray ionisation using single reactant monitoring (SRM, m/z 499→99). The internal standard (1H, 1H, 2H, 2H perfluorooctane sulfonic acid) was measured under the same conditions (SRM, m/z 427→81). The dwell time was 0.1 s. The ES-capillary voltage was set at –3.5 kV and the cone voltage was 24V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10^{-5} mm Hg (Ar).

1.3.7 Calculation of safety factors

Threshold concentrations for biomarker responses were calculated after regression analysis with Datafit software (Oakdale Engineering, USA) using the mean liver concentrations of the different exposure groups after 5 days of exposure. Calculated EC_{10}-values were defined as the values for which the effect was 10 % higher than the control value.

Safety factors (SFs) for PFOS-specific effects were defined as the ratio between the biomarker based liver EC_{10}-values and the maximal environmental body burdens of PFOS in mink, bald eagle and carp as reported earlier (Giesy and Kannan, 2001).

1.3.8 Statistical analysis

Data were subjected to Bartlett's test to test the homogeneity of variance. If the criterium of homogeneity was met, a one way ANOVA was used followed by Dunnett's test as post-hoc criterium. If there was no homogeneity of variance, the non-parametric Kruskal-Wallis test was applied with Dunn's test to assign significant differences between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to the control).

1.4 Results

Fish mortality was not observed. As presented in Table 1, no significant effects were observed for cholesterol, triglyceride and TAA, when the animals were exposed to PFOS. For the 864 ng/g treatment group, however, the mean values for cholesterol and triglyceride content decreased after 1 day of exposure although this effect was not significant (12 % and 26 % respectively, relative to the control). This trend was not seen after 5 days of exposure.

Table 1. Serum lipid levels and oxidative status in carp exposed to PFOS.

PFOS treatment group (ng/g)	PFOS nominal dose (ng/g)	exposure period (days)	cholesterol (% of control)	triglyceride (% of control)	TAA (% of control)
16	2000	1	104 ± 12 (4)	119 ± 21 (4)	136 ± 83 (5)
		5	97 ± 11 (6)	90 ± 23 (6)	94 ± 46 (6)
270	25000	1	94 ± 3 (6)	97 ± 30 (6)	132 ± 64 (6)
		5	98 ± 10 (6)	100 ± 34 (6)	94 ± 27 (4)
864	75000	1	88 ± 17 (6)	74 ± 12 (6)	113 ± 39 (6)
		5	104 ± 5 (5)	98 ± 10 (5)	110 ± 40 (6)

The values are expressed as percentages relative to the control value (mean ± SD). The control values for serum cholesterol, triglyceride and TAA for 1 day exposure are 223 ± 14 mg/dl, 144 ± 30 mg/dl and 111 ± 54 µM respectively. For an exposure period of 5 days the control values are 228 ± 19 mg/dl, 137 ± 30 mg/dl and 179 ± 103 µM, respectively. The number of animals is indicated in parentheses.

In Table 2 it is shown that the PFOS treatment had no apparent effects on catalase activity in the liver after 1 and 5 days of exposure although for the 864 ng/g treatment group a non-significant decreasing trend in mean activity was measured (respectively 33 % and 20 %, relative to the control). For palmitoyl CoA oxidation activity no significant changes in mean activity were shown.

Table 2. Liver catalase and palmitoyl CoA oxidase activities in carp exposed to PFOS.

PFOS treatment group (ng/g)	PFOS nominal dose (ng/g)	exposure period (days)	catalase (% of control activity)	palmitoyl CoA oxidase (% of control activity)
16	2000	1	91 ± 25	98 ± 39
		5	112 ± 30	131 ± 47
270	25000	1	119 ± 45	115 ± 30
		5	107 ± 43	125 ± 49
864	75000	1	67 ± 17	126 ± 51
		5	80 ± 32	120 ± 40

The values are expressed as percentages relative to the control value (mean ± SD). The control values for catalase for the 1 and 5 day exposure are 0.041 ± 0.013 μmol/mg.min and 0.096 ± 0.004 μmol/mg.min. For palmitoyl CoA oxidase the control values are 4.29 ± 2.96 nmol/mg.min and 1.63 ± 0.74 nmol/mg.min for 1 and 5 days, respectively. For each group 5-6 animals were used.

After 1 day of exposure the serum ALT activity was not significantly different from the non-exposed population. After 5 days of exposure, the serum ALT activity was characterized by a clear and dose-dependent increase in function of PFOS liver concentration (Fig. 1).

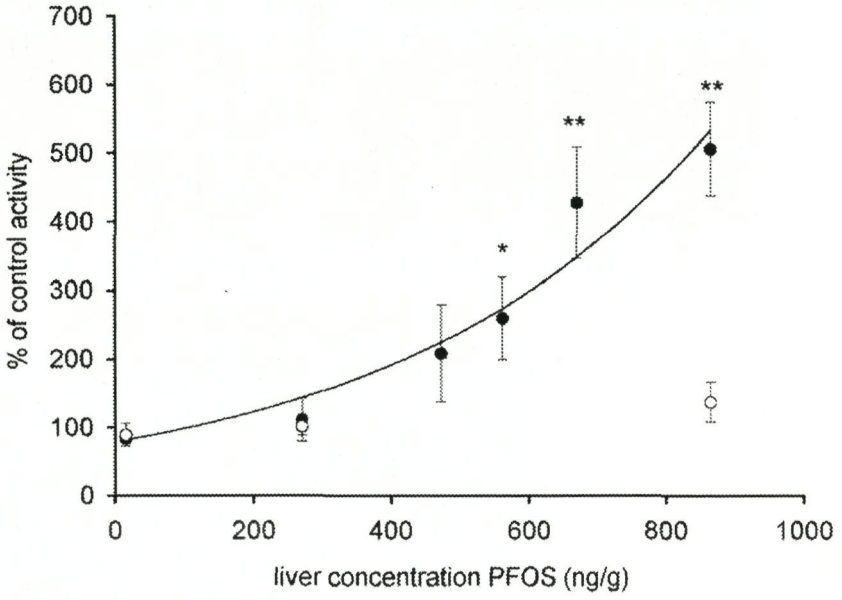

Fig. 1. Effect of PFOS on the serum ALT activity for an exposure of 1 day and 5 days.

The values are expressed as percentages relative to the control value (mean ± SD). The number of animals per group was 3-6. The white and black symbols represent the 1 and 5 day exposure respectively. The control values for 1 and 5 days are 0.43 ± 0.24 U/g and 0.63 ± 0.42 U/g respectively. The curve is the regression curve for the experimental data of the 5 day exposure period ($y = 79.15*1.002^x$ where "x" represents the liver concentration of PFOS in ng/g and "y" the ALT activity expressed relative to the control, $p < 0.001$).

Also the AST activity showed a PFOS dependent increase, with a slight drop in the mean enzymatic activity for the 670 ng/g group (Fig. 2).

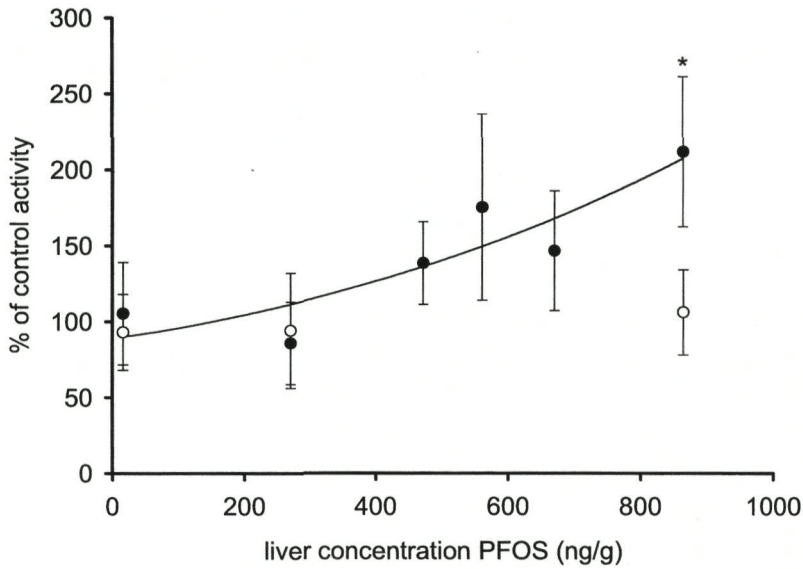

Fig. 2. Effect of PFOS on the serum AST activity for an exposure of 1 day and 5 days.

The values are expressed as percentages relative to the control value (mean ± SD). The number of animals per group was 3-6. The white and black symbols represent the 1 and 5 day exposure respectively. The control values for 1 and 5 days are 6.1 ± 3.6 U/g and 5.2 ± 2.9 U/g respectively. The curve is the regression curve for the experimental data ($y = 89.56*x^{(0.000144*x)}$ where "x" represents the liver concentration of PFOS in ng/g and "y" the AST activity expressed relative to the control, $p < 0.05$).

Both serum enzyme levels increased significantly compared to the control for the 864 ng/g treatment groups but only the serum ALT level was significantly different from the control for the 561 and 670 ng/g group. In general, the increase of serum ALT activity was higher than that for AST,

the ratio of the activities ranging between 0.8 and 2.4 if all the treatment groups were considered. The increase of the aminotransferase activities was not parallelled by a MPO activity increase (Table 3).

Table 3. Effect of PFOS exposure on the hepatic MPO activity (5 days).

PFOS treatment group (ng/g)	PFOS nominal dose (ng/g)	MPO (% of control activity)
561	50000	115 ± 35
670	60000	114 ± 68
864	75000	102 ± 43

The values are expressed as percentages relative to the control value (mean ± SD). The MPO control value was 0.027 ± 0.013 ΔOD/mg.min. The number of exposed animals per group was 3-6.

As shown in Figure 3, a significant increase in the average DNA basepair length (ABPL) was already seen after 1 day of exposure for the 270 ng/g and 864 ng/g groups while for the 16 ng/g treatment group a significant response was also seen but only after 5 days of exposure. The observed increases for 1 day of exposure relative to the control were 10.9 % and 13.4 % for the 270 ng/g and 864 ng/g treatment groups respectively. For the 5 day exposure experiment, this increase was less, ranging from 8.1 % to 9.8 % relative to the control.

Histologic investigation of the hepatic tissue did not reveal peroxisome proliferation or abnormalities in subcellular structure.

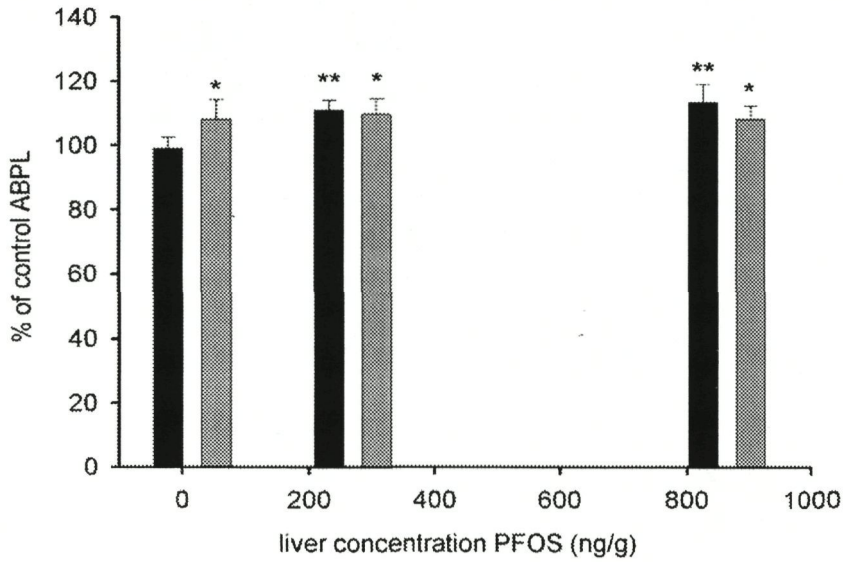

Fig. 3. Effect of PFOS on the hepatic ABPL for an exposure of 1 day and 5 days.

The black and gray bars represent the 1 and 5 days of exposure respectively. The values for the hepatic ABPL are expressed as percentages relative to the control value (mean ± SD). The control values for the ABPL for 1 and 5 days were 1026 ± 43 and 973 ± 59 respectively. The number of animals in each group was 6, $p < 0.001$.

1.5 Discussion

With the present study we tried to further elucidate the toxicological mode of action of PFOS on selected biochemical endpoints of carp after a short exposure period. Concerning the blood endpoints, no significant changes in serum triglyceride and cholesterol levels were observed after 1 and 5 days exposure to PFOS. This finding is clearly different from the

strong hypolipemic effect of PFOS described for rats (Haughom and Spydevold, 1992). These authors showed that PFOS administration (0.02 % in the diet) to rats after 24 hours of treatment caused a reduction in serum cholesterol to about 70 % of the control and further reduction if the treatment was prolonged for 2 weeks. After 1 and 2 weeks of treatment, the serum triacylglycerols decreased to about 50 % and 30 % relative to the control.

Despite the fact that PFOS is a peroxisome proliferator in rat and mouse as judged by the use of multiple enzymatic markers (Ikeda et al., 1987; Sohlenius et al., 1993), the peroxisomal catalase and palmitoyl CoA oxidase activities did not change significantly in PFOS-exposed carp what might indicate that PFOS is only a weak peroxisomal proliferator in carp.

Taken together, these results corroborate the overall but limited amount of information on the reported low sensitivity of fish species towards peroxisome proliferators. Pretti et al. (1999) showed that after 2 weeks of treatment of sea bass (*Dicentrarchus labrax*) with 35 and 70 mg/kg/day clofibrate, a known peroxisome proliferator in rats (Lake et al., 1987), no specific effects could be observed in the gill, liver or kidney of the organisms. Palmitoyl CoA oxidase and several other peroxisomal, mitochondrial and microsomal markers for peroxisome proliferation were unaffected although induction of glutathion S-transferase and reduction in epoxide hydrolase activities were observed but only at the highest dose. Only a mild increase in rainbow trout fatty acyl CoA oxidase activity at doses of 46, 87 and 152 mg/kg/day gemfibrozil and a significantly increased liver to body weight ratio at the highest injected dose after injection for 2 weeks were found. For Japanese medaka a significant increase of peroxisomal bifunctional enzyme activity after waterborne

gemfibrozil exposure (1.25, 2.5 and 5 mg/kg) was found after 2 weeks but also at the highest dose only (Scarano et al., 1993). The weak responsiveness of rainbow trout towards peroxisome proliferators was also reported by Yang et al. (1990). These authors showed only a weak significant induction of peroxisomal acyl CoA oxidase activity for a dose of 35 mg/kg ciprofibrate after intraperitoneal injection every other day for 3 weeks. Relatively mild inductions of PPA-80, a polypeptide that is believed to be induced specifically by peroxisome proliferators, were observed at doses of 25 and 35 mg/kg. A significant increase in catalase activity was apparent for the 15 and 25 mg/kg doses after 3 weeks and after 2 and 3 weeks for the 35 mg/kg group. The relatively short exposure period (1 to 5 days) and the single injection used in the present experiment, however, make comparison with data available for other fish species difficult and might account for the lack of a clear peroxisome proliferating response in PFOS exposed carp.

For the exposure groups with the highest PFOS liver concentrations (561 and 864 ng/g for ALT and 561, 670 and 864 ng/g for AST) a clear leakage of hepatocyte-specific enzymes after a 5 day exposure period was seen as shown by the increased dose-dependent serum AST and ALT activities. This suggests that PFOS has deleterious effects on the membrane integrity of the liver and might cause necrosis of (mainly) liver cells (although other organs may be damaged too since AST in carp can also be found in other tissues such as in muscle). ALT, however, is almost exclusively found in the liver with only small amounts in the muscles and kidneys (Toth et al., 1996).

Furthermore, since the presence of AST and ALT in mitochondria and cytosol has been demonstrated for teleost fish (Srivastava et al., 1998; Vaglio and Landriscina, 1999), it is possible that the increase of ALT and

AST activities in carp serum reflects disruption of both mitochondrial and cellular membranes of hepatocytes.

The present results correspond to former reports suggesting that PFOS has several membrane-specific effects such as uncoupling of oxidative phosphorylation in rat mitochondria (Gadelhak, 1992), increase of membrane fluidity in a rat liver hepatoma cell line and carp and chicken red blood cells (Hu et al., 2000) and inhibition of gap junction intercellular communication in rat liver and dolphin kidney epithelial cell lines (Hu et al., 2001). Recently, induction of a regulatory control element involved in the translation of a major outer membrane protein of *E. coli* was reported (De Coen et al., 2001). The observed ALT and AST leakage however is the first, *in vivo*, report supporting the current insight that PFOS can influence membrane structure and suggests there might be a common underlying mechanism for these observed membrane-related phenomena.

Taken into account the amphipatic structure of PFOS, the compound could physically disturb the structure of membranes as is the case for many detergent-like compounds (Schreier et al., 2000). It is unlikely that the observed leakage results from enhanced lipid peroxidation as the increases in serum enzymes were not associated with significant changes of serum TAA or liver peroxisomal catalase and palmitoyl CoA oxidase activities. This is in contrast with other compounds for which exposure resulted in both lipid peroxidation and a simultaneous increase in serum AST activity (e.g. Ahmed et al., 2001) or papers that report negative correlations between serum AST and ALT activity and levels of biomolecules that are involved in oxidative protection, such as hepatic and serum glutathione S-transferase (El-Demerdash, 2001). Since MPO activity in carp has been shown to increase not only in the case of natural infection (Stosik and Deptula, 1997) but also upon exposure to

inflammatory organic chemicals (Sakai et al., 1987), the absence of a significant increase in MPO activity in perfused liver suggests that no inflammatory response was induced in the liver because migration of neutrophils into the liver did not take place. This suggests that there is no direct or indirect relationship between inflammation and the obvious membrane leakage in the liver.

The observation that the average basepair length (ABPL) increased for the 16, 270 and 864 ng/g treatment groups after 5 days and for the 270 and 864 ng/g groups after 1 day is in correspondence with data obtained for *Daphnia magna* neonates exposed to 0.5 and 3 mg/l linear alkyl sulfonic acid, another sulfonic acid with surfactant properties (De Coen, 1999). Based on the results of the present study, the increase of the ABPL suggests that PFOS might induce DNA repair and/or affect DNA breakdown thereby disturbing the homeostasis of the "overall DNA metabolism". In this respect PFOS might be similar to its structural analogue perfluorooctanoate that also interferes with DNA structure by inducing DNA fragmentation in human hepatoblastoma HepG2 cells (Shabalina et al., 1999). Present results also corroborate data from an *in vitro* gene fusion experiment that showed that PFOS can induce *DinD* activity, a gene that is inducible by DNA damage in *E.coli* (De Coen et al., 2001). In a broader context, however, present results differ from *in vivo* studies in rats that have reported the absence of DNA damaging properties for several other peroxisome proliferators. Elliot et al. (1987) found no induction of DNA strand breaks upon oral gavage with mono-ethylhexyl phtalate, di-ethylhexyl phtalate (DEHP), clofibrate or methyl clofenapate. Even long-term oral administration of DEHP, clofibrate and bezafibrate did not result in apparent DNA strand breaks (Tamura et al., 1991).

The current results indicate that significant effects on the ABPL can be found for lower doses and a smaller exposure period than the membrane disruption effect. This follows from the finding that for the 270 ng/g treatment group the increases in ABPL are already significant after 1 day while for the aminotransferases there is only a significant increase in the serum for the 561 ng/g treatment group after an exposure of 5 days. It is possible that the cellular damage observed in the present study is related to the increase of the ABPL although this possible link remains to be elucidated.

Histological investigation did not show peroxisome proliferation. This was supported by the unaltered peroxisomal catalase and palmitoyl CoA oxidase activities observed in this study. The absence of apparent laesions seems to be in contradiction with the significantly increased ALT and AST activities. Although the reason for this discrepancy is unclear, it might be possible that the relatively short PFOS exposure period after intraperitoneal injection resulted in tissue damage in the outer part of the liver only.

1.6 Conclusion

Taken together this first assessment of the toxicity of PFOS suggests that short term PFOS exposure does not have clear effects on lipid metabolism and oxidative status of liver and serum of common carp. Also, PFOS does not induce inflammation of the liver but two other effects are proposed to contribute to PFOS' toxicity. The first one is induction of membraneous damage associated with cell necrosis. The second effect involves disruption of the equilibrium between DNA damage and its repair processes.

In order to assess the environmental relevance of the increase in serum transaminase levels, EC_{10}-values for the increase of both enzyme activities in serum were compared with tissue concentrations from different fish species and two top-predators. In Fig. 4 it can be seen that most of the observed exposure concentrations from various organisms in the environment are close to the doses which caused enzymatic leakage in the laboratory (after short exposure period and through injection).

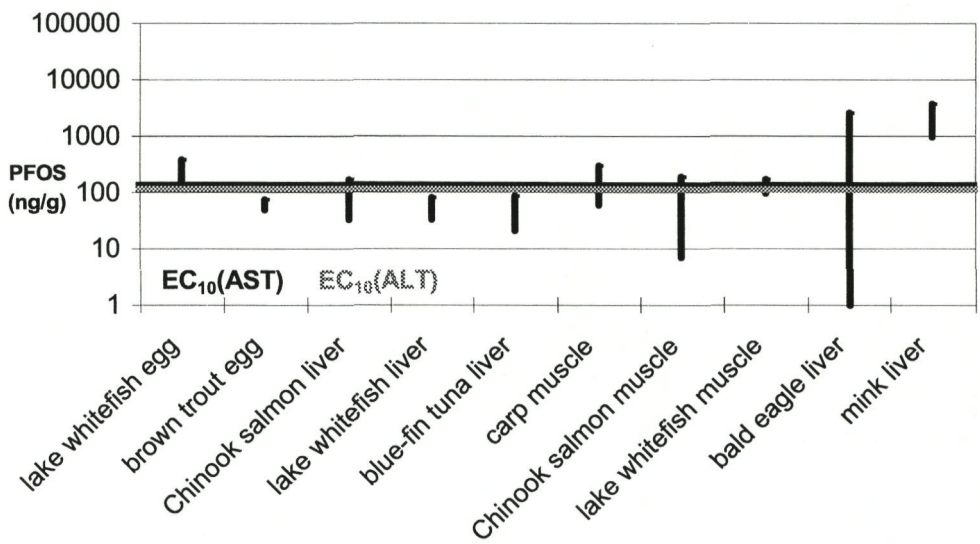

Fig. 4. PFOS concentration ranges for a selection of wildlife species.

Vertical bars represent the range between minimal and maximal tissue values as published elsewhere (Giesy and Kannan, 2001). Horizontal lines represent EC_{10}-values for both transaminases in serum.

Moreover, the calculated liver EC_{10}-values for both aminotransferases (EC_{10}(AST) = 258 ng/g wet tissue, EC_{10}(ALT) = 164 ng/g wet tissue for liver) are lower than the maximum PFOS concentrations found in environmental samples from eagle, carp and mink resulting in low calculated aminotransferase-specific SF values (Table 4).

It is important to consider, however, that these factors are approximations of the hazard associated with environmental exposure of PFOS since they do not take into account the interspecies differences in sensitivity for enzyme leakage, differences in exposure route and exposure period that occur in the environment. In conclusion, these preliminary safety factors suggest that the current environmental exposure levels of PFOS detected in wildlife might disrupt the hepatic integrity although further refinement of the hazard assessment for PFOS is required.

Table 4. AST and ALT safety factors for three different species in the environment.

tissue	maximum environmental concentration (ng/g)	SF(AST) EC_{10}(AST)/max. env. conc.	SF(ALT) EC_{10}(ALT)/max. env. conc.
carp muscle	300	0.860	0.547
eagle plasma	2570	0.100	0.064
mink liver	3680	0.070	0.045

The safety factors (SFs) were defined as the ratios of EC_{10}-values for ALT and AST and the maximal environmental concentrations in the tissues (Giesy and Kannan, 2001).

Chapter 2

Isolation of perfluorooctane sulfonic acid induced hepatic genes in the common carp (*Cyprinus carpio*) using micro-array and real-time PCR technology.

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in preparation for publication

2.1 Abstract

Juvenile carp (*Cyprinus carpio*) was exposed to perfluorooctane sulfonic acid (PFOS) through water for two weeks, resulting in a hepatic PFOS concentration of 31.5 ± 13.3 µg/g wet weight (mean ± SD). After suppressive subtractive hybridization of PFOS exposed and unexposed RNA pools, a hepatic cDNA library was obtained with 700 clones. Micro-array analysis suggested a PFOS-mediated overexpression of two cDNA fragments showing > 90 % nucleotide identity with zebrafish (*Danio rerio*) liver fatty acid binding protein and chymotrypsinogen. The fold-inductions (relative to control) of these cDNAs were 1.53 and 1.87, respectively. Also two cDNA clones with corresponding amino-acid sequences that were 67 % identical to rainbow trout (*Oncorhynchus mykiss*) toxin-1 and 41 % identical to arctic lamprey (*Lethenteron japonicum*) serum lectin, were respectively overexpressed 2.34 and 2.03 times, respectively. Real-time PCR analysis did not confirm PFOS-mediated upregulation of these transcripts. Future work should be devoted to further unravel the molecular mode of action of this chemical. More detailed information is needed on the biological function of these genes in the common carp. Furthermore, it is essential to characterize the relationship between changes in gene expression and overall fish health.

2.2 Introduction

Perfluorooctane sulfonic acid (PFOS) is a widespread pollutant in aquatic ecosystems. Marine as well as freshwater fish species from various geographic locations have been shown to have tissue PFOS concentrations ranging from < 7 up to 9031 ng/g wet weight (Giesy and Kannan, 2001; Hoff et al., 2003a; Kannan et al., 2002b; Martin et al.,

2004a; Taniyasu et al., 2003). The highest tissue PFOS concentrations are usually measured in more populated and industrialized locations and have also been suggested to result from accidental releases (Moody et al., 2002).

Biochemical effects of PFOS exposure are mainly studied in liver and serum of mammalian species and include effects on the lipid metabolism (Berthiaume and Wallace, 2002; Ikeda et al., 1987; Sohlenius et al., 1993; Haughom and Spydevold, 1992), intercellular communication (Hu et al., 2002), neuroendocrine effects (Austin et al., 2003), carboxylesterase acticity (Derbel et al., 1996) and alanine aminotransferase activity (Seacat et al., 2003). PFOS-mediated induction of biochemical effects in fish species have hardly been addressed. In juvenile carps (*Cyprinus carpio*) exposed to PFOS, inflammation-independent leakage of liver cells and disturbance of DNA metabolism homeostasis in liver tissue has been demonstrated (Hoff et al., 2003b).

In order to further characterize the biochemical effect pattern of PFOS in liver tissue of juvenile carp, fishes were exposed to PFOS via water. Liver was selected as a target tissue because PFOS is known to bioconcentrate and bioaccumulate in liver, a primary target organ in fish, as was demonstrated for rainbow trout (*Oncorhynchus mykiss*) under controlled conditions (Martin et al., 2003a, 2003b). The effect of PFOS exposure on gene transcription in liver tissue was assessed by constructing a liver cDNA library enriched for cDNAs that were differentially expressed between PFOS-exposed and control carp. For confirmation of differential expression, micro-array hybridization and real-time PCR analysis were used.

2.3 Materials and methods

2.3.1 Animals and treatment

Juvenile carp (*Cyprinus carpio*) of 6 months old was acclimatised at 17°C for 2 weeks in plastic 20 l aquaria filled with fully aerated OECD water medium hard fresh water (OECD, 1993) containing $CaCl_2$ (2.0 mM), $MgSO_4$ (0.5 mM), $NaHCO_3$ (770 µM), KCl (77 µM), pH 7.7, hardness 250 expressed as mg/l $CaCO_3$. Seven carps were housed in each aquarium. Water was changed daily and carps were fed 1 % (w/w) Tetra Pond sticks (Tetra, Germany) once per day. The carps were exposed to PFOS at a final concentration of 1 mg/l during two weeks while the control carps were kept in OECD water for the exposure period. After 2 weeks, the fishes were killed by an overdose of MS-222. The liver was immediately removed and frozen in liquid nitrogen.

2.3.2 Determination of liver PFOS concentrations

The PFOS concentrations in liver tissue were measured using combined high pressure liquid chromatography-mass spectrometry according to Giesy and Kannan (2001). High pressure liquid chromatography was done on a CapLC system (Waters, Milford, MA, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm inner diameter, Alltech, Deerfield, IL, USA). The analysis was performed on a Betasil C18 column (50 mm x 1 mm inner diameter, Keystone Scientific, San Jose, CA, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH_4OAc (A) / CH_3OH (B). A gradient elution was used starting at 45 % B and going to 90 % B in 3 min. After 5 min

initial conditions were resumed. PFOS was measured under negative electrospray ionization using single reactant monitoring (m/z 499→99). The internal standard (1H, 1H, 2H, 2H-perfluorooctane sulfonic acid) was measured under the same conditions (m/z 427→81). The dwell time was 0.1 s. The electrospray-capillary voltage was set at –3.5 kV and the cone voltage was 24V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10^{-5} mm Hg (Ar).

2.3.3 Suppressive subtraction hybridization

Liver RNA was isolated from two pools of liver sample consisting of liver tissue of PFOS exposed and unexposed carp with the Totally RNA Kit (Ambion, Austin, Texas). RNA quality was checked on a denaturing formaldehyde-agarose gel. The SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) was used for cDNA preparation. PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Penzbzerg, Germany). Suppressive subtraction hybridization (SSH) was performed with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer´s instructions. Forward and reverse subtraction were performed. cDNA fragments of the enriched library were cloned into the pGEM-T vector (Promega, Madison, WI) used to transform competent JM 109 E. coli cells (Promega, Madison, WI). Blue/white selection of transformed cells was performed according to the manufacturer´s guidelines on agar LB medium supplemented with isopropyl-β-D-thiogalactopyranoside, ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Transformed cells were grown in 96 well microtiter plates with liquid LB medium and ampicillin. After dilution (1:10), cells were lysed by heating

(10 min, 96 °C) and centrifuged (4000rpm, 5 min). Clone inserts were amplified by PCR. The PCR reaction mixture consisted of 2.5µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM each), 5µl PCR buffer (Fermentas, St. Leon-Rot, Germany), 0.5 µl Taq polymerase (500 U), 1 µl SP6 and T7 primer (1µM each), 2 µl lysate supernatant and 37 µl H_2O. 35 PCR cycles were applied (96 °C for 5 min, 68 °C for 30 s, 75 °C for 1.5 min). PCR products were purified according to Werle et al. (1994). Specificity of amplification was checked with agarose gel electrophoresis.

2.3.4 Micro-array

Aminosilane coated Genorama™ Microarray Slides (Asper Biotech, Tartu, Estonia) were used to spot 700 clones from the enriched cDNA library in triplicate. cDNA representing carp β-actin was also spotted on the array. β-actin cDNA was prepared via PCR. The PCR reaction mixture consisted of 1 µl $MgCl_2$ (25 mM), 0.5 µl dNTPs (10 mM each), 1.5 µl PCR buffer (Fermentas, St. Leon-Rot, Germany), 1 µl Taq polymerase (500 U), 1 µl actin-specific forward (GATGATGAAATTGCCGCAC, 10 µM) and reverse primer (ATCCAGACAGAGTATTTACGCTCA, 10 µM), 10 µl H_2O and 2µl liver cDNA solution prepared with the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). 39 PCR cycles were applied (94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min). Specificity of amplification was checked with agarose gel electrophoresis.

Before spotting PCR products were purified using Montage $PCR_{\mu 96}$ Plates (Millipore, Billerica, MA, USA) and transferred to 384-well plates (Genetix, Hampshire, UK) in 50 % dimethylsulfoxide at a concentration of ± 150 ng/µl. A Qarray Mini Robot (Genetix, Hampshire, UK) with 8-

solid pin-head was used to spot each clone in triplicate. After spotting, cDNA was fixated by UV cross-linking (300 mJ) after rehydration and drying with a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA).

2.3.5 Hybridization

Two cDNA probes were prepared for hybridization starting from two pools of liver total RNA that was isolated using the Totally RNA Kit (Ambion, Austin, Texas) as described above. Single strand cDNA was prepared with aminoallyl-dUTP (Sigma-Aldrich, Bornem, Belgium) with random hexamer primers (Invitrogen, Merelbeke, Belgium) using Superscript II Reverse Transcriptase (Invitrogen, Merelbeke, Belgium) according to the instructions of the manufacturer. Remaining RNA was hydrolized by heating (65 °C, 15 min) in the presence of 10 µl NaOH (1 M) and 10 µl EDTA (0.5 M). After purification with the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) the aminoallyl labelled cDNA from unexposed and PFOS exposed carp was covalent coupled with Cy3 and Cy5 mono NHS esters (Amersham Biosciences, Roosendaal, the Netherlands), respectively. Unincorporated Cy dyes were removed by purification with the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) and labelling efficiency was determined by spectrophotometry (NanoDrop, NanoDrop Technologies, Rockland, DE, USA).

Micro-arrays were incubated in a coplin jar at 42°C for 60 min with prehybridization solution consisting of 50 % formamide, 5X SSC (175.3 g/l NaCl, 88.2 g/l sodium citrate, pH 7), 0.1 % SDS and 0.1 mg/ml BSA. After washing with deionized water and isopropanol, the arrays were immediately dried with compressed N_2. Vacuum dried probes were resolved in hybridization solution (50 % formamide, 5X SSC, 0.1 %

SDS, 0.1 mg/ml BSA, 0.1 mg/ml salmon sperm) and incubated (95 °C, 5 min). A probe volume corresponding with 150 pmol of incorporated dye was applied on the array. The arrays were covered with coverslips (Glas Menzel, Bad Wildungen-Altwildungen, Germany) and placed overnight in a hybridization chamber (Genetix, Hampshire, UK) at 42 °C. After incubation, slides were washed with the following wash buffers: 2X SSC and 0.1% SDS (42 °C for 1 and 5 min), 0.1X SSC and 0.1 % SDS (room temperature for two times 10 min), 0.1X SSC (room temperature for 15 seconds, twice 2 min and 1 min) and 0.01X SSC (room temperature for 15 s). Finally slides were rinsed with deionized water and isopropanol and dried with compressed N_2.

2.3.6 Scanning and data analysis

Micro-arrays were scanned at 532 and 635 nm using the Genepix Personal 4100A Scanner (Axon Instruments, USA). The images were analyzed by means of the Genepix pro Software (Axon Instruments, Union City, CA, USA) for spot identification and for quantification of the fluorescent signal intensities. The fluorescent signal intensity for each DNA spot (average of intensity of each pixel present within the spot) was calculated using local background subtraction. Spots for which the mean foreground signal was smaller than the mean background signal + 2 SD and saturated signals were filtered out. The ratio (Cy5/Cy3) was calculated for each spot, transformed into a logarithmic value (log_2), and normalized using Locally Weighed Scatterplot Smoothing (Lowess, Yang et al., 2002). Clones with a mean dye ratio of three replicates (Cy5/Cy3) < 0.5 and > 1.5 were retained for sequencing and real-time PCR. Dye ratios of these latter clones were also calculated after normalization for β-actin. Three hybridization experiments were performed.

2.3.7 Sequencing and selection of clones

The selected PCR products were sequenced using the Ceq™Dye Terminator Cycle Sequencing Kit on the CEQ™8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Only clones with homologies characterized by *E*-values < 10^{-21} in BLASTX and/or BLASTN (http://www.ncbi.nlm.nih.gov/BLAST) were considered.

2.3.8 Real-time PCR

Real-time PCR was carried out on a Roche Molecular Biochemicals LightCycler 3.5 with the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit according to the manufacturer's instructions. Reverse transcription was performed using the First Strand cDNA Synthesis Kit with random hexamer primers (Fermentas, St. Leon-Rot, Germany). RNA from individual fishes was isolated using the SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA). Relative quantification was calculated using β-actin for normalization. β-actin was chosen because of its apparent lack of differential expression as demonstrated by micro-array analysis (1.09 ± 0.08 fold-induction relative to control, mean ± SD, n = 3). Primers for real-time PCR were designed using Roche Molecular Biochemicals LightCycler Probe Design Software version 1.0 (Roche Diagnostics, Penzbzerg, Germany) and were based on the cDNA sequences of the enriched library clones suggested to be upregulated by micro-array analysis. Primers for carp β-actin were designed using the previously reported nucleotide coding sequence of carp β-actin (accession number M24113). The PCR reaction consisted of the following steps: denaturation (95°C for 10 min), amplification (95°C for 10 s, 55°C for 10

s, 72°C for 12 s). The melting curve program consisted of a heating step of 0.1°C per second (55-95°C) and was followed by a cooling step (40°C). Primer sequences used for amplification of the target transcripts suggested to be upregulated as shown by micro-array analysis were as follows: chymotrypsinogen B1 homologuous transcript: forward primer TTGTTACCGGCTACGC, reverse primer GCTCGCCAAGGATAACG); toxin-1 homologuous transcript: forward primer GTGATTAGCGTGGTCG, reverse primer GATCAAGCGTTGCACA; basic liver fatty acid binding protein 10 transcript: forward primer ATTCGATTTCGAGCGG, reverse primer CCTCGTAGTTCTCCTGC, serum lectin homologuous transcript: forward primer GGCAGGTACTACGACAT, reverse primer TCATACACCACCGGAC and β-actin: forward primer TGCAAAGCCGGATTCG, reverse primer: CACGCAGCTCGTTGTA. The relative expression of the target transcript sequences was calculated as reported by Pfaffl et al. (2002) including efficiency corrections for each transcript. The formation of non-specific PCR products was checked by melting curve analysis.

For each fish, three real-time PCR analysis was performed. For each experiment, mean inductions were calculated for the PFOS exposed and non-exposed fishes.

2.3.9 Statistical analysis

For real-time PCR analysis, relative expressions were calculated using the Pair Wise Fixed Reallocation Randomisation Test© as described by Pfaffl et al. (2002, http://www.wzw.tum.de/gene-quantification/).

2.4 Results

The resulting hepatic PFOS concentration of the exposed juvenile carps was 31.5 ± 13.3 μg/g wet weight (mean ± SD) after 14 days of exposure. Those genes that showed differential expression at the selected cut-off values were isolated and sequenced. Only 4 genes showed differential expression and are shown in Table 1.

Table 1. Sequence homologies of cDNAs representing PFOS-induced hepatic genes.

identification (accession number)	function	BLASTN		BLASTX	
		E-value (species)	x/y nucleotides (% identity)	*E*-value (species)	x/y amino acids (% identity)
chymotrypsinogene B1 (NM 212618, AAH21198)	protease precursor	e-21 (*Danio rerio*)	61/63 (96)	0.003 (*Danio rerio*)	19/21 (90)
toxin-1 (AAM21198)	unknown	ND	ND	4e-26 (*Oncorhynchus mykiss*)	51/76 (67)
basic liver fatty acid binding protein 10 (NM 152960)	fatty acid binding	e-123 (*Danio rerio*)	356/359 (90)	ND	ND
serum lectin (BAB32787)	carbohydrate binding	ND	ND	e-31 (*Lethenteron japonicum*)	62/148 (41)

ND: not determined because no strong similarities were found (*E*-value ≥ 2e-0.8). Accession numbers are given in brackets.

The fold-inductions of the isolated cDNA clones after micro-array analysis (with and without normalization for β-actin) and real-time PCR analysis are reported in Table 2. Table 2 shows no statistically significant differences in induction or repression of the isolated transcripts after real-time PCR analysis ($p > 0.05$) and the independence of actin normalization for the outcome of the micro-array analyses.

Table 2. Fold inductions (relative to control) of transcription of PFOS-mediated hepatic genes based on micro-array (n = 3) and real-time PCR analysis (n = 3, mean ± SD).

	micro-array (without β-actin normalization)	micro-array (with β-actin normalization)	real-time PCR (with β-actin normalization)
chymotrypsinogene B1 (NM 212618, AAH21198)	1.87 ± 0.11	1.77 ± 0.18	1.02 ± 0.34 (p = 0.7)
toxin-1 protein (AAM21198)	2.34 ± 0.32	2.44 ± 0.25	2.04 ± 0.67 (p = 0.1)
basic liver fatty acid binding protein 10 (NM 152960)	1.53 ± 0.12	1.66 ± 0.19	1.02 ± 0.30 (p = 0.6)
serum lectin (BAB32787)	2.03 ± 0.22	1.99 ± 0.20	1.30 ± 0.41 (p = 0.5)

Accession numbers are given in brackets, p = p-value.

2.5 Discussion

The study of PFOS-mediated gene transcription has received only little attention. Giesy et al. (2003) identified some genes that were responsive to PFOS exposure in the rat. These genes consisted mainly of fatty acid metabolizing genes, cytochrome P450s and genes involved in hormone regulation. Shipley et al. (2004) recently reported that PFOS exposure

could activate human and mouse peroxisome proliferator-activated receptor α (PPARα) *in vitro*. No information, however, was available on PFOS-mediated gene induction and repression in fish species so far.

The present study addresses this issue for the first time in fish. Array results indicate that PFOS might induce fatty acid binding protein (FABP) transcription in liver tissue of the common carp. The PFOS binding affinity for the rat liver FABP protein was previously reported by Luebker et al. (2002) at an average hepatic PFOS concentration of 31.5 µg/g wet weight. The induction of this protein might provide a link to the PPARα activating capacity of PFOS demonstrated by Shipley et al. (2004). Indeed, Poirier et al. (2001) showed that bezafibrate-induced transcription of liver FABP was abolished in PPARα-null mice in liver tissue, suggesting a link between the expression of genes.

A previous study in the common carp (Hoff et al., 2003b) showed that hepatic peroxisomal fatty acid β-oxidation (PFAO) was not affected by PFOS exposure at hepatic concentrations up to 864 ng/g ww. Considering the coordinate peroxisome proliferator-mediated induction of liver FABP and PFAO observed in mammals (Kaikaus et al., 1993), the observed absence of PFAO induction in the study by Hoff et al. (2003b) and the induction of FABP in the present study seem to be in contrast. Comparison of both studies is difficult, however, because of the higher hepatic PFOS concentrations in the latter study and the different routes of PFOS exposure and exposure times. Furthermore, the isolated FABP clone reported in this study is strongly homologuous with basic liver-type FABPs (Lb-FABP) which are only found so far in non-mammalian vertebrates (Denovan-Wright et al., 2000). Therefore, comparison with current studies on mammalian liver FABPs is problematic.

Aside from the suggested PFOS-mediated induction of Lb-FABP transcription, the results of the present study also suggest upregulation of lectin transcription. Several biological functions have been proposed for these molecules involving the regulation of immune functions such as the recruitment of leukocytes to inflammatory sites (McEver et al., 1995), maturation and proliferation of lymphocytes (Vannecke et al., 1995), opsonisation of pathogens for phagocytosis (Arason, 1996) and activation of mast cells and eosinophiles (Liu, 1993). Lectins can also mediate induction of apoptosis (Perillo et al., 1995; Telford et al., 1992).
The demonstrated upregulation of a chymotrypsinogen-like gene might result in the modulation of a number of functions in which chymotrypsin or chymotrypsin-like proteins are known to be involved. These functions include: digestion of dietary proteins (Simon et al., 1999), innate immunity (Concha et al., 2004), sperm motility (Inaba and Morisawa, 1991) and proteasome function (Adams et al., 2003).

It should be noted that it is unclear at present whether the cDNA clones that were isolated in this study represent mRNAs specifically upregulated by PFOS exposure or whether exposure to other chemical compounds might elicit a similar response. The need for specificity testing in fish toxicology is clearly illustrated for FABP, for example, which was not only shown to be upregulated in this study but also in liver tissue of mummichog (*Fundulus heteroclitus*) from a polycyclic aromatic hydrocarbon polluted site (Peterson and Bain, 2004).

Furthermore, these results of this initial attempt to unravel the molecular mode of action of PFOS should be seen as a preliminary result. Due to the limited number of concentrations and exposure periods that were measured, no clear insight can be gathered on the dose-response

relationship and the time trend of the expression of these genes. Due to the test-design and the amount of starting material needed for the micro-array analysis samples were pooled for these measurements. The real time PCR analysis was, however, performed on individual fishes.

Although micro-array analysis suggested that chymotrypsinogen B1, toxin-1, basic liver fatty acid binding protein 10 and serum lectin homologuous liver transcripts were upregulated by PFOS exposure in carp, this was not confirmed by real-time PCR analysis. It has been reported earlier that discrepancies exist between the expression of transcripts when micro-array analysis or real-time PCR analysis are used and that the reason for this discrepancies can at least partly be ascribed to the different analytical methodologies and normalization procedures used (Pfaffl et al., 2003). In the latter paper, modulation of gene expression was only significantly confirmed by real-time PCR analysis for micro-array based fold-inductions of at least 3.33 (at the $p < 0.01$ level). Considering the maximal fold induction observed in the present study (2.34 fold upregulation for the toxin-1 homologuous transcript) it might not be surprising that the upregulations observed after micro-array analysis were not confirmed by real-time PCR analysis.
Individual variation in expression of the isolated cDNA clones due to individual differences in hepatic PFOS concentration might also account for the different outcome of the micro-array and real-time PCR analyses.

The liver PFOS concentrations at which induction of chymotrypsinogen B1, toxin-1, basic liver fatty acid binding protein 10 and serum lectin homologuous liver transcripts was suggested (31.5 ± 13.3 μg/g wet weight, mean ± SD) was higher than the maximal hepatic PFOS concentrations measured in feral freshwater fish (1.8 μg/g in carp and 9.0

μg/g in eel, Hoff et al., 2005). Whether the genes that were observed in the present study would also be affected in feral freshwater fishes remains to be elucidated. Furthermore, it is important to further characterize the induction levels of these genes in relation to the PFOS body burdens as well as their function in the organism. At present, is remains unclear what the consequences of the transcription modulation might be at the whole organism level.

2.6 Conclusion

This study suggests that PFOS exposure can elicit upregulation of some carp hepatic genes involved in a number of physiological processes by using micro-array analysis although this was not confirmed by real-time PCR analysis. It is clear that the present study should be considered as a first attempt to characterize PFOS-mediated biochemical effects on gene expression in carp liver demonstrating the usefulness of micro-array and real-time PCR analysis for the unravelling of transcriptional effects elicited by PFOS exposure.

Chapter 3

Perfluorooctane sulfonic acid in bib (*Trisopterus luscus*) and plaice *(Pleuronectes platessa*) from the Western Scheldt and the Belgian North Sea: distribution and biochemical effects

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3.1 Abstract

A biomonitoring campaign was conducted in the Belgian part of the North Sea and in the Western Scheldt (The Netherlands) with the primary goal to assess the perfluorooctane sulfonic acid (PFOS) contamination and distribution in different biota. This study covers the results obtained for bib (*Trisopterus luscus*) and plaice (*Pleuronectes platessa*) and includes the assessment of some stress-related biochemical endpoints. Analysis of liver and muscle PFOS concentrations of both species provided evidence for the existence of a PFOS pollution gradient along the Western Scheldt with higher levels at the upstream locations and a relatively low degree of PFOS pollution at the marine locations.

Cellular necrosis was studied by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum. Serum ALT, but not serum AST was shown to correlate positively with the PFOS liver concentration in bib ($r = 0.44$, $p < 0.05$), indicating that PFOS might contribute to the induction of hepatic damage in bib in the area of study. Analysis of total carbohydrate, lipid and protein content of bib liver tissue revealed a positive correlation between the protein content and the PFOS liver concentration ($r = 0.55$, $p < 0.01$), suggesting induction of compensatory mechanisms, detoxification or repair processes.

3.2 Introduction

The widespread character of perfluorooctane sulfonic acid (PFOS) in wildlife was only recently demonstrated by Giesy and Kannan (2001). Other reports confirmed the presence of PFOS in marine mammals (Kannan et al., 2001b) and fish-eating water birds (Kannan et al., 2001a).

PFOS tissue concentrations in aquatic organisms and their predators were mainly described for species in the USA, Canada and the Pacific region and there is a lack of data on the degree of PFOS pollution and distribution in aquatic wildlife tissues in Western Europe.

In this context the knowledge of PFOS tissue burdens and distribution in wildlife species in the North Sea and the Western Scheldt are of major interest considering the high ecological value of the Western Scheldt as a nursery (Beyst et al., 1999; Cattrijsse et al., 1997; Hostens, 2000; Hostens and Mees, 1999) and the reported biologically relevant effects on wildlife as a result of pollution (De Wolf et al., 2001; Hoare et al., 1995; Mees and Reijnders, 1994). Furthermore it is known that the Belgian part of the North Sea is under the continuous threat of anthropogenic pollutants such as heavy metals (Van Alsenoy et al., 1993) and PCBs (Roose et al., 1998). The importance of assessing coastal and estuarine ecosystems for PFOS exposure is emphasized by the observation that PFOS concentrations in animal tissues from more densily populated and industrialized locations is higher than for animals at more remote marine locations (Giesy and Kannan, 2001).

The degree of PFOS pollution in the Western Scheldt estuary (The Netherlands) is of particular interest since an important fluorochemical production plant is located in Antwerp (Belgium), a city upstream of the Western Scheldt estuary. Next to PFOS discharges into this estuary, other fluorochemicals that have been discharged may transform metabolically to PFOS as an end-stage metabolite and might possibly cause increased PFOS levels in tissues of the estuarine and marine fauna (Olsen et al., 1999).

Although PFOS is not characterised very well on a toxicological level, a substantial number of effects have been documented. PFOS was shown to have membrane-related effects in *in vitro* studies such as increase of membrane fluidity (Hu et al., 2000) and inhibition of gap junction intercellular communication (Hu et al., 2001).

In vivo experiments showed that PFOS affects lipid metabolism in rodents (Haughom and Spydevold, 1992, Ikeda et al., 1987, Sohlenius et al., 1993). Furthermore, PFOS induces reduced maternal body weight gain and feed consumption, increased abortions and reduced fetal weights in rabbits (Case et al., 2001).

Recently a short term *in vivo* exposure study with the common carp (*Cyprinus carpio*) showed that PFOS induced an increase in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels indicative for leakage of hepatocytes and possibly liver necrosis (Hoff et al., 2003b).

The first aim of the present study was to determine PFOS concentrations in muscle and liver of two common fish species (bib and plaice) at various locations on the Belgian continental shelf and in the Western Scheldt in order to obtain preliminary information about the severity of PFOS pollution and its distribution in this ecosystem. Analysis of the relation between the PFOS liver concentrations and biological endpoints such as fork length, serum activity of ALT and AST allowed a preliminary effect evaluation of PFOS on fish in an estuarine/marine environment. The overall effect of PFOS on the major components of the energy metabolism were investigated by assessing correlations between liver PFOS burdens and liver total protein, lipid and carbohydrate content.

3.3 Materials and methods

3.3.1 Sampling

Fishes were sampled with a 3 m beam trawl with fine-meshed nets (6 x 6 mm) in the Belgian part of the North Sea and the Western Scheldt (the South-west of the Netherlands) in October and November 2001 using the research vessel "Zeeleeuw". Trawling was carried out with the tide with a speed of 1.5-2 knots for about 30 minutes. The locations at which trawling was initiated were location 1 (Zeebrugge coastal area, N 51°22′ E 03°16′), location 2 (East of the Spijkerplaat, N 51°25′ E 03°36′), location 3 (East of Terneuzen, N 51°21′ E 03°45′) and location 4 (Hansweert, N 51°25′ E 04°00′) for bib and location 5 (Nieuwpoort coastal area, N 51°09′ E 02°40′), location 6 (Nieuwpoort marine area, N 51°24′ E 02°38′), location 7 (Zeebrugge marine area, N 51°32′ E 02°55′) and location 8 (West of Terneuzen, N 51°20′ E 03°51′) for plaice. The distance from these sites to Antwerp was calculated as the shortest distance over water (Fig.1).

The number of fishes caught at each location ranged between 4 and 8 for bib and 4 and 7 for plaice. The captured fishes were kept in a tank with aerated seawater until they were killed by a blow on the head. Blood was taken immediately after killing via caudal puncture. Serum was prepared on board by centrifugation at room temperature (4000 rpm, 5 min) and collection of the supernatant. Serum, liver and muscle tissue was stored on board at - 20°C until further analysis.

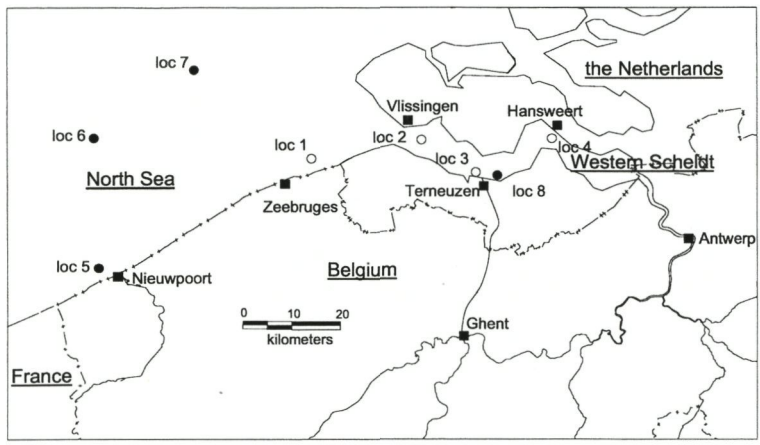

Fig. 1. Area of study and sampling sites.

The open circles represent the sampling locations for bib while the black circles show the plaice sampling locations. Cities are indicated with a black squares.

3.3.2 Determination of PFOS concentrations

The PFOS concentrations in the liver and muscle tissue of the animals were measured using combined liquid chromatography-mass spectrometry according to Giesy and Kannan (2001) performed on a CapLC system (Waters, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, Sercolab, Belgium). The analysis was performed on a Betasil C18 column (50 mm x 1 mm i.d., Keystone Scientific) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH_4OAc (A) / CH_3OH (B). A gradient elution was used starting at 45 % B and going to 90 % B in 3 min. After 5 min initial conditions were resumed. PFOS was measured under (-) electrospray ionisation using single reactant monitoring (SRM, m/z 499→99). The internal standard (1H, 1H, 2H, 2H-perfluorooctane sulfonic acid) was

measured under the same conditions (SRM, m/z 427→81). The dwell time was 0.1 s. The ES-capillary voltage was set at –3.5 kV and the cone voltage was 24V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10^{-5} mm Hg (Ar).

3.3.3 Determination of serum aminotransferase activities

Serum alanine aminotransferase and aspartate aminotransferase activities were determined by the spectrophotometric methods described in Bergmeyer et al. (1986a) and Bergmeyer et al. (1986b), respectively.

3.3.4 Determination of lipid, carbohydrate and protein concentrations in the liver

Liver samples were homogenised on ice with an MSE 150 Watt ultrasonic disintegrator (MSE Scientific instruments, England). Lipid and carbohydrate determination was carried out according to De Coen and Janssen (1997).
The Bio-Rad Protein Assay (Bio-Rad, Germany) was used for assessing the total liver protein concentration.

3.3.5 Statistical analysis

The mean intraspecies bib liver PFOS concentrations for each location were compared with one way ANOVA and Tukey´s test as post-hoc criterium. Homogeneity of variance was confirmed with Bartlett´s test. The non-parametric Kruskal-Wallis test was applied with Dunn´s test as post-hoc criterium at a significance level of $p = 0.05$ for comparison of the mean plaice PFOS liver concentrations, the serum ALT and AST

activity and the liver protein content at the sampling locations. To investigate the associations between the PFOS liver content and the distance from Antwerp, the serum ALT and AST activity, the fork length, the liver protein, carbohydrate and lipid content, Pearson product-moment correlation analysis was used since the normality of these variables was confirmed with Kolmogorov-Smirnov's test. The same methodology was used for investigation of the association between the distance of the sampling locations from Antwerp and the AST and ALT activities and for analysis of the correlation between the serum aminotransferase activities.

3.4 Results

The serum ALT and AST activity and the liver protein contents for plaice and bib are plotted in Fig. 2 and Fig. 3, respectively. For plaice, no significant differences between the locations were observed although the mean serum ALT activity in the Western Scheldt (location 8) was higher than those at the marine locations. The mean liver protein content was lower at location 8. For bib, increased values of both aminotransferase activities and the liver protein content were observed along the Westerscheldt axis. The mean serum AST activity and the mean liver protein content at location 4 were significantly higher than those at location 1 while the mean ALT activity at location 4 was significantly higher than at location 2.

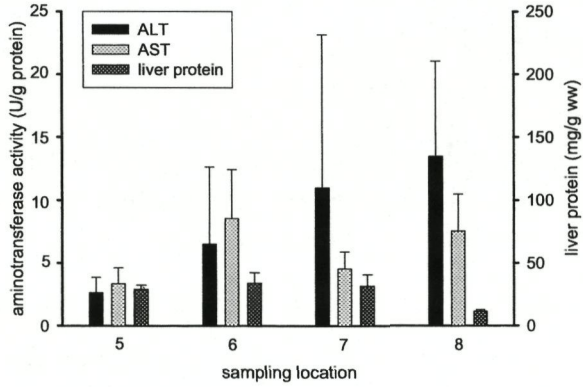

Fig. 2. Serum aminotransferase activities and total liver protein content in plaice at each sampling location.

The numbers of fish were 7, 6, 5 and 4 for locations 5, 6, 7 and 8, respectively.

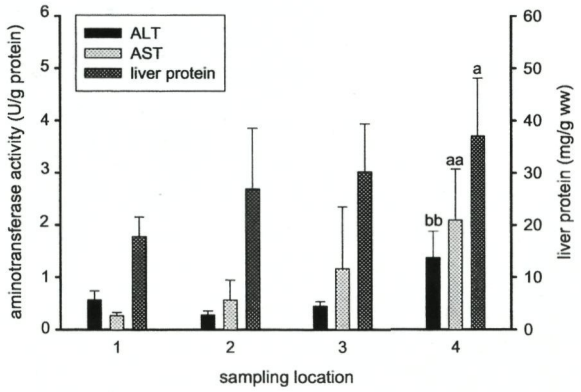

Fig. 3. Serum aminotransferase activities and total liver protein content in bib at each sampling location.

The lower cases a and b indicate significant differences from locations 1 and 2, respectively (a: $p < 0.05$; aa, bb: $p < 0.01$). The numbers of fish were 4, 5, 8 and 4 for locations 1, 2, 3 and 4, respectively.

The PFOS concentrations measured in liver of plaice and bib are shown in Fig. 4 and 5, respectively. All liver concentrations were above the detection limit (10 ng/g ww). For plaice liver no significant differences in mean PFOS liver content between sampling locations were recorded (Fig. 4). At the only estuarine sampling location where plaice was caught three specimens out of four had extremely high liver concentrations: 1286 ng/g ww, 1744 ng/g ww and 7760 ng/g ww accounting for the high variation at that site (Fig. 4). Fig. 4 also shows that the mimimal, maximal, mean and median marine liver concentrations in plaice from the North Sea were all lower than the minimum, maximum, mean and median at the estuarine location (location 8).

In bib, the mean PFOS concentration at location 4 was found to be significantly higher than the mean at location 1. As can be seen in Table 1, a significant positive correlation was found between the distance from the sampling locations to Antwerp and the PFOS liver content in bib. The maximum and mean PFOS concentrations in bib liver at the Western Scheldt locations were always higher than those at location 1, the only marine sampling location where bib was sampled (Fig. 5).

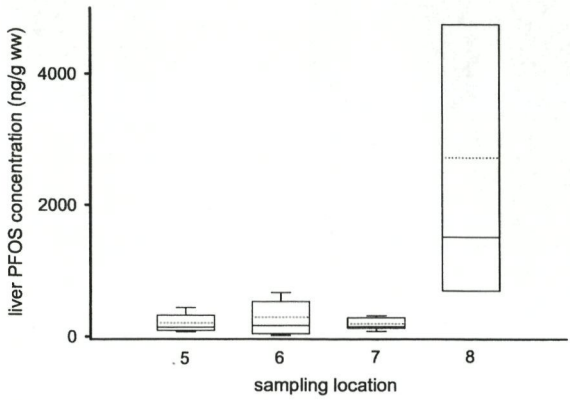

Fig. 4. Liver PFOS concentrations in plaice at each sampling location.

The straight line is the median and the dotted line represents the mean. The 25th and 75th percentiles define the boxes. The whiskers represent the 10th and 90th percentiles. The numbers of plaice for locations 5, 6, 7 and 8 were 7, 6, 5 and 4, respectively.

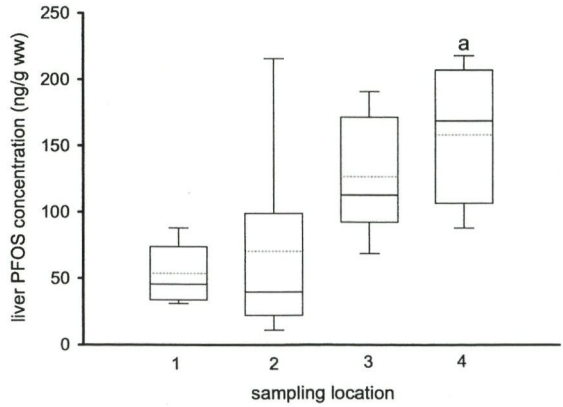

Fig. 5. Liver PFOS concentrations in bib at each sampling location.

The straight line is the median and the dotted line represents the mean. The 25th and 75th percentiles define the boxes. The whiskers represent the 10th and 90th percentiles. The lower case a indicates a significant difference between the means of location 1 and location 4 ($p < 0.05$). The numbers of bib for locations 1, 2, 3 and 4 were 4, 5, 8 and 4, respectively.

Table 1. Results of the correlation analysis between the liver PFOS content and the endpoints investigated and between some endpoints.

correlation	bib (n = 21)	plaice (n = 22)
PFOS liver concentrations vs. distance towards Antwerp	r = -0.61 ***p = 0.004	ND
serum AST activity vs. distance towards Antwerp	r = -0.59 ***p = 0.004	ND
serum ALT activity vs. distance towards Antwerp	r = -0.51 *p = 0.02	ND
PFOS liver concentrations vs. serum ALT activity	r = 0.44 *p = 0.047	r = 0.043 p = 0.85
PFOS liver concentrations vs. serum AST activity	r = 0.31 p = 0.155	r = 0.11 p = 0.62
PFOS liver concentrations vs. lipid content liver	r = 0.24 p = 0.28	r = -0.027 p = 0.91
PFOS liver concentrations vs. carbohydrate content liver	r = -0.24 p = 0.29	r = -0.034 p = 0.87
PFOS liver concentrations vs. protein content liver	r =0.55 **p = 0.008	r = -0.081 p = 0.72
PFOS liver concentrations vs. fork length	r = -0.66 ***p = 0.004	r = 0.24 p = 0.28
serum ALT activity vs. serum AST activity	r = 0.60 ***p = 0.004	ND

Pearson product-moment correlation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. ND = not determined, r = correlation coefficient, p = p value, n = number of fish.

A clear reduction in the percentage of plaice with PFOS muscle concentrations below the detection limit was observed when the estuarine sampling location (location 8) was compared with the marine locations

(locations 5, 6 and 7) as can be seen in Table 2. For plaice the maximal marine muscle concentrations were 2.22 to 6.25 times lower than the highest estuarine muscle concentration determined for that species.

Table 2. Ranges of PFOS muscle concentrations in plaice.

sampling location number plaice	5	6	7	8*
PFOS muscle concentration (ng/g ww)	< 10-39	< 10-17	< 10-14	< 10-87
percentage of total fish of sampling location below detection limit muscle (< 10 ng/g ww)	71	83	80	25
ratio between maximal PFOS muscle concentration of sampling location and the maximal Western Scheldt muscle concentration	0.45	0.20	0.16	1

The Western Scheldt (The Netherlands) sampling location is marked with an asterisk. The marine locations are not marked. The numbers of plaice analyzed for locations 5, 6, 7 and 8 were 7, 6, 5 and 4, respectively.

For bib an increasing trend was observed for the minimum and maximum PFOS muscle concentrations when sampling was done at locations closer to Antwerp (Table 3). The ratios in Table 3 show that the various maximal estuarine muscle concentrations in bib are between 2.3 and 3.7 times higher than the maximal marine concentration measured.

The liver PFOS content of bib was significantly correlated with the liver protein content and the serum ALT activity (Table 1). The PFOS content of bib liver was not found to be significantly associated with the liver

lipid and carbohydrate content and no significant correlation was found between the liver PFOS content and the serum AST activity. Furthermore, a significant negative correlation between the liver PFOS content and the fork length of bib was shown. For plaice none of these endpoints were found to correlate with the liver PFOS content. The serum AST and ALT activity in bib were both significantly correlated with the distance towards the city of Antwerp. The serum activities of ALT and AST were shown to correlate significantly (Table 1).

Table 3. Ranges of PFOS muscle concentrations in bib.

sampling location number bib	1	2*	3*	4*
PFOS muscle concentration (ng/g ww)	< 10-30	< 10-76	15-111 (55)	41-107 (64)
Percentage of total fish of sampling location below detection limit muscle (< 10 ng/g ww)	50	40	0	0
ratio between maximal PFOS muscle concentration of sampling location and the maximal North Sea PFOS muscle concentration	1	2.3	3.7	3.6

The Western Scheldt (The Netherlands) sampling locations are marked with an asterisk. The marine locations is not marked. The numbers of bib analyzed for locations 1, 2, 3, and 4 were 4, 5, 8 and 4, respectively.

3.5 Discussion

In the present study we report for the first time on the exposure levels of PFOS in the Western Scheldt and the Belgian North Sea. The significant positive correlation observed between the bib liver PFOS content and the proximity of the sampling locations to Antwerp suggests discharge in the Western Scheldt upstream from location 4. The increasing trends observed for the minimum and maximum muscle concentrations in bib that was caught closer to Antwerp illustrates furthermore that PFOS might indeed be present as a gradient along the Western Scheldt decreasing towards the sea. This distribution pattern was previously shown for several metals and volatile chlorinated compounds in the Scheldt (Van Alsenoy et al., 1993; Dewulf et al., 1998; Paucot and Wollast, 1997). Interestingly, the presence of these compounds in the ecosystem is also reflected in the tissue concentrations of aquatic biota as was shown by De Wolf et al. (2000). These authors observed a concentration gradient for a number of metals in the soft body tissues of the periwinkle (*Littorina littorea*) parallelling their Western Scheldt pollution gradient. However, this is the first time that such a pattern is documented for PFOS.

Although the PFOS tissue concentrations might reflect the distribution of PFOS in the Western Scheldt, a tissue dilution effect could contribute, however, to the decreasing trends for liver and muscle PFOS concentrations observed in bib when these fish were caught further away from Antwerp. Indeed, not only the PFOS levels increased at the various sampling sites but also the fork length of the fish decreased closer to the city of Antwerp. As a consequence of this type of dilution, PFOS could be more concentrated in smaller tissue volumes as a mere consequence of

the lower degree of diffusion in a smaller volume of tissue compared to a larger one. Watanabe et al. (1999) suggested that tissue dilution probably accounted for the decrease of polychlorinated biphenyl and hexachlorocyclohexane isomer levels in blubber of immature Caspian seals when the animals had larger body lengths. Solé et al. (2001) found lower concentrations of several organic pollutants in muscle tissue of deep-sea fish with larger sizes and suggested that tissue dilution might be responsible for this phenomenon. Seasonal variation in eelpout (*Zoarces viviparus*) liver mercury burden results largely from the dilution of similar burdens by a seasonally growing and shrinking liver illustrating the possible impact of tissue dilution on concentrations of toxicants in fish tissues (Mathieson et al., 1996). Tissue dilution as a result of changes in nutritional status can also greatly influence tissue concentrations as was illustrated for cadmium exposure of dogwhelks (*Nucella lapillus*, Leung and Furness, 2001). Since the liver PFOS concentration has the tendency to be higher in fishes with a smaller fork length as suggested by the significant correlation between both endpoints, tissue dilution might contribute to the explanation of the PFOS gradient observed.

Differences in diet of small and large bibs might also affect PFOS tissue burdens leading to higher PFOS concentrations in smaller fish. Papers on the feeding patterns of bib, however, suggest that a difference in feeding habits is probably not an important factor. Hamerlynck and Hostens (1993) reported that the feeding pattern of small bib in a coastal area of the South-west Netherlands changed to a regime of almost exclusively shrimp and small fish when they were about 100 mm in length. In the mesohaline zone of the Western Scheldt estuary Hostens and Mees (1999) found that bib showed a diet shift at 50 mm and 130 mm. Since the smallest bib in the present study measured 130 mm it can be

reasonably assumed that the nutritional habits of the examined fish were similar.

The high PFOS liver concentrations found in plaice caught at the inflow of the canal Ghent-Terneuzen (1286 ng/g ww, 1744 ng/g ww and 7760 ng/g ww) suggests that substantial amounts of PFOS are discharged via this canal. Paper mill factories along the canal using PFOS for surface treatment might contribute to the observed PFOS pollution. Other studies show that organic pollutants might be present at relatively high concentrations at Terneuzen. Steen et al. (2001) showed that this canal is a source for inflow of several pesticides into the Western Scheldt. Polychlorinated biphenyl levels in the blood of common terns (*Sterna hirundo*) were reported to be significantly elevated at Terneuzen compared to two other reference sites (van den Brink and Bosveld, 2001). Because Terneuzen is the most inland location at which plaice was caught, however, these high PFOS concentrations might also reflect PFOS inflow in the Western Scheldt from locations that are situated further upstream. The much higher percentage of plaice with muscle PFOS concentrations below the detection limit at the marine sampling locations also suggests that the marine locations are less polluted which is probably a consequence of dilution of PFOS in the North Sea. This supports the data on bib liver and muscle PFOS burdens that also suggest higher PFOS concentrations in tissues at locations further upstream in the Western Scheldt.

Giesy and Kannan (2001) reported PFOS concentrations in the livers of 41 fish from various species and sampling locations ranging from < 7 ng/g ww to 170 ng/g ww while the PFOS tissue concentrations in the Western Scheldt in the present study ranged from 11 ng/g ww to 217 ng/g

ww liver in bib and from 107 ng/g ww to 7760 ng/g ww in plaice. The measured muscle tissue concentrations in the Western Scheldt ranged between < 10 ng/g ww and 111 ng/g ww for bib and < 10 ng/g ww and 87 ng/g ww for plaice while concentrations between < 6 ng/g ww and 300 ng/g ww have been reported for fish muscle tissue (Giesy and Kannan, 2001). The highest Western Scheldt liver PFOS concentrations in plaice were 1286 ng/g ww, 1744 ng/g ww and 7760 ng/g ww; higher than any fish liver concentration reported before. The maximum concentration in plaice liver (7760 ng/g ww) is about two times higher than the maximum concentration ever reported for an animal tissue (3680 ng/g ww) and the three highest liver concentrations are between 7.5 and 46 times higher than the maximum PFOS concentration in fish liver documented so far (Giesy and Kannan, 2001). Although there are differences between the fish species of the present study and those studied by Giesy and Kannan (2001), the PFOS liver concentrations of both fish species presented here show that the PFOS pollution level in the Scheldt estuary can be considered among the highest ever reported. Additional research should be conducted to assess the full impact of this pollution problem.

In a previous study Hoff et al. (2003b) observed significant dose-dependent increases of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum of common carp (*Cyprinus carpio*) exposed to PFOS suggesting PFOS-mediated induction of cellular necrosis.

In the present study a significant correlation was observed between the bib liver PFOS content and the serum ALT levels. Since the serum AST activity was significantly correlated with the proximity to Antwerp but not with the liver PFOS content in bib, it could be possible that tissue necrosis-inducing compounds different from PFOS are present as a

gradient along the longitudinal axis of the Western Scheldt. For both AST and ALT are significantly correlated in this study, it might be that these unspecified compounds are also (partly) responsible for the significant correlation observed between the serum ALT activity and the proximity to Antwerp. Therefore, the significant association between the serum ALT activity and the liver PFOS content in bib, which also has a gradient-like profile along the Western Scheldt, might not have an unambiguous interpretation, especially since an increase of serum AST and ALT activity might not (exclusively) be linked to toxicant exposure but might as well be influenced by environmental factors varying along the Western Scheldt (e.g. salinity).

The finding that the PFOS content in bib liver did not correlate with the serum AST level but that a correlation was observed with the serum ALT activity can be due to a less pronounced PFOS-mediated increase of serum AST compared to serum ALT in the field. This relatively weak increase of serum AST activity compared to serum ALT activity was observed in carp exposed to PFOS under laboratory conditions (Hoff et al., 2003b).

A second biochemical effect reported was the significant linear correlation between the PFOS content and the bib liver protein content. Increases of protein content in fish liver as a result of toxicant exposure have been reported before and can be concurrent with the induction of detoxification mechanisms (Brumley and Haritos, 1995). In addition, increased protein synthesis in fish subjected to a toxic challenge might be linked to chronic repair processes (Wilson et al., 1996). Compensatory induction of protein synthesis as a result of toxicant-mediated inhibition is also possible. Differences in dietary habits between sampling locations,

however, could also affect the liver protein content. As discussed earlier, this is not very likely.

Although a significant correlation was found between the liver PFOS content and the serum ALT activity and between the liver PFOS content and the liver protein content in bib, such a correlation was not found in plaice. This could be due to a lower sensitivity of this species to PFOS' toxicity, differences in uptake, elimination or metabolism. Most importantly, it should be considered that the sampling locations for plaice were different than those for bib suggesting different environmental conditions for the two fish species. Four out of five plaice sampling locations were marine locations while for bib only one sampling location out of four was situated in the coastal zone. Furthermore, differences in feeding pattern of bib and plaice have been described (Hostens and Mees, 1999; van den Broek, 1978) and could account for differences in response of the two species investigated.

3.6 Conclusion

In conclusion, the current results suggest the existence of a gradient of PFOS exposure along the Western Scheldt estuary. A general decrease in PFOS contamination was observed downstream and results in a pollution level of the Belgian part of the North Sea which is lower than that of the Western Scheldt. On the biological level, the PFOS liver content in bib was found to be positively correlated with the liver protein content, the serum ALT activity and negatively with the fork length. Assessment of confounding factors such as tissue dilution, salinity and other pollutants would allow a more profound interpretation of the observations made and

thus lead to a better understanding of the hazard in aquatic ecosystems linked to PFOS.

Chapter 4

Perfluorooctane sulfonic acid and organohalogen pollutants in liver of three freshwater fish species in Flanders (Belgium): relationships with biochemical and organismal effects

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4.1 Abstract

A perfluorooctane sulfonic acid (PFOS) assessment was conducted on gibel carp (*Carassius auratus gibelio*), carp (*Cyprinus carpio*), and eel (*Anguilla anguilla*) in Flanders (Belgium). The liver PFOS concentrations in fishes from the Ieperlee canal (Boezinge, 250-9031 ng/g wet weight) and the Blokkersdijk pond (Antwerp, 633-1822 ng/g wet weight) were higher than at the Zuun basin (Sint-Pieters-Leeuw, 11.2-162 ng/g wet weight) and among the highest in feral fishes worldwide. Eel from the Oude Maas pond (Dilsen-Stokkem) and Watersportbaan basin (Ghent) had PFOS concentrations ranging between 212 and 857 ng/g wet weight. The hepatic PFOS concentration was significantly and positively related with the serum alanine aminotransferase activity, and negatively with the serum protein content in eel and carp. The hepatic PFOS concentration in carp correlated significantly and negatively with the serum electrolyte concentrations whereas a significant positive relation was found with the hematocrite in eel. Although 13 organochlorine pesticides, 22 polychlorinated biphenyl (PCB) congeners and 7 polybrominated diphenyl ethers (PBDEs) were also measured in the liver tissue, only PCB 28, PCB 74, γ-hexachlorocyclohexane (γ-HCH) and hexachlorobenzene (HCB) were suggested to contribute to the observed serological alterations in eel.

4.2 Introduction

PFOS is a widely used chemical with applications as wetting and foaming agent and as precursor of surfactants and pesticides (Abe and Nagase, 1982). It was recently shown to be a widespread environmental contaminant in aquatic and terrestrial biota. Top predators were shown to

generally have the highest tissue concentrations suggesting biomagnification of PFOS (Giesy and Kannan, 2001). PFOS concentrations in feral fish are scarcely documented. In lake whitefish eggs from Michigan waters (USA), PFOS concentrations up to 380 ng/g wet weight have been measured and the maximal muscle and liver tissue PFOS concentrations reported in the USA were 300 ng/g wet weight and 170 ng/g wet weight, respectively. Tissue PFOS concentrations comparable to the highest ever measured (5140 ng/g wet weight in mink liver, Kannan et al., 2002d) were found recently in the liver from plaice (*Pleuronectes platessa*) captured in the Western Scheldt estuary (The Netherlands) where the maximal liver PFOS concentration measured was 7760 ng/g wet weight (Hoff et al., 2003a). Also in fish from Etobicoke creek (ON, Canada) elevated PFOS liver concentrations in common shiner (*Notropus cornutus*) have been recorded ranging from 2 to 72.9 μg/g wet weight after an accidental spill of fire retardant foam at a nearby airport (Moody et al., 2002).

The biochemical effects of PFOS exposure have mainly been studied in mammalian model species in which PFOS was shown to be an inducer of peroxisomal β-oxidation (Ikeda et al., 1987; Sohlenius et al., 1993) and a hypolipemic agent (Haughom and Spydevold, 1992; Lau et al., 2001; Seacat et al., 2003). PFOS can also increase membrane fluidity (Hu et al., 2000) and inhibit gap junction intracellular communication (Hu et al., 2002). Effects on carboxylesterase expression (Derbel et al., 1996) have also been demonstrated in addition to developmental effects (York et al., 2000). An increase in serum alanine aminotransferase (ALT) activity was demonstrated in rhesus monkeys and in rats (Goldenthal et al., 1978c, 1978b; Seacat et al., 2003). Hoff et al. (2003b) suggested that PFOS interferes with homeostasis of DNA metabolism and that PFOS induces

liver damage in carp, as assessed by the serum ALT activity, while peroxisomal β-oxidation was not shown to be induced.

While preliminary PFOS biomonitoring campaigns have been conducted for wood mice (*Apodemus sylvaticus*, Hoff et al., 2004) and plaice and bib (*Trisopterus luscus*) in an estuarine environment (Hoff et al., 2003a), information for freshwater fish species in this context is lacking. Therefore, the liver PFOS concentrations, the serum ALT activity, the serum protein content, the hematocrite value, serum electrolyte levels, condition and growth rate have been measured in feral gibel carp, carp and eel. This allowed us to characterize the PFOS pollution degree at a number of freshwater locations in Flanders (Belgium) for the first time and investigate the relation between these biological endpoints and the liver PFOS concentration.

Aside from PFOS, the liver concentrations of 13 organochlorine pesticides, 22 polychlorinated biphenyl congeners and 7 polybrominated diphenyl ethers were measured because laboratory controlled experiments suggest that these compounds can possibly affect the endpoints under investigation in this study. This has been demonstrated for hexachlorobenzene exposed rats and workers in which an ALT activity increase has been observed (Almeida et al., 1997; Queiroz et al., 1998). Also PCB 126 exposure has been shown to induce the plasma ALT activity in birds (Hoffman et al., 1996) and Aroclors 1254 and 1260 have been demonstrated to be associated with increased serum ALT activities in rats (Mayes et al., 1998). The measurement of these organohalogens provided us with information on their possible involvement in the modulation of endpoints suggested to be affected by PFOS in this study.

4.3 Materials and methods

4.3.1 Sampling

In September and October 2002, eels that were in their yellow, pre-migratory stage were captured in the Ieperlee canal (Boezinge), the ponds Oude Maas (Dilsen-Stokkem), Blokkersdijk (Antwerp) and the Watersportbaan (Ghent) and Zuun basins (Sint-Pieters-Leeuw) in Flanders (Belgium). Carps were captured in the Blokkersdijk pond and the Zuun basin. Gibel carps were also collected at the latter location and in the Ieperlee canal (Fig. 1).

Fykes were set up two days before the fishes were collected. The numbers of fishes captured are given in Table 1. The captured fishes were anaesthetised with ethyl 3-aminobenzoate, weighed and the fork length was determined. Scales were taken behind the head along the longitudinal axis. Blood was taken with caudal puncture. Serum was prepared by centrifugation (4000 rpm, 5 min) and frozen in liquid nitrogen. The liver was dissected and also stored in liquid nitrogen. Le Cren´s condition factors were calculated and the age of the carps and gibel carps was determined by counting the scale annuli. The fish growth rate was calculated as described by the Fraser-Lee method (Bagenal and Tesch, 1978).

4.3.2 Serum biochemical assays

The serum alanine aminotransferase activity was determined by the spectrophotometric method of Bergmeyer et al. (1986a). The serum Cl^-, Na^+, Ca^{2+} and K^+ levels were determined with ion-selective electrodes on a 9180 Electrolyte Analyzer (AVL Scientific, Roswell, GA, USA). The

serum protein content was determined with the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). For the determination of the hematocrite, the relative red blood cell volume was determined after centrifugation of heparinized blood in sealed capillaries (2000 rpm, 5 min). For all these measurements, values for duplicate analyses varied maximally 14 %.

4.3.3 Determination of liver PFOS concentrations

The PFOS concentration in liver tissue (100-500 mg) was measured using combined high pressure liquid chromatography-mass spectrometry according to Giesy and Kannan (2001). High pressure liquid chromatography was done on a CapLC system (Waters, Milford, MA, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm inner diameter, Alltech, Deerfield, IL, USA). The analysis was performed on a Betasil C18 column (50 mm x 1 mm inner diameter, Keystone Scientific, San Jose, CA, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH_4OAc (A) / CH_3OH (B). A gradient elution was used starting at 45 % B and going to 90 % B in 3 min. After 5 min initial conditions were resumed. PFOS was measured under negative electrospray ionization using single reactant monitoring (m/z 499→99). The internal standard (1H, 1H, 2H, 2H-perfluorooctane sulfonic acid) was measured under the same conditions (m/z 427→81). The dwell time was 0.1 s. The electrospray-capillary voltage was set at –3.5 kV and the cone voltage was 24V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10^{-5} mm Hg (Ar). The PFOS concentrations were determined with an unextracted calibration curve. Data quality assurance included

laboratory blanks and continuing calibration verification. Repeatability and reproducibility were 85 and 80 %, respectively.

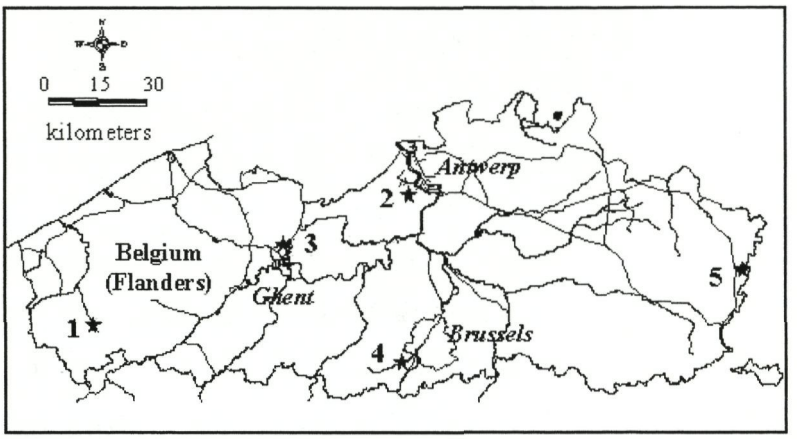

Fig. 1. Area of study and sampling locations.

1: Ieperlee canal, 2: Blokkersdijk pond, 3: Watersportbaan basin, 4: Zuun basin, 5: Oude Maas pond.

4.3.4 Determination of liver concentrations of polychlorinated and polybrominated pollutants

The organochlorine pesticides under investigation were α-, β-, γ- isomers of hexachlorocyclohexane, p,p'-dichlorodiphenylethane (p,p′-DDE), p,p'-dichlorodiphenyldichloroethane (p,p′-DDD), o,p'-dichlorodiphenyltrichloroethane (o,p′-DDT) and p,p'-dichlorodiphenyltrichloroethane (p,p′-DDT), pentachlorobenzene (QCB), hexachlorobenzene, oxychlordane (OxC), *trans*-nonachlor (TN), *trans*-(TC) and *cis*-chlordane (CC). The following polychlorinated biphenyl congeners (International Union of Pure and Applied Chemistry numbers) were targeted: 28, 31, 74, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149,

153, 156, 163, 170, 180, 183, 187, 194 and 199. Polybrominated biphenyl ether congeners 28, 47, 99, 100, 153, 154 and 183 were also included. The method used for sample preparation and analysis was described in detail by Jacobs et al. (2002) and Voorspoels et al. (2003). Briefly, the available amount of tissue (50–500 mg) was ground with Na_2SO_4, internal standards were added and the mixture was extracted for 2 h with 75 ml hexane:acetone = 3:1 (volumetric ratio) into a hot Soxhlet manifold. After concentration, the extract was subjected to clean-up on acidified silica and analytes were eluted with 15 ml n-hexane followed by 10 ml dichloromethane. The eluate was concentrated to 80 µl and transferred to an injection vial. PCBs were determined on a HP 6890 gas chromatograph with electron capture detection (Hewlett Packard, Palo Alto, CA, USA) equipped with a 50 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE Scientific, Zulte, Belgium). PBDEs and organochlorine pesticides were determined on a HP 6890 gas chromatograph-5793 mass spectrometer (Hewlett Packard, Palo Alto, CA, USA) which was operated in negative chemical ionization and selected ion monitoring and was equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column. Instrumental operating conditions and quality control were detailed presented by Jacobs et al. (2002) and Voorspoels et al. (2003). Briefly, daily check of calibration curves, regular analysis of procedural blanks and of certified material CRM 350 (PCBs in mackerel oil) were included in the quality assurance protocol. Additionally, the method was tested by participation in several interlaboratory tests. Recoveries of target compounds ranged between 72 and 103 %. Method limits of detection for individual PCB congeners ranged between 0.5 and 1 ng/g wet weight, while for organochlorine pesticides and PBDEs, they were 0.2 and 0.1 ng/g wet weight, respectively.

4.3.5 Statistical analysis

The non-parametric Mann-Whitney U test was used for comparison of the gibel carp and carp hepatic PFOS concentrations and body weights at the different sampling locations. This test was also used to compare the gibel carp and eel hepatic PFOS concentrations and body weights from the Ieperlee canal. The eel liver PFOS concentrations and body weights at each location were compared with the non-parametric Kruskal-Wallis test with Dunn´s test as post hoc criterion. The latter tests were also used to compare the liver PFOS concentrations and body weights of gibel carp, carp and eel from the Zuun basin.

The relationship between the measured pollutants and the biological endpoints was investigated with Partial Least Squares Analysis (PLS). The variables for which a numeric value was obtained in < 30 % of the observations and for which the variance was close to zero were not used for PLS analysis. If PLS did not yield a relevant model, correlation analysis was used to study the relation between a selected set of organohalogens and biological endpoints. Correlation analysis was carried out for those compounds which were quantifiable in > 50 % of the individuals.

4.4 Results

The measured hepatic PFOS concentrations of the three fish species are shown in Figure 2. The hepatic PFOS concentration in eels and gibel carps from the Ieperlee canal were significantly higher than in eels and gibel carps from the Zuun basin, respectively. Also the liver PFOS concentration in carps from Blokkersdijk was significantly elevated in comparison to carps from the Zuun basin. The liver PFOS concentrations

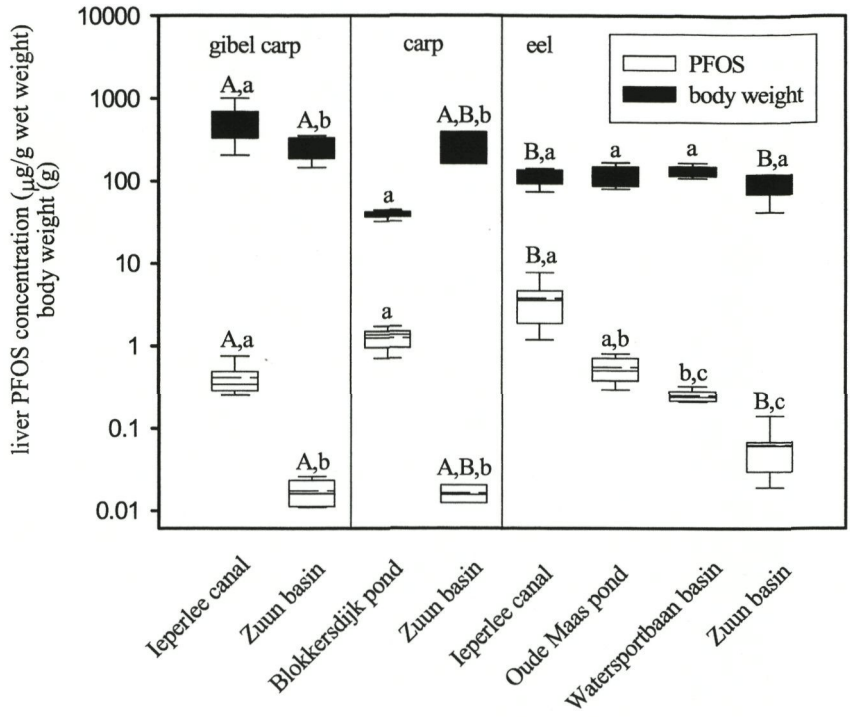

Fig. 2. Liver PFOS concentrations and body weight of gibel carps, carps and eels at each sampling location.

The straight line is the median and the dotted line represents the mean. The 25th and 75th percentiles define the boxes. The whiskers represent the 10th and 90th percentiles. The lower cases a, b and c indicate significant differences within species. The capitals A and B indicate significant differences between species for identical sampling locations. Boxes having different letters are significantly different ($p < 0.05$).

in eels from the Zuun basin were found to be lower than at the Oude Maas pond. Eels from the Watersportbaan basin had lower hepatic PFOS concentrations than eels from the Ieperlee canal. Gibel carps from the Ieperlee canal and the Zuun basin had significantly lower liver PFOS concentrations than eels from these respective locations. The hepatic PFOS concentration of carps and gibel carps from the Zuun basin did not

differ significantly, neither did the hepatic PFOS concentration of carps and eels from the Zuun basin.

The gibel carp body weight at the Ieperlee canal was significantly higher than at the Zuun basin while the body weight for carp at the latter location was significantly higher than at the Blokkersdijk pond. The eel body weight did not differ significantly among locations. Gibel carps from the Ieperlee canal and the Zuun basin had significantly higher body weights than eels from these respective locations. The body weight of carps and gibel carps from the Zuun basin did not differ significantly, neither did the body weight of carps and eels from the Zuun basin. Table 1 gives an overview of the hepatic organohalogen concentrations measured.

The PLS models describing the relations between the measured pollutant levels and the assessed biological endpoints, showed poor relationships for eel, carp and gibel carp ($Q^2 = 0.11$, $Q^2 = 0.10$, $Q^2 = 0.15$, respectively). Table 2 shows the relationships between the liver PFOS concentration and the biological endpoints.

Table 1. Ranges and mean concentrations (in brackets) expressed in ng/g wet weight for organohalogens measured in liver of freshwater fishes.

organohalogen	gibel carp ($n = 13$)	carp ($n = 12$)	eel ($n = 28$)
PFOS	11.2-781 (201)	11.3-1822 (934)	17.3-9031 (1387)
α-HCH	< LOD	< LOD-0.3	< LOD-0.3
β-HCH	< LOD	< LOD-0.2	< LOD
γ-HCH	< LOD-0.7	< LOD-0.6	0.2 2.3
p,p′-DDE	< LOD-2.5	4.3-56.0 (14.6)	1.8-122.7 (13.8)

p,p´-DDD	< LOD-2.0	3.7-14.0 (6.2)	0.6-26.0 (4.8)
p,p´-DDT	< LOD-0.4	< LOD-0.8	< LOD-4.8
o,p´-DDT	< LOD-0.9	< LOD-5.3	< LOD
QCB	< LOD	< LOD	< LOD
HCB	< LOD-0.8	< LOD-2.4	0.2-3.7 (1.3)
OxC	< LOD-0.6	< LOD-0.8	< LOD-5.3
TN	< LOD-2.8	< LOD-3.1	< LOD-5.5
TC	< LOD-1.8	< LOD-2.7	< LOD
CC	< LOD-1.1	< LOD-1.7	< LOD
PCB 28	< LOD-26.7	< LOD-6.8	< LOD-52.4
PCB 31	< LOD-16.3	< LOD-5.7	< LOD-14.2
PCB 74	< LOD-20.3	1.3-5.8 (3.4)	< LOD-19.8
PCB 95	< LOD-13.5	2.0-28.4 (11.9)	< LOD-18.0
PCB 99	< LOD-14.2	6.6-18.6 (11.9)	4.9-130 (47.4)
PCB 101	< LOD-12.8	11.3-23.4 (17.4)	2.2-40.0 (4.3)
PCB 105	< LOD-10.3	3.1-10.9 (5.0)	< LOD-33.1
PCB 110	< LOD-18.8	7.0-19.7 (14.8)	2.5-30.4 (11.2)
PCB 118	< LOD-29.0	11.2-23.2 (19.8)	6.8-81.9 (32.4)
PCB 128	< LOD-9.3	4.5-13.6 (7.4)	1.6-23.3 (5.5)
PCB 132	< LOD-7.5	3.2-9.1 (6.9)	< LOD-10.7
PCB 138	< LOD-34.5	15.3-69.5 (27.0)	7.0-104 (21.6)
PCB 149	< LOD-14.3	8.4-21.2 (16.6)	3.2-88.9 (31.4)
PCB 153	< LOD-53.8	22.4-126 (41.9)	14.7-257 (66.2)
PCB 156	< LOD-4.38	0.8-14.1 (3.2)	1.0-13.1 (2.2)
PCB 163	< LOD-11.7	4.0-21.5 (7.7)	0.8-29.5 (6.1)
PCB 170	< LOD-7.1	0.9-27.5 (6.4)	1.5-26.4 (4.5)
PCB 180	< LOD-18.1	3.8-62.3 (14.3)	3.0-91.7 (12.7)
PCB 183	< LOD-5.0	1.3-14.6 (3.5)	2.0-30.8 (6.2)
PCB 187	< LOD-9.4	< LOD-36.4	3.0-60.1 (11.9)
PCB 194	< LOD-0.9	< LOD-7.4	< LOD-9.7
PCB 199	< LOD-1.4	< LOD-7.9	< LOD-12.2
PBDE 28	< LOD-0.2	< LOD-0.7	< LOD-0.2
PBDE 47	< LOD-10.3	0.3-4.6 (0.9)	0.6-33.1 (3.9)
PBDE 99	< LOD	< LOD-0.1	< LOD-0.4
PBDE 100	< LOD-0.9	< LOD-0.7	0.2-6.0 (1.1)
PBDE 153	< LOD-0.2	< LOD	< LOD-0.3
PBDE 154	< LOD-0.4	< LOD-1.2	< LOD-0.4
PBDE 183	< LOD	< LOD	< LOD

n = number of fish, LOD = limit of detection

Table 2. Correlation analysis of the relationship between the hepatic PFOS concentration and the biological endpoints investigated.

correlation	gibel carp ($n = 13$)	carp ($n = 12$)	eel ($n = 28$)
liver PFOS concentration	$r = 0.16$	$r = 0.70$	$r = 0.64$
versus	$p = 0.62$	$*p = 0.014$	$***p = 0.0003$
serum ALT activity (U/g protein)			
liver PFOS concentration	$r = 0.18$	$r = 0.03$	$r = 0.63$
versus	$p = 0.58$	$p = 0.93$	$*** p = 0.0007$
serum ALT activity (U/l)			
liver PFOS concentration	$r = -0.035$	$r = -0.74$	$r = -0.41$
versus	$p = 0.92$	$**p = 0.0078$	$*p = 0.029$
serum protein content			
liver PFOS concentration	$r = -0.50$	$r = -0.21$	$r = 0.53$
versus	$p = 0.081$	$p = 0.56$	$*p = 0.020$
hematocrite			
liver PFOS concentration	$r = -0.36$	$r = -0.73$	$r = -0.34$
versus	$p = 0.25$	$*p = 0.013$	$p = 0.068$
serum Cl^- concentration			
liver PFOS concentration	$r = -0.41$	$r = -0.79$	$r = -0.31$
versus	$p = 0.18$	$**p = 0.0033$	$p = 0.13$
serum Na^+concentration			
liver PFOS concentration	$r = -0.007$	$r = -0.86$	$r = -0.026$
versus	$p = 0.99$	$***p = 0.0005$	$p = 0.90$
serum Ca^{2+} concentration			
liver PFOS concentration	$r = 0.50$	$r = -0.33$	$r = 0.39$
versus	$p = 0.10$	$p = 0.30$	$p = 0.055$
serum K^+ concentration			
liver PFOS concentration	$r = -0.46$	$r = -0.49$	$r = 0.0059$
versus	$p = 0.11$	$p = 0.13$	$p = 0.98$
Le Cren´s condition factor			
liver PFOS concentration	$r = -0.083$	$r = 0.59$	ND
versus	$p = 0.84$	$p = 0.061$	
growth rate			

n = number of fish, p = p value, r = correlation coefficient, ND= not determined

In carp, the liver PFOS concentration was positively and significantly related to the serum ALT activity (expressed in U/g protein), and significant negative relations were found with the serum protein concentration and the serum Cl^-, Na^+ and Ca^{2+} concentrations. In eel, significant positive relationships were observed between the hepatic PFOS concentration and the serum ALT activity (expressed in U/g protein and U/l) and the hematocrite and a significant negative relationship was observed with the serum protein concentration. Graphical representations of these relationships are shown in Fig. 3 and 4.

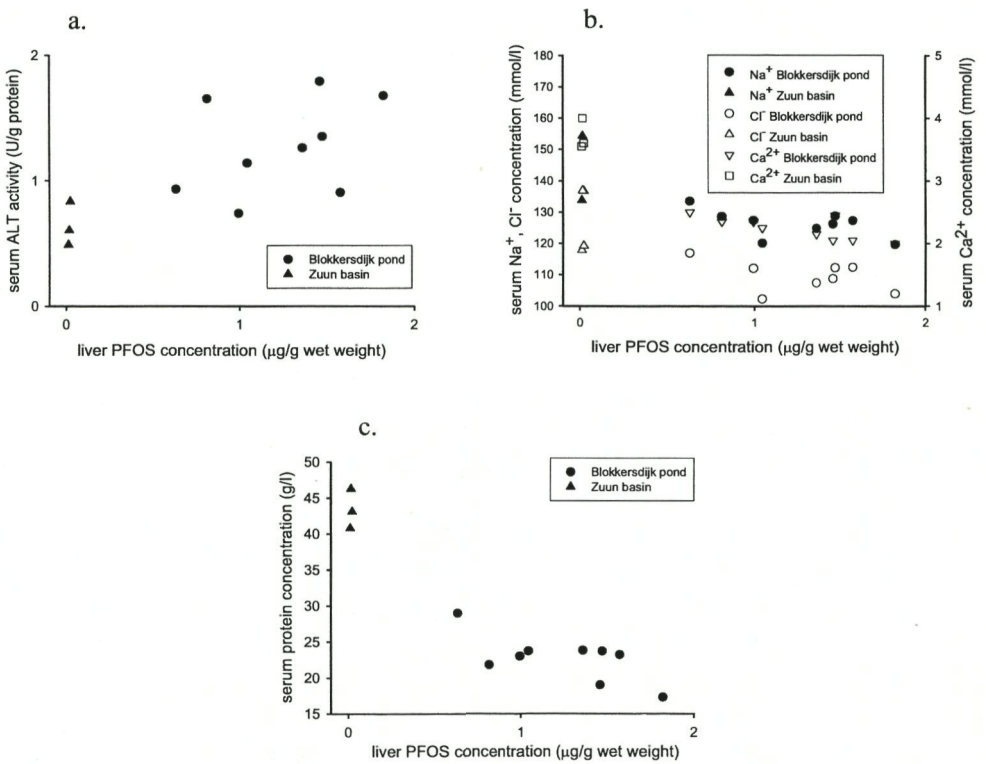

Fig. 3. The relationship between the hepatic PFOS concentration and the serum ALT activity (a), electrolyte concentrations (b) and serum protein concentration (c).

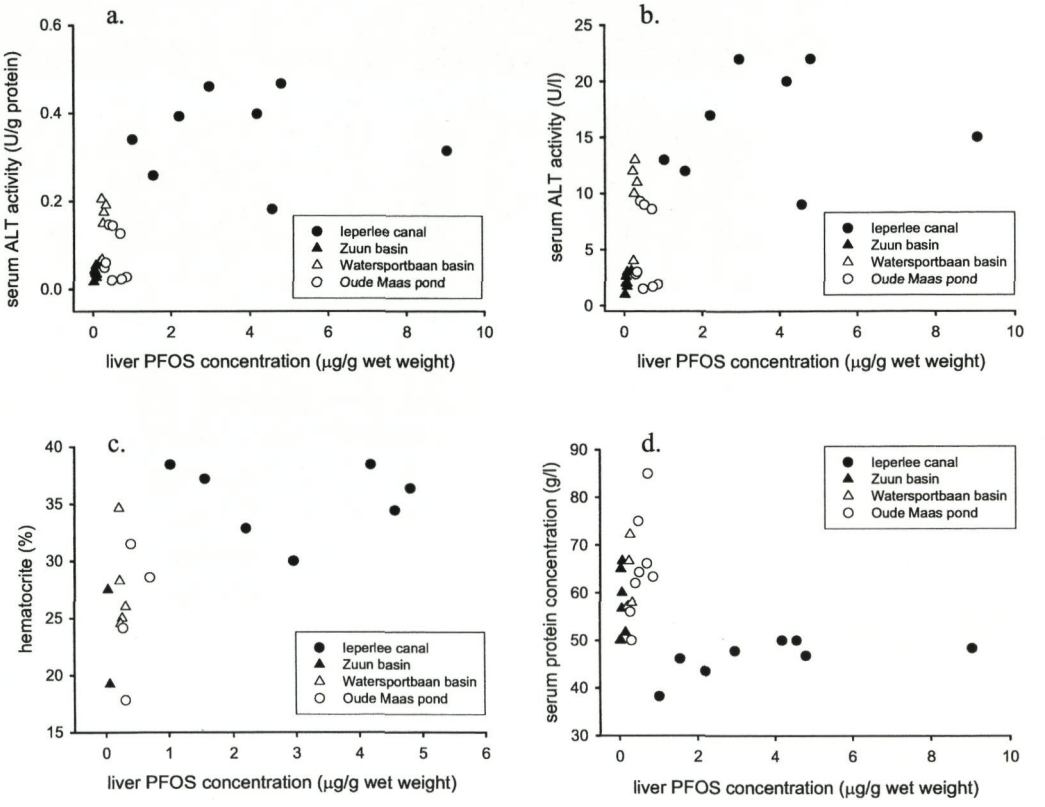

Fig. 4. The relationships between the eel liver PFOS concentration and the serum ALT activity (a, b), hematocrite (c) and serum protein concentration (d).

Table 3 shows the relations between the PCB 28, PCB 74, γ-HCH and HCB concentrations and the biological endpoints shown to correlate significantly to the liver PFOS concentration in eels. This analysis shows that latter organohalogens are significantly related to these biological endpoints. For carp, none of the measured organohalogens aside from PFOS correlated significantly with the endpoints that correlated significantly with PFOS.

Table 3. Correlation analysis of the relationship between the hepatic PCB 28, PCB 74, γ-HCH and HCB concentrations and the biological endpoints investigated in eel.

	serum ALT activity (U/g protein)	serum ALT activity (U/l)	hematocrite	serum protein content
PCB 28	$r = 0.80$	$r = 0.77$	$r = 0.80$	$r = -0.45$
($n = 22$)	$^{***}p < 0.0001$	$^{***}p < 0.0001$	$^{***}p < 0.0001$	$^{*}p = 0.040$
PCB 74	$r = 0.76$	$r = 0.77$	$r = 0.65$	$r = -0.47$
($n = 21$)	$^{***}p < 0.0001$	$^{***}p < 0.0001$	$^{**}p = 0.0066$	$^{*}p = 0.035$
γ-HCH	$r = 0.63$	$r = 0.64$	$r = 0.67$	$r = -0.48$
($n = 28$)	$^{***}p = 0.0005$	$^{***}p = 0.0002$	$^{**}p = 0.0016$	$^{**}p = 0.0099$
HCB	$r = 0.72$	$r = 0.65$	$r = 0.64$	$r = -0.65$
($n = 28$)	$^{***}p < 0.0001$	$^{***}p < 0.0001$	$^{**}p = 0.0029$	$^{***}p = 0.0002$

n = number of fish, p = p value, r = correlation coefficient, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

4.5 Discussion

The measurement of the liver PFOS levels showed that the concentrations in gibel carp and eel captured in the Ieperlee canal at Boezinge ranged from 250 to 781 ng/g wet weight and from 1024 to 9031 ng/g wet weight, repectively. Carps from the Blokkersdijk pond (Antwerp) had liver PFOS concentrations ranging between 633 and 1822 ng/g wet weight, an observation that is in good accordance with the high liver PFOS concentrations (mean and median values of 26.18 and 5.06 μg/g wet

weight, respectively) measured in wood mice from Blokkersdijk (Hoff et al., 2004). The PFOS concentrations in the liver tissue of fishes from the Ieperlee canal and the Blokkersdijk pond are higher than the highest PFOS liver concentration measured in fish liver tissue in the USA (170 ng/g wet weight in Chinook salmon liver captured in the Great Lakes, Giesy and Kannan, 2001) and are comparable with the highest liver PFOS concentration measured in wildlife to date (5140 ng/g wet weight in mink liver, Kannan et al., 2002d). The elevated liver PFOS concentrations in the Ieperlee fishes show that high tissue PFOS concentrations are not only found in the proximity of fluorochemical production units as is the case for the nature reserve Blokkersdijk, but might also occur in industrialized areas with no apparent perfluorochemical production activity. This is clearly the case for the Ieperlee canal at Boezinge since this sampling location is located downstream of the industrial zone of the city of Ypres suggesting PFOS (or its precursors) release into the Ieperlee canal via industrial and/or household wastewater discharges.

The observed differences in hepatic PFOS concentration between fish species at the same sampling location and within species for different sampling locations could be explained by differences in PFOS concentrations in water and sediment within and between locations, differences in PFOS tissue dilution extent, species-specific differences in nutritional habits, uptake/depuration and differences in ecological characteristics. Eel is an epibenthic species, for example, while carp and gibel carp are pelagic species. Also the nutritional habits of the investigated species differ: eel is carnivorous and carp and gibel carp are omnivorous species (Vandelannoote et al., 1998). It is currently unknown, however, how these factors could affect PFOS accumulation in liver tissue.

The strongly significant correlations between the liver PFOS concentration and the serum ALT activity in eel and carp observed in this study suggest that PFOS may induce hepatic damage in these species in the field. In an earlier report, PFOS has been demonstrated to be significantly related to the serum ALT activity in juvenile bibs from the Western Scheldt although the relation was weak ($r = 0.44$, $p < 0.05$, Hoff et al., 2003a). The relations found in the present study are possibly stronger because of the larger liver PFOS concentration ranges (11.3-1822, 17.3-9031 ng PFOS/g wet weight in carp and eel, respectively) compared to bibs for which the liver PFOS concentrations ranged between 11 and 217 ng PFOS/g wet weight. Although the liver hepatic concentrations in plaice from the North Sea and the Western Scheldt ranged between 107 and 7760 ng/g wet weight, which is similar to the PFOS concentration range in eel measured in this study, no significant relation was found between the hepatic PFOS concentration and the serum ALT activity in plaice what could be due to differences in species sensitivity. The positive significant relationship between the liver PFOS concentration and the serum ALT activity in eel and carp is consistent with a PFOS-mediated serum ALT activity increase observed in a short term exposure experiment in carp (Hoff et al., 2003b). It should be noted that the lowest observed effect concentration for serum ALT activity increase in the latter experiment was 561 ng PFOS/g wet weight. Comparison between this tissue value and the liver PFOS concentrations measured in this study, however, is difficult because intraperitoneal injection was used. An increase of serum ALT activity after PFOS exposure was also shown in a subacute rhesus monkey and two rat studies (Goldenthal et al., 1978b, 1978c; Seacat et al., 2003). Seacat et al. (2003) showed that the mean liver PFOS no observed adverse effect level for male and female rats for an increase in serum ALT activity was 364 μg

PFOS/g wet weight after 14 weeks of PFOS exposure. In the present study, the hepatic PFOS concentration was found to be significantly correlated to the serum ALT activity for PFOS concentration ranges well below the rat no observed effect level (11.3-1822, 17.3-9031 ng PFOS/g wet weight in carp and eel, respectively). Differences in species sensitivity or route of exposure could account for this observation. It should also be noted that the duration of exposure of the carps could be much longer than the experimental rat exposure as the age range for carps from the Blokkersdijk pond was 1.9-3.6 years. Also for the yellow eels captured in this study the exposure period might have been relatively long as feral eels can remain in the yellow pre-migratory stage for 7-20 years (Langston et al., 2002).

Other toxicants may have contributed to the alteration in serum ALT activity in eel and carp. In eel, the possibility that other organohalogenated contaminants but PFOS might have affected the serum ALT activity, the total protein concentration and the hematocrite is supported by the significant correlations found between the liver concentrations of PCB 28, PCB 74, γ-HCH, HCB and these endpoints. It is possible that these compounds could have contributed to the induction of the serum ALT activity because earlier studies report increased plasma ALT activities observed in HCB exposed rats (Almeida et al., 1997) and increased serum ALT activities in HCB exposed workers (Queiroz et al., 1998). Also PCB congeners could affect the ALT activity. PCB 26, for example, can induce the plasma ALT activity in birds (Hoffman et al., 1996) and Aroclors 1254 and 1260 have been demonstrated to be associated with increased serum ALT activities in rats (Mayes et al., 1998).

The significant relations between the hepatic PFOS concentration and the serum Cl^-, Na^+ and Ca^{2+} concentrations in carp suggest that PFOS could induce ionregulatory distress by disrupting membrane structure and/or function of gill cells which play a key role in osmoregulation and regulation of ion homeostasis (Wendelaar Bonga and Lock, 1992). Although not being investigated under laboratory conditions, PFOS has been shown to have several effects on the membrane level: increasing of proton leakage of the inner mitochondrial membrane (Starkov and Wallace, 2002), enhancing of membrane fluidity in a rat liver hepatoma cell line and carp and chicken red blood cells (Hu et al., 2000) and inhibition of gap junction communication between cells (Hu et al., 2002). PFOS has an amphipatic structure and could physically disturb the structure of membranes as is the case for many detergent-like compounds (Schreier et al., 2000). Moreover, PFOS has been shown to induce hepatocyte damage *in vivo* (Hoff et al., 2003b) what might be indicative for a more general cytotoxic capacity, that of the gills included.

PFOS-mediated disturbance of gill structure in carp, suggested by the decrease in serum Cl^-, Na^+ and Ca^{2+} concentrations, can lead to hemodilution due to an increase in osmotic water uptake (Wendelaar Bonga and Lock, 1992). This could be an explanation for the observed decrease in serum total protein content associated with PFOS exposure as serum proteins might have been diluted in increased serum volumes. Hemodilution could also explain the lack of any significant relation between the hepatic PFOS concentration in carp and the serum ALT activity, expressed in U/l serum; an increase in serum volume due to hemodilution, could conceal an increase in ALT activity. This increase in ALT activity is suggested by the significant positive relation with the hepatic PFOS concentration when the ALT activity is expressed in U/g

protein. It is also possible, however, that the latter relation indicates a decrease in total serum protein content and an unaltered ALT activity.

At present it is difficult to explain the significant negative correlations between the hepatic PFOS concentration and the total serum protein content observed in carp and eel. It has been shown that PFOS binds to serum proteins and mainly to serum albumin (Jones et al., 2003) but the relation between total protein serum levels and hepatic PFOS concentrations has not been studied under controlled conditions.

In carp, the serum endpoints that correlated significantly with the liver PFOS concentration, did not correlate significantly with the other measured organohalogens. This could imply that the latter compounds are relatively unimportant determinants in the prediction of the serum ALT activity, the serum Cl^-, Na^+ and Ca^{2+} concentrations and the serum protein concentration which were shown to be significantly associated with the liver PFOS concentration.

In contradiction to carps, the lack of changes in serum electrolyte concentrations in eel suggests that PFOS did not induce gill dysfunction in that species. Therefore, the mechanism underlying the observed serum protein concentration decrease in eel is most probably not due to hemodilution caused by gill dysfunction. The probable lack of hemodilution in eels could also account for the observation that the correlation between the liver PFOS concentration and the serum ALT activity, expressed relative to the serum volume, is significant in eels but not in carps.

The PFOS-associated hematocrite increase in eels, which was found to be associated with increased liver PFOS concentrations, might reflect swelling of erythrocytes, increase of erythrocyte numbers or dehydration. It would be speculative, however, to elaborate on the likelihood of any of these hypotheses.

Although some biological endpoints were suggested to be altered by PFOS exposure in this study, no indications were obtained for a decrease in fish condition or a reduced growth capacity mediated by PFOS exposure. The different correlation patterns observed between fish species could suggest differences in PFOS toxicity mechanisms between species. The present study, however, does not allow to draw sound conclusions concerning this issue as capturing locations and PFOS liver concentrations were not similar for all the fish species.

4.6 Conclusion

This study shows that the Blokkersdijk pond (Antwerp), situated in a nature reserve neighbouring a fluorochemical production unit, and the Ieperlee canal at Boezinge (Ypres), a location downstream of an industrial area without apparent perfluorochemical production activity, are hot spots for freshwater fish PFOS pollution in Flanders (Belgium). At these locations the hepatic PFOS concentrations are among the highest concentrations ever reported for wildlife liver tissue. In eel and carp, the liver PFOS concentration correlated significantly and positively with the serum ALT activity, a marker for hepatic damage, showing that PFOS might induce liver damage in freshwater fish under field conditions. A decrease of the total serum protein content in carp and eel, disturbance of ion homeostasis in carp suggesting gill damage, and an increase of

hematocrite levels in eel were also suggested to be PFOS-mediated although other measured organohalogens might be (partly) responsible for the observed biochemical alterations in eel. The hepatic PFOS levels were not shown to be significantly linked to fish growth or condition.

Chapter 5

Biochemical effect evaluation of perfluorooctane sulfonic acid polluted wood mice (*Apodemus sylvaticus*)

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5.1 Abstract

Wood mice (*Apodemus sylvaticus*) were captured at Blokkersdijk, a nature reserve in the immediate vicinity of a fluorochemical plant in Antwerp (Belgium) and at Galgenweel, three kilometers further away. The liver perfluorooctane sulfonic acid (PFOS) concentrations in the Blokkersdijk mice were extremely high (0.47 to 178.55 μg/g wet weight). Perfluorononanoic, perfluorodecanoic, perfluoroundecanoic and perfluorododecanoic acid were found sporadically in the liver tissue of the Blokkersdijk mice. The liver PFOS concentrations at Galgenweel were significantly lower than at Blokkersdijk (0.14 to 1.11 μg/g wet weight). Further results suggest sex-independence of the liver PFOS levels, increased levels of PFOS bioaccumulation in older mice and maternal PFOS transfer to the young. Several liver endpoints were significantly elevated in the Blokkersdijk mice: the liver weight, relative liver weight, the peroxisomal β-oxidation activity, the microsomal lipid peroxidation level and the mitochondrial fraction protein content. For the mitochondrial fraction catalase activity no significant difference between locations was found. The liver weight, relative liver weight and the liver microsomal lipid peroxidation level increased significantly with the liver PFOS concentration. No indications for PFOS-mediated effects on the serum triglyceride, cholesterol or potassium levels were obtained. The liver PFOS concentration was negatively related to the serum alanine aminotransferase activity.

5.2 Introduction

Anthropogenic perfluorinated acids and related perfluorinated compounds were only recently shown to be present in a great diversity of aquatic

wildlife species and fish eating mammals and birds. In these animals perfluorooctane sulfonic acid (PFOS) was demonstrated to be the predominant perfluorinated pollutant for which concentrations up to 3.68 μg/g liver tissue in top predators have been reported. Even at remote locations perfluorochemicals are present in animal tissues but the levels are usually higher in more populated and industrialized regions (Giesy and Kannan, 2001; Kannan et al., 2001a). Although the available literature provides only little information on perfluorochemical distribution in terrestrial mammalian species, available data show that these chemicals might be widespread in the terrestrial mammalian fauna. PFOS was detected in livers of polar bears (0.18-0.68 μg/g wet weight), minks (0.97-3.68 μg/g wet weight) and river otters (0.034-0.99 μg/g wet weight) (Giesy and Kannan, 2001; Kannan et al., 2002d).

A discrepancy exists between the scarcity of information on the presence and distribution of perfluorochemicals in terrestrial mammalian wildlife species and the relatively larger number of reports on *in vivo* toxicological effects of perfluorochemicals assessed under laboratory conditions in mammalian species. Known *in vivo* effects of perfluorochemical exposure are an increase of the relative liver weight in the rat, the mouse and the cynomolgus monkey, the induction of peroxisomal fatty acid β-oxidation and effects on several biochemical endpoints related to oxidative stress in the rat and the mouse (Ikeda et al., 1985, 1987; Permadi et al., 1992, 1993; Sohlenius et al., 1993). Other documented effects are the induction of hypolipemia in the rat, the mouse and the cynomolgus monkey (Haughom and Spydevold, 1992; Lau et al., 2001; Seacat et al. 2002), the inhibition of gap junction intercellular communication (Hu et al., 2002) and effects on carboxylesterase

expression in the rat (Derbel et al., 1996). Developmental and maternal effects in the rabbit, rat and mouse (Lau et al., 2001; York et al., 2000) and promotion of carcinogenesis in the rat (Abdellatif et al., 1990) have also been reported.

The aim of the present study was to evaluate the effects of PFOS exposure in wood mice (*Apodemus sylvaticus*) under field conditions. For the latter purpose, animals were trapped at a nature reserve next to a fluorochemical production plant and a location away from this potential pollution source. The liver concentrations of PFOS and some perfluorinated fatty acids were measured in order to establish possible differences in exposure between both locations. Afterwards, biological and biochemical effect endpoints were studied. Therefore, the liver weight, relative liver weight, the liver peroxisomal fatty acid β-oxidation activity, the degree of microsomal lipid peroxidation and the total protein content and catalase activity in the liver mitochondrial fraction were assessed. In order to study the hypolipemic effect the serum triglyceride and cholesterol levels were measured. The serum alanine aminotransferase (ALT) activity and the serum potassium level were respectively assessed as general markers for monitoring possible hepatic damage (Morgan et al., 2002) and renal failure (Vricella et al., 1992). The relationship between the individual PFOS levels and the latter endpoints was assessed. Potential age and gender effects on the PFOS bioaccumulation and the different endpoints were taken into account.

5.3 Materials and methods

5.3.1 Sampling

In September 2002 wood mice (*Apodemus sylvaticus*) were captured at Blokkersdijk (n = 21) and Galgenweel (n = 21), located in the city of Antwerp (Belgium, Figure 1). Both areas are artificial sand dune habitats with willow (Salix spp.) groves. Sherman live traps were set up at dusk. The trapped animals were brought to the laboratory the next morning. Upon arrival, the animals were anaesthetized with ether and blood was taken using the retro-orbital puncture method. Serum was prepared by centrifugation at room temperature (4000 rpm, 5 min) and frozen in liquid nitrogen. After sacrificing the animals, sex, body and liver weight were determined. The eye lenses were collected and fixed in 10 % formaldehyde. The liver was dissected, weighed and stored in liquid nitrogen for further analysis.

5.3.2 Age determination

The eye lenses were dried at 80°C during 24 hours and immediately weighed with an accuracy of 0.1 mg. The age of the animals (expressed in days) was calculated using the equation: exp((weight of both lenses in mg+15.213)/6.568) (Vandorpe and Verhagen, 1979).

5.3.3 Liver biochemical assays

Liver samples were homogenized on ice in 0.25 M sucrose with an MSE 150 Watt ultrasonic disintegrator (MSE Scientific Instruments, UK). Mitochondrial and microsomal fractions were prepared from liver

homogenate in 0.25 M sucrose according to Meijer et al. (1987). In the final step of the microsomal fraction preparation the sucrose was washed away with 0.15 M KCl. The catalase activity in the mitochondrial fraction was measured fluorometrically by a coupled reaction measuring resorufin formation (λex 540 nm, λem 590 nm) with the Amplex Red Catalase Kit (Molecular Probes, The Netherlands). The peroxisomal β-oxidase activity was also measured on the mitochondrial fraction using fluorometric measurement of the β-oxidation rate based on the peroxidase-linked oxidation of hydroxyphenylacetic acid (λex 318 nm, λem 405 nm) according to Kvannes and Flatmark (1991) with minor changes. The lipid peroxidation level of the microsomal fraction was determined by assessing malondialdehyde-thiobarbituric acid complex formation fluorometrically (λex 515 nm, λem 555 nm) according to Yagi (1976) with slight modifications. The protein content of the mitochondrial and microsomal fractions was determined with the Bio-Rad Protein Assay (Bio-Rad, Germany).

5.3.4 Serum biochemical assays

The serum alanine aminotransferase (ALT) activity was determined by the spectrophotometric method described by Bergmeyer et al. (1986a). Cholesterol concentrations were measured spectrophotometrically at 500 nm according to Allain et al. (1974) and the triglyceride concentration was assessed spectrophotometrically at 640 nm as described by Spayd et al. (1978). The serum potassium levels were measured with an ion-selective electrode on a 9180 Electrolyte Analyzer (AVL Scientific Corporation, USA). The protein content of the serum was determined with the Bio-Rad Protein Assay (Bio-Rad, Germany).

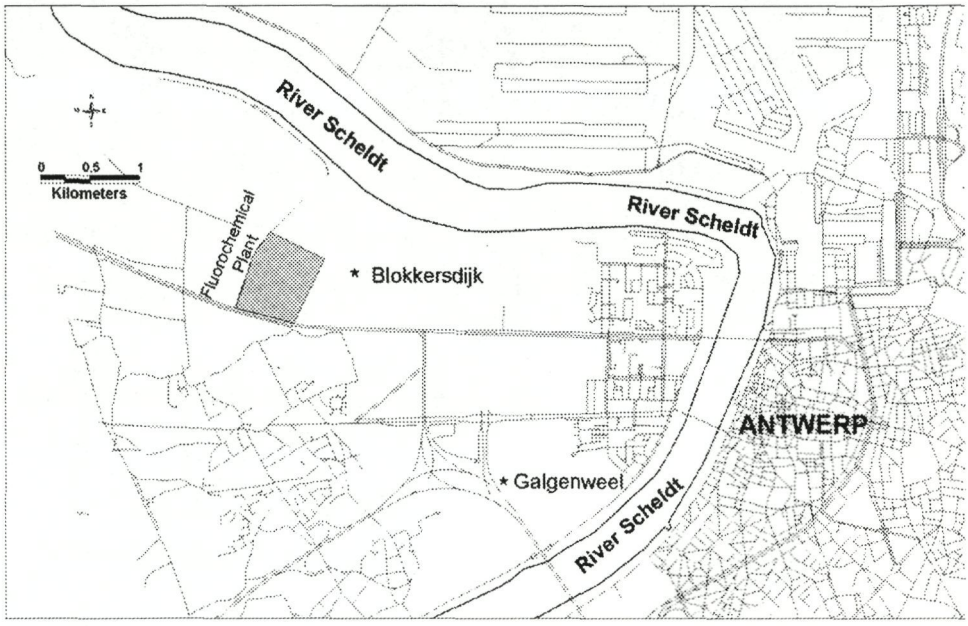

Fig. 1. Study area and sampling sites.

5.3.5 Determination of perfluorochemical concentrations

The liver concentrations of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorooctane sulfonic acid (PFOS), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUA) and perfluorododecanoic acid (PFDOA) were measured using combined liquid chromatography-mass spectrometry according to Giesy and Kannan (2001) using a CapLC system (Waters, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm i.d., Alltech, USA). The analysis was performed on a Betasil C18 column (50 mm x 1 mm i.d., Keystone Scientific, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH4OAc (A) / CH3OH (B). A

gradient elution was used starting at 10 % B and going to 90 % B in 8 min. At 10 min the initial conditions were resumed. PFOA, PFNA, PFOS, PFDA, PFUA and PFDOA were measured under (-) electrospray ionisation using the respective transitions 413→369, 463→419, 499→99, 513→469, 563→519 and 613→569. The internal standard (1H,1H,2H,2H-perfluorooctane sulfonic acid) was measured under the same conditions (427→81). The dwell time was 0.1 s. The ES-capillary voltage was set at –3.5 kV and the cone voltage was 24 V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10-5 mm Hg (Ar). The PFOS concentrations were calculated using an unextracted calibration curve. The standard deviations of replicate analyses were maximally 17 %. Limit of detection (LOD) values for PFNA, PFDA, PFUA and PFDOA were respectively 0.09, 0.05, 0.03 and 0.04 μg/g wet weight.

5.3.6 Statistical analysis

An ANOVA analysis was used in order to test for differences in liver PFOS concentrations, age structure and weight among the study populations. In second instance, we carried out a general linear model analysis (GLM) in SAS (1999) in order to determine whether gender and age influenced the PFOS concentrations. Sex, location and age and the interaction between latter variables were used as independent variables and the log transformed liver PFOS concentrations as dependent variable. We also tested whether there was a sex, location or age effect for the different biological and biochemical endpoints using general linear model analysis. The logarithmic transformed values of the biochemical

endpoints were considered as dependent variables, while sex, location and age and all interactions were considered as independent variables.

The relationship between the different biological and biochemical endpoints and the PFOS concentrations was investigated with linear mixed model analysis (LMM) in SAS (1999). The logarithmic transformed values of the biochemical endpoints were considered as dependent variables, while PFOS was used as independent variable. Location was treated as random effect in order to account for potential differences among sites. For the analysis of the relationship between PFOS and liver weight, we also considered body weight as covariate in the analysis. As graphical investigation suggested that the relationship between the hepatic PFOS concentration and the relative liver weight or the liver microsomal lipid peroxidation level were not linear, we fitted non-linear regression equations through these data using DATAFIT software (Oakdale Engineering, USA).

All statistical analyses, except for the non-linear regression analyses, were performed with the PROC MIXED module in SAS (1999). We used a stepwise backwards selection procedure in order to remove all insignificant interactions from the regression model, starting with the least significant terms. The need of the random terms was assessed with the Akaike information criterion. The degrees of freedom of the fixed effects F-test were adjusted for statistical dependence using Satterthwaite formulas. Variance components were estimated by restricted maximum likelihood.

5.4 Results

The perfluorooctane sulfonic acid (PFOS) liver concentrations at Blokkersdijk ranged from 0.47 to 178.55 μg/g wet weight, while those at Galgenweel ranged from 0.14 to 1.11 μg/g wet weight (Table 1). Mean PFOS liver concentrations at Blokkersdijk differed significantly from Galgenweel. The median and liver PFOS concentration was also higher at Blokkersdijk. Table 1 also shows that perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUA) and perfluorododecanoic acid (PFDOA) were sporadically detected in respectively 5 %, 10 %, 29 % and 38 % of the Blokkersdijk mice but not in the Galgenweel mice. The latter perfluorocarboxylates were generally present at detectable concentrations in those mice with the highest PFOS concentrations. Perfluorooctanoic acid (PFOA) was not detected above the detection limit (0.11 μg/g) in any of the mice.

Mean mice age did not differ significantly ($F_{1,34} = 0.36$, $p > 0.05$) between the Blokkersdijk (70 ± 36 days, mean ± SD) and the Galgenweel population (71 ± 22 days). It is therefore unlikely that the differences in PFOS liver concentration are due to a different age structure of the two populations. This is confirmed by the GLM analysis which shows that PFOS concentrations still differ between Blokkersdijk and Galgenweel when age and sex are included in the model (Table 2). This analysis also showed an age-related increase of PFOS liver concentration but no difference among sexes was observed. The interaction terms were not significant. Body weight did not differ significantly ($F_{1,34} = 0.59$, $p > 0.05$) between Blokkersdijk (14.1 ± 3.7 g) and Galgenweel mice (14.8 ± 3.3 g).

Table 1. Liver perfluorochemical concentrations (μg/g wet weight).

	number	PFOS	PFNA	PFDA	PFUA	PFDOA
Blokkersdijk	1	15.34	0.27	< LOD	< LOD	0.04
	2	4.94	< LOD	< LOD	< LOD	< LOD
	3	2.01	< LOD	< LOD	< LOD	< LOD
	4	1.55	< LOD	< LOD	< LOD	< LOD
	5	7.71	< LOD	< LOD	< LOD	0.04
	6	52.65	< LOD	< LOD	0.15	0.10
	7	0.47	< LOD	< LOD	< LOD	< LOD
	8	2.35	< LOD	< LOD	< LOD	< LOD
	9	2.89	< LOD	< LOD	< LOD	< LOD
	10	56.78	< LOD	< LOD	0.07	0.21
	11	45.66	< LOD	0.13	0.06	0.22
	12	98.41	< LOD	< LOD	0.04	0.11
	13	1.73	< LOD	< LOD	< LOD	< LOD
	14	2.10	< LOD	< LOD	< LOD	< LOD
	15	9.19	< LOD	< LOD	< LOD	< LOD
	16	25.25	< LOD	0.19	0.08	0.09
	17	28.07	< LOD	< LOD	0.08	0.10
	18	4.71	< LOD	< LOD	< LOD	< LOD
	19	5.06	< LOD	< LOD	< LOD	< LOD
	20	4.30	< LOD	< LOD	< LOD	< LOD
	21	178.55	< LOD	< LOD	< LOD	< LOD
	minimum	0.47	< LOD	< LOD	< LOD	< LOD
	maximum	178.55	0.27	0.19	0.15	0.22
	median	5.06				
	mean* ± SD	26.18 ± 43.12				
Galgenweel	22	0.37	< LOD	< LOD	< LOD	< LOD
	23	0.19	< LOD	< LOD	< LOD	< LOD
	24	0.42	< LOD	< LOD	< LOD	< LOD
	25	0.14	< LOD	< LOD	< LOD	< LOD
	26	0.15	< LOD	< LOD	< LOD	< LOD
	27	1.11	< LOD	< LOD	< LOD	< LOD
	28	0.28	< LOD	< LOD	< LOD	< LOD
	29	0.36	< LOD	< LOD	< LOD	< LOD
	30	0.87	< LOD	< LOD	< LOD	< LOD
	31	0.24	< LOD	< LOD	< LOD	< LOD
	32	0.23	< LOD	< LOD	< LOD	< LOD
	33	0.47	< LOD	< LOD	< LOD	< LOD
	34	0.23	< LOD	< LOD	< LOD	< LOD
	35	0.19	< LOD	< LOD	< LOD	< LOD
	36	0.22	< LOD	< LOD	< LOD	< LOD
	37	0.24	< LOD	< LOD	< LOD	< LOD
	38	0.36	< LOD	< LOD	< LOD	< LOD
	39	0.30	< LOD	< LOD	< LOD	< LOD
	40	0.39	< LOD	< LOD	< LOD	< LOD
	41	0.23	< LOD	< LOD	< LOD	< LOD
	42	0.30	< LOD	< LOD	< LOD	< LOD
	minimum	0.14	< LOD	< LOD	< LOD	< LOD
	maximum	1.11	< LOD	< LOD	< LOD	< LOD
	median	0.28				
	mean* ± SD	0.35 ± 0.23				

*significantly difference between Blokkersdijk and Galgenweel ($p < 0.001$).

Table 2. General linear model analyses of the effects of sex, location, and age on the liver PFOS concentration (μg/g wet weight) in wood mice.

variables	F-value	df	*p*-value
location	100.94	1, 32	< 0.001
sex	0.03	1, 32	NS
age	8.74	1, 32	0.006

NS = not significant. All two-way and the three-way interaction terms were insignificant and were removed from the model.

The relative liver weight, the mitochondrial fraction protein content, the peroxisomal β-oxidation activity and the microsomal lipid peroxidation level were significantly higher at Blokkersdijk than at Galgenweel (Table 3). The liver weight was positively related to body weight and was higher at Blokkersdijk (0.77 ± 0.23 g) than at Galgenweel (0.69 ± 0.16 g) (Location: $F_{1,33} = 11.17$, $p = 0.002$; Body weight: $F_{1,33} = 139.66$; $p < 0.001$).

The mitochondrial fraction catalase activity showed no significant location effect. The mitochondrial fraction protein content and the liver lipid peroxidation level were shown to be sex-dependent (Table 3). Both endpoints were higher in females than in males, but the difference among the liver lipid peroxidation levels of both sexes was larger at Blokkersdijk than at Galgenweel as indicated by the significant interaction between sex and location. None of the latter endpoints was related to age. The relative liver weight and the liver microsomal lipid peroxidation level showed a significant relationship with the liver PFOS concentration (Figure 2 and

3, Table 3). Liver weight, with body weight as covariate, was also positively related to liver PFOS concentration (PFOS: $F_{1,32.7} = 4.98$, $p = 0.033$; Body weight: $F_{1,32.5} = 127.36$, $p < 0.001$; Log_{10} Liver weight = 2.4287 + 0.000559 * PFOS + 0.02821 * Body weight).

The values of the different serum endpoints did not differ among locations or sexes. The serum triglyceride concentration was positively related to the age (Table 4). For the serum endpoints, a significant positive relation with the liver PFOS concentration could be shown for the triglyceride concentration and a significant negative relation for the alanine aminotransferase activity (Table 4).

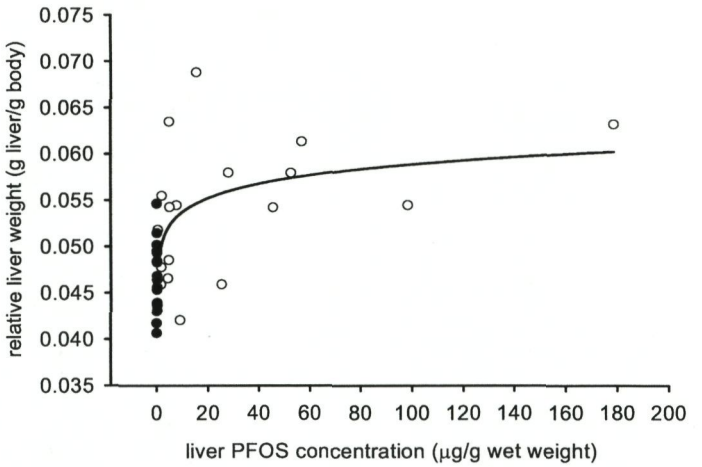

Fig. 2. Regression curve describing the relationship between the relative liver weight and the liver PFOS concentration.

The black and white dots represent the Blokkersdijk and Galgenweel mice, respectively. The regression equation is $y = 0.04901 * x^{0.03982}$ where "y" represents the relative liver weight in g/g wet weight and "x" the hepatic PFOS concentration in µg/g wet weight ($R^2 = 0.42$, $p < 0.001$).

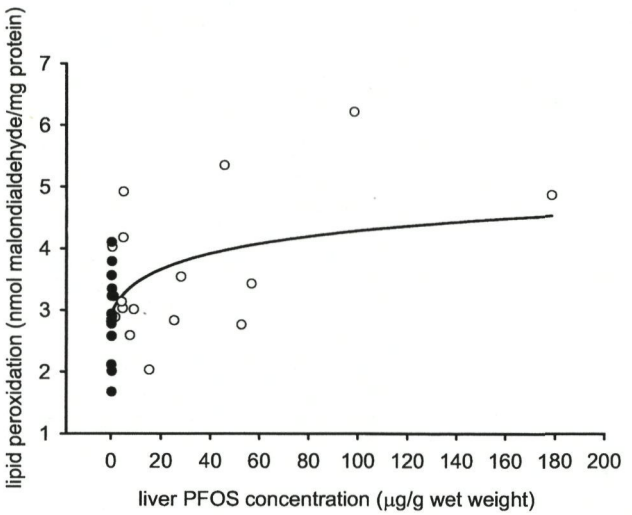

Fig. 3. Regression curve describing the relationship between the liver microsomal lipid peroxidation level and the liver PFOS concentration.

The black and white dots represent the Blokkersdijk and Galgenweel mice, respectively. The regression equitation is $y = \log(19.5489 + 0.7360 * x)$ where "y" represents the liver microsomal lipid peroxidation level in nmol malondialdehyde/mg protein and "x" the hepatic PFOS concentration in µg/g wet weight ($R^2 = 0.30$, $p < 0.001$).

Table 3. Liver endpoints (mean ± SD) for the different study sites.

	relative liver weight (mg liver/g body)	mitochondrial fraction protein content (mg protein/g liver)	peroxisomal β-oxidation activity (10^{-4} nmol H_2O_2/mg protein x min)	mitochond. fraction catalase activity (nmol H_2O_2/mg protein x min)	microsomal lipid peroxidation (nmol malondialdehyde/mg protein)
Blokkersdijk					
total	54 ± 7	2.27 ± 0.80	93.42 ± 35.06	10.09 ± 3.78	3.68 ± 1.15
males	53 ± 8	2.07 ± 0.64	92.69 ± 40.30	10.29 ± 4.01	3.03 ± 0.52
females	56 ± 7	2.66 ± 1.01	94.88 ± 24.55	9.63 ± 3.61	5.11 ± 0.75
Galgenweel					
total	46 ± 4	1.76 ± 0.95	58.76 ± 23.23	8.08 ± 2.57	2.91 ± 0.62
males	46 ± 3	1.45 ± 0.70	63.97 ± 21.66	9.12 ± 2.16	2.82 ± 0.72
females	48 ± 5	2.34 ± 1.14	49.21 ± 24.87	6.68 ± 2.56	3.03 ± 0.47
GLM					
location	$F_{1,32}$ = 16.41***	$F_{1,31}$ = 5.77*	$F_{1,31}$ = 11.67**	$F_{1,26}$ = 2.10	$F_{1,28}$ = 15.56***
sex	$F_{1,32}$ = 1.13	$F_{1,31}$ = 5.14*	$F_{1,31}$ = 0.84	$F_{1,26}$ = 2.94	$F_{1,28}$ = 13.80***
age	$F_{1,32}$ = 0.00	$F_{1,31}$ = 0.12	$F_{1,31}$ = 0.56	$F_{1,26}$ = 2.23	$F_{1,28}$ = 0.13
location x sex					$F_{1,28}$ = 6.56*
LMM					
statistics model	$F_{1,33.9}$ = 5.46* $Log_{10}Y$ = 1.6895 + 0.000554 x PFOS	$F_{1,31.8}$ = 0.13	$F_{1,33.6}$ = 0.14	$F_{1,23.6}$ = 0.36	$F_{1,16.3}$ = 8.43** $Log_{10}Y$ = 0.4728 + 0.001578 x PFOS

GLM = General linear model analysis of the effect of sex, location and age on the liver endpoints. LMM = Linear mixed model analysis of the relationship between the liver PFOS concentration (μg/g wet weight) and the liver endpoints. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4. Serum endpoints (mean ± SD) for the different study sites.

	triglyceride (mg/dl)	cholesterol (mg/dl)	potassium (mmol/l)	ALT (U/g protein)	ALT (U/dl)
Blokkersdijk					
total	106 ± 76	132 ± 30	4.14 ± 0.97	1.93 ± 0.66	12.5 ± 4.5
males	85 ± 52	139 ± 42	4.12 ± 0.65	1.80 ± 0.46	11.6 ± 3.1
females	148 ± 105	151 ± 17	4.17 ± 1.42	2.15 ± 0.94	14.0 ± 6.5
Galgenweel					
total	102 ± 55	143 ± 35	4.13 ± 0.65	2.21 ± 0.64	14.2 ± 3.8
males	108 ± 65	124 ± 28	4.17 ± 0.69	2.21 ± 0.64	14.1 ± 4.1
females	92 ± 33	145 ± 31	4.07 ± 0.66	2.21 ± 0.68	14.3 ± 3.6
GLM					
location	$F_{1,28} = 0.23$	$F_{1,31} = 1.13$	$F_{1,29} = 0.00$	$F_{1,30} = 2.37$	$F_{1,30} = 2.44$
sex	$F_{1,28} = 0.00$	$F_{1,31} = 1.44$	$F_{1,29} = 0.00$	$F_{1,30} = 0.01$	$F_{1,30} = 0.00$
age	$F_{1,32} = 6.47$*	$F_{1,31} = 2.50$	$F_{1,29} = 0.84$	$F_{1,30} = 3.00$	$F_{1,30} = 3.83$
LMM					
statistics model	$F_{1,25.4} = 6.58$* $Log_{10}Y = 1.9102 + 0.002683$ x PFOS	$F_{1,33.0} = 2.69$	$F_{1,31.0} = 2.06$	$F_{1,32.0} = 3.65$	$F_{1,32.0} = 4.71$* $Log_{10}Y = 1.1257 - 0.00139$ x PFOS

GLM = General linear model analysis of the effect of sex, location and age on the serum endpoints. LMM = Linear mixed model analysis of the relationship between the liver PFOS concentration (µg/g wet weight) and the serum endpoints. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5.5 Discussion

The assessment of the liver PFOS concentrations showed that the mean and median liver PFOS concentrations in the Blokkersdijk mice (26.18 µg/g and 5.06 µg/g wet weight, respectively) exceed the maximum liver PFOS concentration reported in wildlife so far (3.68 µg/g wet weight in mink liver, Giesy and Kannan, 2001). The liver PFOS concentration range in Blokkersdijk mice (0.47 to 178.55 µg/g wet weight) has a higher minimum and maximum than serum PFOS concentration ranges which have been reported for fluorochemical production employees in Decatur (AL, USA) and Antwerp (Belgium) which were 0.06-10.06 µg/ml and 0.04-6.24 µg/ml, respectively (Olsen et al., 2003a). A similar liver concentration range (2.00 to 72.9 µg PFOS/g wet weight) has also been reported in fish following an accidental release of 22000 l fire retardant foam into a nearby creek (Moody et al., 2002). This suggests that the Blokkersdijk mice were also most probably subjected to massive perfluorochemical exposure by the fluorochemical plant nearby. PFOS was not the only perfluorinated contaminant present in the liver tissue of the Blokkersdijk mice since PFNA, PFDA, PFUA and PFDOA could also be detected, although only sporadically. The perfluorocarboxylates were generally present at lower concentrations than PFOS supporting the observation that PFOS is usually the perfluorochemical that is present at the highest concentrations in animal tissues (Kannan et al., 2002a, 2002b, 2002d; Moody et al., 2002) probably because it might be a breakdown product of several perfluorochemicals (Canadian Environmental Protection Act, 1999). The perfluorocarboxylates could have the same origin as PFOS since they tend to be present in the mice with the highest PFOS concentrations. At Galgenweel, the measured

perfluorocarboxylates were present at levels below the detection limit suggesting a lower degree of perfluorinated pollution at this location. Also the liver PFOS concentrations at Galgenweel were lower (0.14 to 1.11 μg/g wet weight) than at Blokkersdijk (0.47 to 178.55 μg/g wet weight) suggesting that Blokkersdijk is a hot spot area for perfluorinated pollution. At present it is difficult to evaluate or to comment on the PFOS pollution degree at Galgenweel due to a lack of reports on background PFOS tissue concentrations in wildlife rodent species in Belgium. Interestingly, a liver tissue PFOS pollution gradient was recently found in bib (*Trisopterus luscus*) and plaice (*Pleuronectes platessa*) in the Western Scheldt (Hoff et al., 2003a) confirming the hypothesis that a point source such as this fluorochemical plant might be an important source of PFOS (precursor) release in the environment.

The age-dependence of the liver PFOS level indicates that PFOS bioaccumulates with age in mouse liver tissue. This could correspond with biomonitoring studies assessing the liver PFOS level in polar bears in which a trend for higher PFOS concentrations has been found in adults compared to subadults ($p = 0.07$) (Kannan et al., 2001b). Also in bald eagles less PFOS was generally found in plasma of nestlings than in adult liver tissue (Kannan et al., 2001a). The results presented do not corroborate with earlier reports on PFOS concentrations in water bird liver (Kannan et al., 2002a), river otter liver (Kannan et al., 2002d) and blood or liver of ringed and gray seals (Kannan et al., 2001b, 2002b) since no age-specific trends or differences in PFOS tissue concentrations were found in these species. The youngest animal captured at Blokkersdijk was 20 days old and had a liver PFOS concentration of 52.65 μg/g wet weight (Nr. 6 in Table 1). Since the lactation duration for wood mice is 18-22 days (Corbet and Harris, 1991) it is suggested that

PFOS could be transferred from the dam to the pup during pregnancy and/or lactation.

In the present study, no significant differences in liver PFOS concentration among sexes were found what is concurrent with earlier data on the liver or plasma PFOS concentrations for several other species (Kannan et al., 2001a, 2001b, 2002a, 2002d). In male gray seals, however, the liver and blood PFOS concentrations were found to be significantly higher than in females (Kannan et al., 2001b, 2002b). Under controlled laboratory conditions sex-related differences in hepatic elimination rate in rats were reported for PFOA (Vanden Heuvel et al., 1991a) and PFDA (Vanden Heuvel et al., 1991b). Also, hepatic PFOA and PFNA concentrations are markedly higher in exposed male than in female rats (Kudo et al., 2000). The present report, however, does not suggest a gender-associated difference in liver PFOS concentrations. Therefore, it might be possible that sex-specific perfluorochemical tissue elimination rates are only confined to some perfluorochemicals and/or to specific rodent species such as the rat.

The observed significant positive relation between the liver PFOS content and the relative liver weight corroborates earlier work on mice orally exposed to PFOS under laboratory conditions as this exposure increased the relative liver weight significantly (Sohlenius et al., 1993).
In the rat, the microsomal NADPH-dependent lipid peroxidation increased after PFOA exposure (Kawashima et al., 1994). This is consistent with the findings in the present study, suggesting an increased oxidative stress level following PFOS exposure of wood mice in the field. It should be noted, however, that the lipid peroxidation level in the mitochondrial fraction of mice was shown to decrease in mice exposed to

PFOA under laboratory conditions (Permadi et al., 1992) but this could be due to differences in response between the mitochondrial and the microsomal fraction.

The lack of any significant predictability of the liver PFOS content towards the peroxisomal β-oxidation activity, the mitochondrial catalase activity and the total protein content in the mitochondrial fraction stands in contrast with the results of previous laboratory studies in mice (Permadi et al., 1993; Sohlenius et al., 1993). It is possible that species sensitivity differences or differences in exposure regime between these laboratory studies could (partially) explain the discrepancies between the results obtained in the field and the laboratory. Also, other perfluorinated pollutants might affect the investigated liver endpoints. The presence of PFNA, PFDA, PFUA and PFDOA clearly shows that other perfluorinated compounds than PFOS are present in the Blokkersdijk mouse liver tissues possibly affecting the liver biochemistry and contributing to the elevated values for the liver endpoints observed at Blokkersdijk. Moreover, Blokkersdijk -although a nature reserve- is situated at the border of a heavily industrialized area underpinning the possibility that the mice might also be contaminated with other (non-perfluorinated) compounds contributing to the observed location-dependence of the liver endpoints (except for the mitochondrial fraction catalase activity).

It has been shown that PFOS exposure drastically decreases the serum triglyceride level in mice exposed in the laboratory (Haughom and Spydevold, 1992). The present results, however, show a significant positive relationship between the liver PFOS concentration and the serum triglyceride concentration. However, the latter relation does probably not reflect an exposure-effect relationship but most likely results from the fact that both the liver PFOS concentration and the serum triglyceride concentration increase with age. This is illustrated by the positive

relationship between the liver PFOS concentration and the age and the positive relationship between the serum triglyceride level and the age.

Perfluorochemical concentrations required to induce triglyceride accumulation in the liver of rats are much higher than those inducing the liver peroxisomal β-oxidation (Kudo and Kawashima, 2003). Haughom and Spydevold (1992) showed that a perfluorochemical-mediated decrease in serum triglyceride concentration can be paralleled by an increase in hepatic triglyceride content in the rat. Taken together this illustrates that the perfluorochemical threshold concentrations in rodents might be different for the serum triglyceride lowering effect than for the induction of hepatic β-oxidation. This difference in threshold could be an explanation for the significantly increased peroxisomal β-oxidation activity and the lack of a serum triglyceride decrease at Blokkersdijk compared to Galgenweel.

As suggested by the absence of a significant relation between the serum cholesterol and the liver PFOS concentration, no indications for a PFOS-mediated serum cholesterol lowering effect were found.

The serum alanine aminotransferase (ALT) activity was shown to have a significant negative relationship with the liver PFOS concentration in the present study. This contrasts with a rat study in which PFOS exposure resulted in increased plasma and serum transaminase levels (Goldenthal et al., 1978c). The reason for this discrepancy is at present not clear but it is possible that differences in exposure route and/or animal species might account for this observation. Moreover, undefined confounding factors in the present study might influence the relation between the liver PFOS concentration and the serum ALT activity.

Since no significant relation between the potassium level and the liver PFOS level was observed PFOS did probably not induce hyperkalemia in the mice, a phenomenon that has been reported for a moribund

cynomolgus monkey chronically exposed to PFOS (Seacat et al., 2002) and that is used as a marker for renal failure (Vricella et al., 1992).

Recently, the liver PFOS critical toxicity value (based on a rat study in which histopathological effects were seen in the liver) was set at 40.8 μg PFOS/g liver (Canadian Environmental Protection Act, 1999). The derived environmental toxicity value for mammals taking into account laboratory to field extrapolation, within- and between species variability, PFOS' bioaccumulative and persistent properties, and accounting for the chronic exposure conditions was defined to be 0.0408 μg/g. In the present study, the theoretical risk quotients for wood mice, (defined as the ratio of their hepatic PFOS concentration and the environmental toxicity value) exceed the value of 1 for all wood mice analysed, suggesting that there might be a toxicological risk associated with PFOS exposure in these animals. The possibility that PFOS-related toxicological effects might have occurred in the studied wood mice is supported by the observed PFOS exposure related biomarker effects found in the present study.

5.6 Conclusion

This study shows that wood mice living in the proximity of a fluorochemical plant in Antwerp are heavily contaminated with PFOS and to a lesser extent with perfluorocarboxylates. This study also suggests that hepatic PFOS bioaccumulation is age-dependent and that maternal PFOS transfer to the young during pregnancy and/or lactation might occur. Among the liver endpoints, the relative liver weight and the microsomal lipid peroxidation level, indicative for oxidative stress, are most apparently related to the liver PFOS concentration. The serum ALT activity was the only measured serum endpoint suggested to be

significantly affected by PFOS exposure. Further study is, however, required, to understand the possible adverse impact of these observed biochemical alterations at a higher level of biological organization.

Chapter 6

Evaluation of biochemical and organismal effects related to perfluorooctane sulfonic acid exposure in organohalogen-contaminated great tit (*Parus major*) and blue tit (*Parus caeruleus*) nestlings.

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Chemosphere, in press

6.1 Abstract

A perfluorooctane sulfonic acid (PFOS) biomonitoring survey was conducted on great tit (*Parus major*) and blue tit (*Parus caeruleus*) nestlings from Blokkersdijk, a bird reserve in the proximity of a fluorochemical plant in Antwerp (Belgium) and Fort IV, a control area. PFOS, together with 11 organochlorine pesticides, 20 polychlorinated biphenyl congeners and 7 polybrominated diphenyl ethers were measured in liver tissue. The hepatic PFOS concentrations at Blokkersdijk (86-2788 and 317-3322 ng/g wet weight (ww) for great and blue tit, respectively) were among the highest ever measured and were significantly higher than at the control area (17-206 and 69-514 ng/g ww for great and blue tit, respectively). The hepatic PFOS concentration was species- and sex-independent and correlated significantly and positively with the serum alanine aminotransferase activity and negatively with the serum cholesterol and triglyceride levels in both species but did not correlate with condition or serum protein concentration. In the great tit, a significant positive correlation was observed between the liver PFOS concentration and the relative liver weight. In the blue tit, the hepatic PFOS concentration correlated positively and significantly with hematocrite values. None of the investigated organohalogen pollutants except for PFOS were suggested to be involved in the observed biological alterations.

6.2 Introduction

PFOS is a perfluorooctanesulfonylfluoride (POSF) based chemical that is mainly produced by the 3M Company using electrochemical fluorination of octanesulfonyl fluoride. In 2000, the volume of PFOS and its salts that

were commercialized as finished products was less than 91 metric tons. PFOS is also used as a chemical intermediate for the production of PFOS-based chemicals. These chemicals can be divided in three main classes: products for surface treatment applications such as textile, leather and carpet production and aftermarket treatment, products for paper protection for food and non-food applications and performance chemicals such as fire fighting foams, insecticides and floor polishes. The estimated global production volume of these PFOS-based chemicals was 4481 metric tons in 2000 although production volumes are expected to decline because 3M announced phasing out the production of POSF-derived chemicals in May 2000 (OECD, 2002).

PFOS is a widespread environmental contaminant that is found in a great diversity of wildlife species with more elevated tissue concentrations in populated and industrialized areas (Giesy and Kannan, 2001). PFOS is generally measured at concentrations < 1000 ng/g in tissues from a great diversity of aquatic birds species from Japan, Korea, Canada, the USA, the Northern Pacific region and Europe although PFOS concentrations up to 2570 ng/ml (bald eagle plasma, USA) have been reported (Giesy and Kannan, 2001; Kannan et al., 2001a, 2002a, 2002b; Rattner et al., 2004).

Hoff et al. (2004) measured extremely elevated hepatic PFOS concentrations up to 178550 ng/g wet weight (ww) in wood mice (*Apodemus sylvaticus*) from Blokkersdijk, in the proximity of a fluorochemical production unit (Antwerp, Belgium). Due to the PFOS contamination degree of the wood mice and to the status of Blokkersdijk as an area protected by the European Council directive 79/409/EEC on the conservation of wild birds (1979), we assessed liver PFOS concentrations in great tit (*Parus major*) and blue tit (*Parus caeruleus*)

nestlings from Blokkersdijk and Fort IV, a park presumed to be a reference area for PFOS contamination. Birds in their pre-fledging stage were chosen because they provide clear advantages for local biomonitoring: their mobility is low in contradiction to adult tits which are basically resident but can make eruptive movements over a distance of several hundreds of kilometers and generally return early in spring to establish breeding territories. The food origin of tit nestlings is another factor contributing to the suitability of tit nestlings as biomonitors because their food, predominantly consisting of lepidopterous larvae, is generally captured by the parents within territorial boundaries in which the parents are foraging (ca. 0.5 ha in deciduous habitats, Cramp and Perrins, 1993). Also, very young wood mice from Blokkersdijk have considerably elevated hepatic PFOS concentrations (Hoff et al., 2004) showing that young animals can be good indicators of PFOS contamination. Common PFOS exposure routes for wood mice and tit nestlings are not excluded because next to plant material, lepidopterous larvae are also food items for wood mice (Corbet and Harris, 1991).

Known *in vivo* effects of PFOS are increased relative liver weight, induced peroxisomal liver fatty acid β-oxidation and lowered serum cholesterol and triglyceride concentrations (Haughom and Spydevold, 1992; Ikeda et al., 1987; Seacat et al., 2002, 2003; Sohlenius et al., 1993). PFOS exposure also increases the serum alanine aminotransferase (ALT) activity, which is a marker for hepatic damage (Hoff et al., 2003b; Seacat et al., 2003). Other *in vivo* effects are the inhibition of gap junction intercellular communication (Hu et al., 2002), the induction of carboxylesterase expression (Derbel et al., 1996), neuroendocrine effects (Austin et al., 2003) and the occurrence of developmental effects (Lau et al., 2004; Thibodeaux et al., 2003).

In order to evaluate some of these effects for PFOS-contaminated tit nestlings, relationships were evaluated between the hepatic PFOS concentration and the following selected endpoints: relative liver weight, serum cholesterol, triglyceride levels and ALT activity. Also body condition, serum protein concentration and hematocrite were assessed because previous PFOS investigations have demonstrated significant correlations between hepatic PFOS levels and the latter two endpoints (Hoff et al., 2005).

In addition to PFOS, 11 organochlorine pesticides (OCPs), 20 polychlorinated biphenyls (PCBs) and 7 polybrominated biphenyl ethers (PBDEs) were measured in liver tissue for two reasons. Firstly, tits have been demonstrated to be contaminated with organochlorine compounds in the Antwerp region (Dauwe et al., 2003). Secondly, laboratory controlled experiments show that organochlorine contamination of juvenile birds can potentially affect a considerable part of the endpoints under consideration in this study. In American kestrel (*Falco sparverius*) nestlings for example, PCB exposure has been shown to induce the liver weight and the serum ALT activity (Hoffman et al., 1996). In PCB fed broiler chicks, the liver weight, serum cholesterol and triglyceride concentrations were increased while the hematocrite was decreased (Kosutzky et al., 1993; Kosutzky and Skrobanek, 1994). Also OCPs, such as hexachlorobenzene and chlordane can potentially alter the liver weight, the serum ALT activity and the serum cholesterol concentration as has been shown in rodents (Almeida et al., 1997; Khasawinah and Grutsch, 1989). In humans, serum cholesterol concentrations have been demonstrated to be significantly and positively correlated with tissue DDE concentrations (Laden et al., 1999).

6.3 Materials and methods

6.3.1 Sampling

Between May 11 and 24, 2004, 48 great tit and 33 blue tit pre-fledging nestlings (17-20 days old) were collected. Great tit nestlings were collected from nest boxes (M1-4) in the nature reserve Blokkersdijk (Antwerp, Belgium), an artificial sand dune habitat with mainly willow and poplar groves situated next to a fluorochemical plant production site and from three nest boxes (M5-7) in Fort IV (Mortsel, Belgium), a park with loam soil and groves with deciduous trees (mainly beech) 10 kilometers south-east. Blue tit nestlings were taken from two boxes in Blokkersdijk (C1-2) and two in Fort IV (C3-4, Fig. 1).

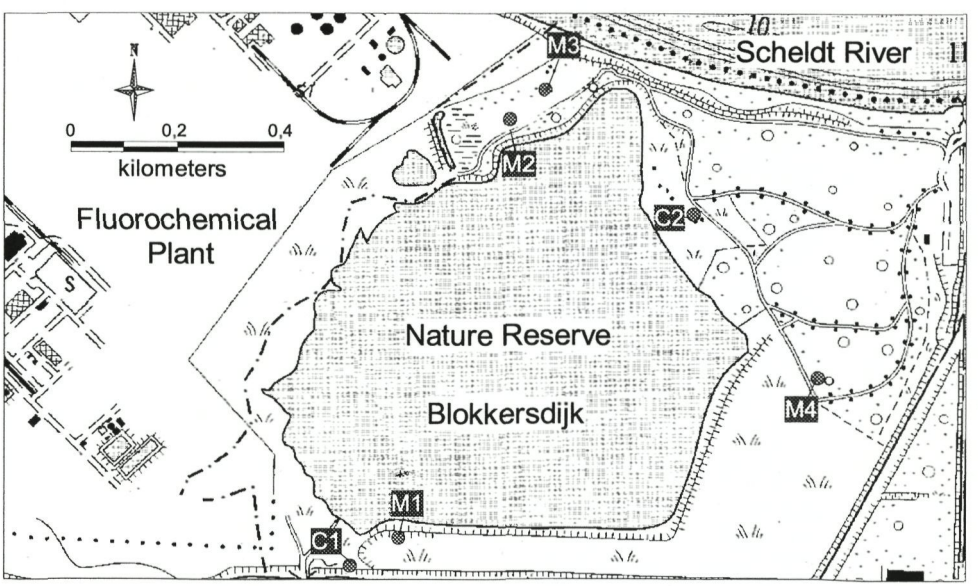


Fig. 1. Area of study and sampling locations.

The Blokkersdijk nest boxes are indicated with black squares. Great tit nest boxes are M1-4, blue tit nest boxes are C1-2.

In Blokkersdijk, nest boxes were closely situated to a pond. In Fort IV, the sampled nest boxes were situated within a radius of about 0.5 km. All nest boxes were located in groves with deciduous trees (willow, beech, poplar). The boxes were installed at least six months before the onset of the breeding season. Before decapitation, the birds were weighed with an accuracy of 0.1g with a Pesola spring balance. After decapitation, blood was collected with glass capillaries and serum was prepared by centrifugation (4000 rpm, 5 min) and stored in liquid nitrogen. For the hematocrite measurement, blood was collected with heparinized capillaries. The liver was immediately excised, weighed and stored in liquid nitrogen. The tarsus length was determined with an accuracy of 0.1 mm with digital callipers. The condition index was calculated as the residual from a linear regression of the tarsus length and body weight and has been shown to predict survival probability relatively well (Merilä, 1997).

6.3.2 Sex determination

DNA was extracted from the shaft of the tail feathers using the Dneasy Tissue Kit (Qiagen, Venlo, The Netherlands). A PCR reaction amplifying the CHD-W and CHD-Z genes was carried out as described in Griffiths et al. (1998) with small modifications.

6.3.3 Biochemical assays

The serum alanine aminotransferase activity was determined by the spectrophotometric method of Bergmeyer et al. (1986). Cholesterol concentrations were measured according to Allain et al. (1974) and the triglyceride concentration was measured according to the method of

Spayd et al. (1978). The serum protein content was determined with the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). For the determination of the hematocrite, the relative red blood cell volume was determined after centrifugation of heparinized blood in sealed glass capillaries (2000 rpm, 5 min).

6.3.4 Determination of liver PFOS concentrations

PFOS extraction and the measurement of PFOS concentrations in liver tissue were done using combined high pressure liquid chromatography-mass spectrometry according to Giesy and Kannan (2001) with minor modifications as previously described by Van de Vijver et al. (2003a). In contradiction to Van de Vijver et al. (2003a), the internal standard (1H, 1H, 2H, 2H-perfluorooctane sulfonic acid) was added to the liver tissue before homogenization. The analytical procedure (amount of added internal standards, solvent volumes used) was adapted according to the available liver tissue masses. The liver tissue masses used for PFOS extraction ranged between 140 and 300 mg. High pressure liquid chromatography was done on a CapLC system (Waters, Milford, MA, USA) connected to a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK). Aliquots of 5 µl were loaded on an Optiguard C18 pre-column (10 mm x 1 mm inner diameter, Alltech, Deerfield, IL, USA). The analysis was performed on a Betasil C18 column (50 mm x 1 mm inner diameter, Keystone Scientific, San Jose, CA, USA) at a flow rate of 40 µl/min. The mobile phase was 2 mM NH_4OAc (A) / CH_3OH (B). A gradient elution was used starting at 45 % B and going to 90 % B in 3 min. After 5 min initial conditions were resumed. PFOS was measured under negative electrospray ionization using single reactant monitoring (m/z 499→99). The internal standard

(1H, 1H, 2H, 2H-perfluorooctane sulfonic acid), was measured under the same conditions (m/z 427→81). The dwell time was 0.1 s. The electrospray-capillary voltage was set at –3.5 kV and the cone voltage was 24V. The source temperature was 80°C. The pressure in the collision cell was 3.3 10^{-5} mm Hg (Ar). PFOS concentrations were calculated using an unextracted calibration curve that was constructed using a dilution series of PFOS in MeOH. Internal standard was added to these serial PFOS dilutions at the same concentration as added to the liver tissue samples. The PFOS concentrations were calculated using a linear calibration curve (R^2 = 0.99) of a plot of log[I(499→99)/I(427→81)] versus log[PFOS concentration] where "I" is the peak area. Repeatibility was 77 %.

Internal standard concentrations in unspiked liver samples were not above the limit of quantification in nestlings from Blokkersdijk or Fort IV (n = 5 for each location). The limit of quantification (LOQ) for PFOS was 10 ng/g ww.

6.3.5 Determination of liver concentrations of polychlorinated and polybrominated pollutants

The OCPs under investigation were α-, β-, γ- isomers of hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), oxychlordane (OxC), *trans*-nonachlor (TN), *trans*-(TC), *cis*-chlordane (CC), p,p′-dichlorodiphenyldichloroethylene (p,p′-DDE), p,p'-dichlorodiphenyldichloroethane (p,p′-DDD) and p,p'-dichlorodiphenyltrichloroethane (p,p′-DDT). The following PCB congeners (IUPAC numbers) were targeted: 28, 52, 74, 99, 101, 105, 110, 118, 128, 138, 149, 153, 156, 167, 170, 180, 183, 187, 194 and 199.

PBDE congeners 28, 47, 99, 100, 153, 154 and 183 were also included. The method used for sample preparation and analysis was described in detail by Dauwe et al. (2003) and Voorspoels et al. (2003). Briefly, the available amount of tissue (150–350 mg) was ground with Na_2SO_4, internal standards were added and the mixture was extracted for 2 h with 75 ml hexane:acetone = 3:1 into a hot Soxhlet manifold. After concentration, the extract was subjected to clean-up on acidified silica and analytes were eluted with 15 ml n-hexane followed by 10 ml dichloromethane. The eluate was concentrated to 80 µl and transferred to an injection vial. PCBs were determined on a HP 6890 gas chromatograph GC-5793 mass spectrometer (MS, Hewlett Packard, Palo Alto, CA, USA) operated in electron impact ionisation mode and equipped with a 30 m x 0.25 mm x 0.25 µm DB-1 capillary column (J&W Scientific, Folsom, CA, USA). PBDEs and OCPs were determined on a HP 6890 GC-MS operated in negative chemical ionisation and equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE Scientific, Zulte, Belgium). Instrumental operating conditions and quality control were detailed presented by Dauwe et al. (2003) and Voorspoels et al. (2003). LOQs for individual PCB congeners ranged between 0.5 and 1 ng/g ww, while for OCPs and PBDEs, they were 0.2 and 0.1 ng/g ww, respectively.

6.3.6 Statistical analysis

The liver PFOS concentrations in great and blue tit nestlings from Blokkersdijk and Fort IV were compared with the non-parametric Mann-Whitney U test. This test was also used for comparison of hepatic PFOS concentrations between sexes and species for Blokkersdijk and Fort IV. For each species, differences in liver PFOS concentration between boxes

were investigated using the non-parametric Kruskal-Wallis test with Dunn's test as post hoc criterion. The values of the biological endpoints investigated (serum ALT activity, cholesterol, triglyceride and protein concentrations, hematocrite and condition index) were compared between males and females for Blokkersdijk and Fort IV with the non-parametric Mann-Whitney U test. Partial Least Square (PLS) analysis models were constructed to establish relationships between the measured pollutants and the biological endpoints. The nestlings for which at least 60 and 56 % (for great tit and blue tit, respectively) of the measured organohalogens were < LOQ and the organohalogens for which the variance was close to zero or the concentration < LOQ were excluded for PLS analysis. Spearman rank correlation analysis was used to establish relationships between the liver PFOS concentration and the relative liver weight, the serum ALT activity, cholesterol, triglyceride, protein concentrations, hematocrite and condition index. Correlation analysis was also used to investigate the relationships between the other compounds and the latter endpoints if these compounds were quantifiable in > 50 % of the nestlings.

6.4 Results

The liver PFOS concentrations in great and blue tit liver were significantly higher in Blokkersdijk compared with Fort IV ($p < 0.001$ for both species). No significant sex or species differences in hepatic PFOS concentrations were observed in Blokkersdijk or Fort IV ($p > 0.05$). In fig. 2a and 2b it can be seen that the PFOS concentrations in nestlings from the same location were not significantly different within one species. However, the hepatic PFOS concentrations in nestlings from nestboxes at the eastside of the Blokkersdijk pond (boxes M4 and C2 for

great and blue tit, respectively) tended to be lower than at the westside (boxes M1-3 for great tit and box C1 for blue tit) of the pond although the differences were not statistically significant.

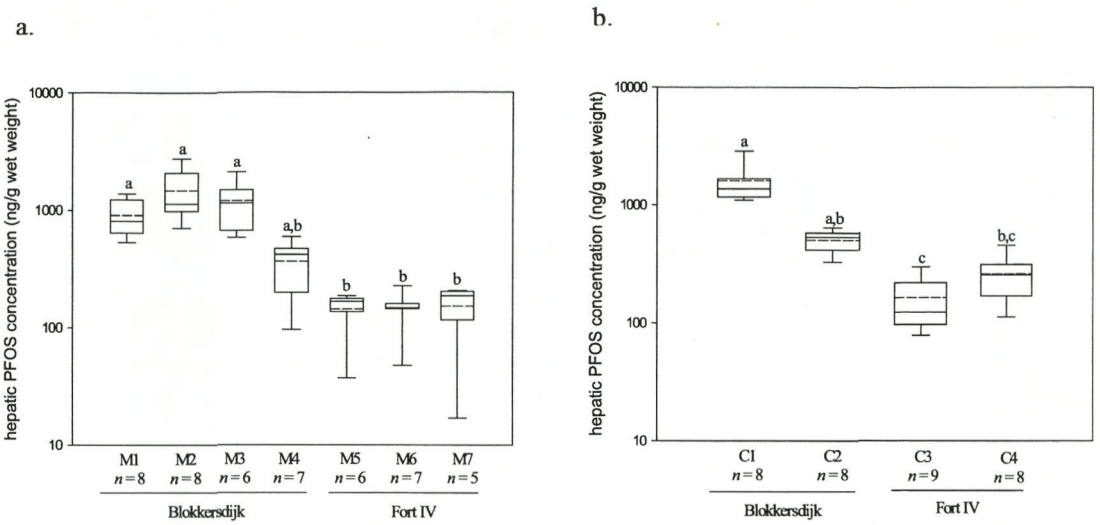


Fig. 2. Hepatic PFOS concentrations in great tit (a) and blue tit (b) nestlings.

The straight line is the median and the dotted line represents the mean. The 25th and 75th percentiles define the boxes. The whiskers represent the 10th and 90th percentiles. Boxes having different letters are significantly different ($p < 0.05$). n = number of nestlings.

The concentration ranges of measured organohalogen pollutants are shown in Table 1. PFOS was present in liver tissue of all nestlings measured at higher concentrations than the other organohalogens.

Table 1. Ranges and mean concentrations (in brackets) expressed in ng/g wet weight for organohalogens measured in liver of great and blue tits nestlings.

compound	great tit Blokkersdijk (n = 29-30)	great tit Fort IV (n = 18)	blue tit Blokkersdijk (n = 16)	blue tit Fort IV (n = 7-19)
PFOS	86-2788 (994)	17-206 (146)	317-3323 (1055)	69-514 (210)
α-HCH	< 0.2	< 0.2	< 0.2	< 0.2
β-HCH	< 0.2	< 0.2	< 0.2	< 0.2
γ-HCH	< 0.2	< 0.2	< 0.2	< 0.2
p,p′-DDE	2.7-9.1 (5.0)	<0.2-1.6	0.8-2.3 (1.7)	< 0.2-1.0
p,p′-DDD	< 0.2	< 0.2	< 0.2	< 0.2
p,p′-DDT	< 0.2	< 0.2	< 0.2	< 0.2
HCB	< 0.2-0.5	< 0.2-0.4	< 0.2	< 0.2-0.3
OxC	< 0.2	< 0.2	< 0.2	< 0.2
TN	< 0.2	< 0.2	< 0.2	< 0.2
TC	< 0.2	< 0.2	< 0.2	< 0.2
CC	< 0.2	< 0.2	< 0.2	< 0.2
PCB 28	< 1.0	< 1.0	< 1.0	< 1.0
PCB 52	< 1.0	< 1.0	< 1.0	< 1.0
PCB 74	< 1.0	< 1.0	< 1.0	< 1.0
PCB 99	1.3-5.1 (2.9)	< 1.0	< 1.0-1.5	< 1.0
PCB 101	1.4-5.0 (2.4)	< 1.0	< 1.0-2.3	< 1.0
PCB 105	< 1.0	< 1.0	< 1.0	< 1.0

PCB 110	1.2-5.5 (2.3)	< 1.0	< 1.0-1.5	< 1.0
PCB 118	1.4-13.9 (5.7)	< 1.0	1.9-6.3 (3.1)	< 1.0-2.9
PCB 128	0.9-3.2 (1.6)	< 0.5	< 0.5-0.9	< 0.5
PCB 138	6.0-35.1 (19.6)	0.8-2.6 (1.5)	5.2-13.2 (8.8)	0.8-2.4 (1.4)
PCB 149	1.5-8.8 (4.9)	< 0.5-1.3	1.4-2.8 (1.9)	< 0.5-0.9
PCB 153	6.7-38.5 (23.0)	0.8-2.8 (2.0)	6.2-15.8 (10.7)	0.9-2.6 (1.5)
PCB 156	< 0.5-2.1	< 0.5	< 0.5-0.9	< 0.5
PCB 167	< 0.5-1.5	< 0.5	< 0.5	< 0.5
PCB 170	1.3-8.4 (4.9)	< 0.5	< 0.5-2.5	< 0.5
PCB 180	4.0-23.7 (14.2)	< 0.5-1.4	2.7-9.3 (5.6)	< 0.5-1.6
PCB 183	1.5-3.0 (2.4)	< 0.5	< 0.5-1.3	< 0.5
PCB 187	2.1-10.7 (6.6)	< 0.5-0.7	1.6-3.6 (2.5)	< 0.5
PCB 194	< 0.5-2.6	< 0.5	< 0.5	< 0.5
PCB 199	< 0.5-2.6	< 0.5	< 0.5	< 0.5
PBDE 28	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 47	< 0.1-1.3	< 0.1-0.9	< 0.1-1.3	< 0.1-6.1
PBDE 99	< 0.1-1.1	< 0.1-0.6	< 0.1-0.5	< 0.1-4.6
PBDE 100	< 0.1-0.2	< 0.1	< 0.1	< 0.1-0.8
PBDE 153	< 0.1-0.2	< 0.1	< 0.1	< 0.1-0.2
PBDE 154	< 0.1	< 0.1	< 0.1	< 0.1-0.2
PBDE 183	< 0.1-0.2	< 0.1	< 0.1	< 0.1

n = number of nestlings

The values of all the biological endpoints did not differ significantly between sexes, neither in Blokkersdijk, nor in Fort IV ($p > 0.05$).

No significant correlations ($p > 0.05$) were found between the hepatic PFOS concentration and the serum protein content ($r = -0.13$, $p = 0.4$, $n = 45$; $r = -0.27$, $p = 0.2$, $n = 26$ for great and blue tits, respectively) or condition index ($r = 0.01$, $p = 0.9$, $n = 45$; $r = 0.02$, $p = 0.9$, $n = 30$ for great and blue tits, respectively). Both in great and blue tits, the liver

PFOS concentration correlated significantly with the serum ALT activity and the cholesterol and triglyceride levels. In the great but not in the blue tit, correlation analysis also showed significant correlations between the hepatic PFOS concentration and the relative liver weight. In blue but not in great tit nestlings, the liver PFOS concentration was positively and significantly related to hematocrite (Fig. 3 and 4).

PLS analysis of the relations between the hepatic pollutant concentrations and the biological endpoints did not result in robust models ($Q^2 < 0.10$ for both species).

No significant correlations were observed between biological endpoints and compounds other than PFOS.

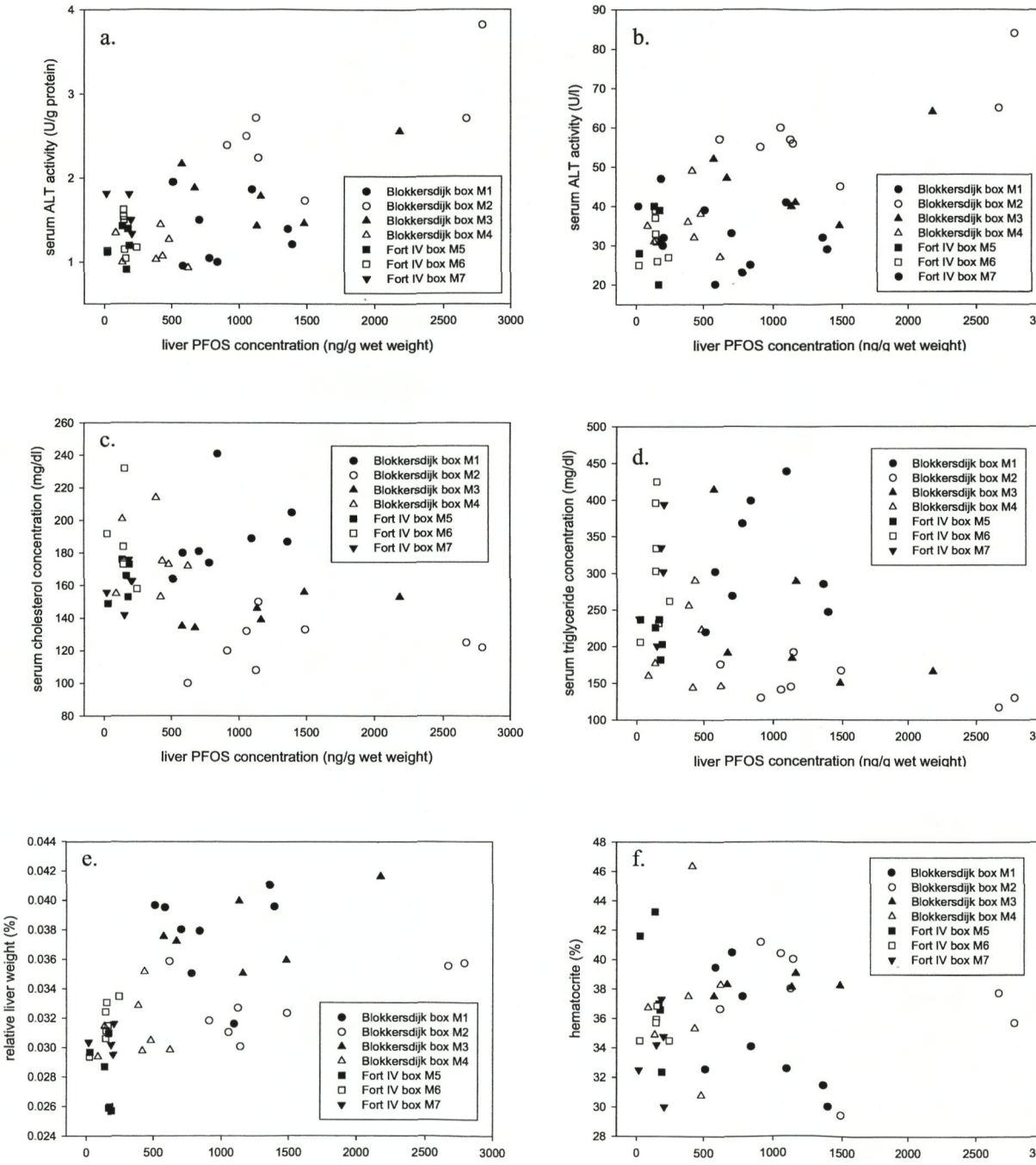
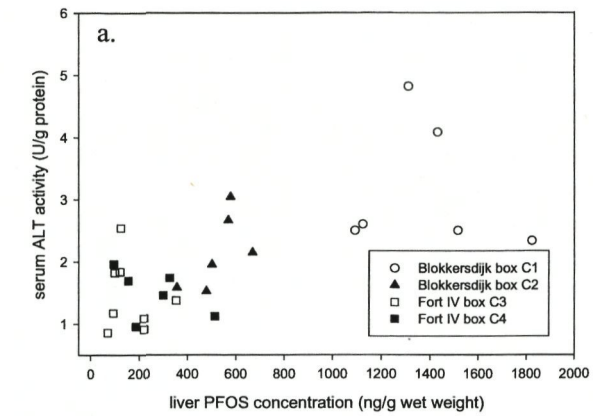
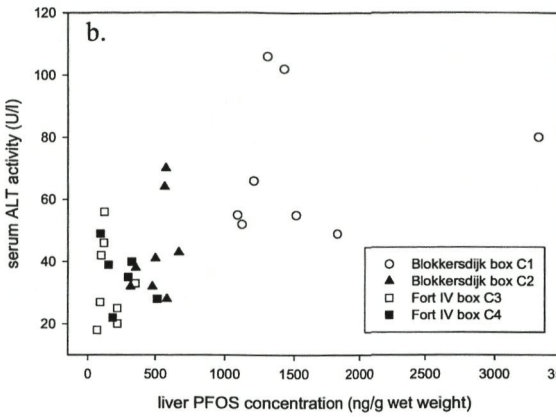
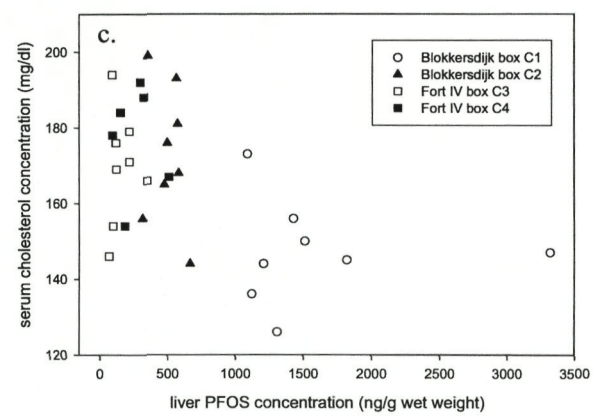


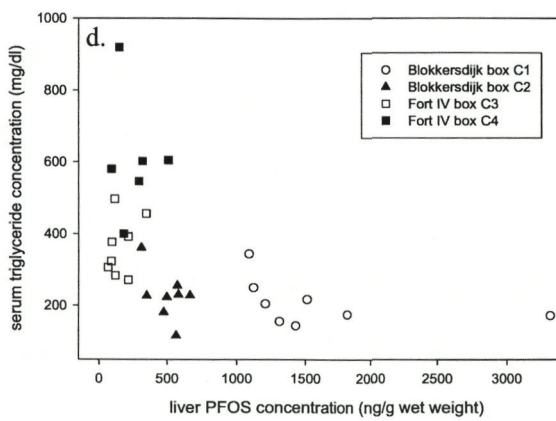
Fig. 3. The relationships between the great tit nestling liver PFOS concentration and the biological endpoints.

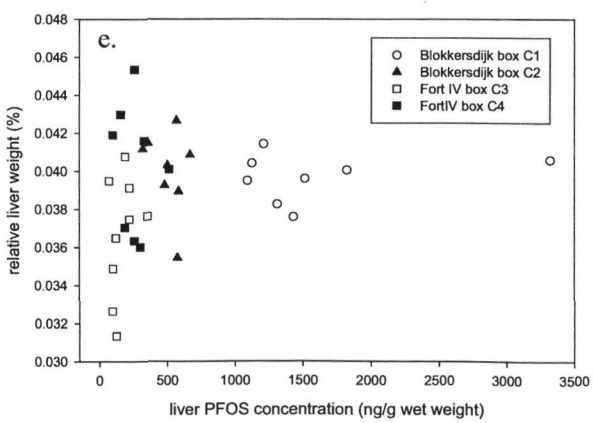
The relationship between the liver PFOS concentration and the serum ALT activity (a, $r = 0.45$, $p = 0.002$**, $n = 45$; b, $r = 0.37$, $p = 0.01$*, $n = 45$), serum cholesterol (c, $r = -0.34$, $p = 0.02$*, $n = 46$), serum triglyceride concentration (d, $r = -0.30$, $p = 0.04$*, $n = 46$), relative liver weight (e, $r = 0.44$, $p = 0.003$**, $n = 46$) and hematocrite (f, $r = 0.10$, $p = 0.5$, $n = 43$). r = correlation coefficient, p = p value, n = number of nestlings, Spearman rank correlation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.











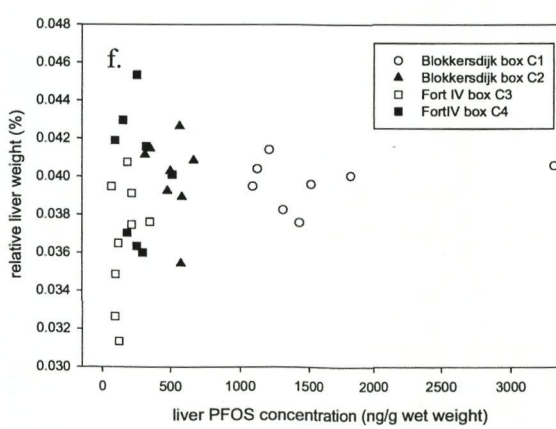


Fig. 4. The relationships between the blue tit nestling liver PFOS concentration and the biological endpoints.

The relationships between the liver PFOS concentration and the serum ALT activity (a, $r = 0.62$, $p = 0.0007$***, $n = 26$; b, $r = 0.61$, $p = 0.0004$***, $n = 30$), serum cholesterol (c, $r = -0.41$, $p = 0.03$*, $n = 30$), serum triglyceride concentration (d, $r = -0.69$, $p < 0.0001$***, $n = 30$), relative liver weight (e, $r = 0.22$, $p = 0.2$, $n = 33$) and hematocrite (f, $r = 0.39$, $p = 0.03$*, $n = 29$). r = correlation coefficient, p = p value, n = number of nestlings, Spearman rank correlation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

6.5 Discussion

The high PFOS concentrations found in the Blokkersdijk tits confirm that the Blokkersdijk nature reserve is severely PFOS polluted what is in agreement with PFOS measurements in Blokkersdijk wood mice and carps (*Cyprinus carpio*) in which the average liver PFOS concentrations were 2618 and 1241 ng/g ww, respectively (Hoff et al., 2004; Hoff et al., 2005).

The measured liver PFOS concentrations in the Blokkersdijk nestlings were significantly higher than in Fort IV. This suggests that the fluorochemical plant might be a source of PFOS release and/or PFOS precursor release in the environment as recent studies have shown that several perfluorinated compounds can be metabolised to PFOS. Xu et al. (2004) showed that N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide can undergo N-deethylation to N-(2-hydroxyethyl)perfluorooctanesulfonamide that can be deethylated to perfluorooctanesulfonamide (PFOSA). PFOSA can undergo metabolisation to PFOS in rat liver slices. In rainbow trout (*Oncorynchus mykiss*) microsomes it has been shown that N-ethylperfluorooctanesulfonamide can be converted to PFOS (Tomy et al., 2004). These precursors could partly be responsible for the presence of

PFOS in liver tissue of Fort IV nestlings because they are more volatile than PFOS. Considering the prevailing wind direction (west, southwest) and the geographical location of Fort IV (10 km southeast from the fluorochemical plant), the plant might be a direct PFOS pollution source for the Fort IV nestlings if airborne pollution is considered.

An indication that the liver PFOS concentration is decreasing rather steeply with increasing distance from the plant, is supported by the observations that nestlings from nestboxes at the eastern side of the Blokkersdijk pond (boxes M4 and C2 for great and blue tit, respectively) had lower hepatic PFOS concentrations than the boxes at the western side although the differences were not significant.

In Europe, PFOS has been measured in liver tissue from only two avian species: the common cormorant (*Phalacrocorax carbo*) from the Italian coast and the white-tailed sea eagle (*Haliaeetus albicilla*) from the Baltic coast. The measured PFOS concentrations in these species ranged from < 3.9 to 150 ng/g ww (Kannan et al., 2002b). In water birds from the USA, Canada and the Northern Pacific, the liver PFOS concentrations ranged between < 30 and 1780 ng/g ww (Giesy and Kannan, 2001; Kannan et al., 2001a). In Japan and Korea, hepatic PFOS levels in fish-eating birds have been reported between < 19 and 650 ng/g ww (Kannan et al., 2002a). In comparison with these hepatic PFOS concentrations, it can be concluded that the PFOS concentrations in Blokkersdijk are among the highest values reported in birds. The PFOS concentrations at Blokkersdijk are comparable to the highest ones measured in top predators worldwide such as the bald eagle (2570 ng/ml in plasma), mink (3680 ng/g ww in liver), bottlenose dolphin (1520 ng/g ww in liver) or polar bear (> 4000 ng/g ww in liver, Giesy and Kannan, 2001; Kannan et al., 2001b; Martin et al.,

2004). Also in bald eagle nestlings, similar PFOS concentrations as those found in the tit nestlings have been measured (> 1000 ng/ml plasma, Kannan et al., 2001a). In the latter study, no sex differences were observed in PFOS contamination levels of the nestlings. That observation is concurrent with the present study because no significant differences in PFOS concentrations were observed between male and female nestlings in Blokkersdijk or Fort IV ($p > 0.05$).

A likely source for PFOS contamination of nestlings is the food. Great and blue tit nestlings have similar nutritional habits. Their diet consists mainly of butterflies and moths (mainly the larval stage) and to a lesser extent of spiders which are generally captured in the vicinity of the nest (Cramp and Perrins, 1993). Therefore, it is very likely that food items for each species have similar PFOS (precursor) contamination levels and contribute at a similar extent to PFOS contamination of the nestlings if uptake/depuration characteristics of PFOS and metabolisation characteristics of its precursors are similar. This seems to be supported by the observation that the hepatic PFOS concentrations were not significantly different between both tit species, as well for Blokkersdijk as for Fort IV ($p > 0.05$).
In addition to PFOS contamination via food items, PFOS (precursor) contamination of nestlings might also occurs via the egg. This possibility is supported by studies in the mallard duck (*Anas platyrhynchos*) and northern bobwhite quail (*Colinus virginianus*, United States Environmental Protection Agency, 2004a, 2004b).

The observed significant relationships between the PFOS liver concentration and the serum alanine aminotransferase (ALT) activity, the relative liver weight and the serum cholesterol and triglyceride

concentrations in the tit nestlings suggest that PFOS might affect hepatic integrity and lipid metabolism. This is in agreement with previous reports on PFOS-induced effects where PFOS has been reported to increase the relative liver weight in rodents (Ikeda et al., 1987; Lau et al., 2003; Seacat et al., 2002; Sohlenius et al., 1993; Thibodeaux et al., 2003) and cynomolgus monkeys (*Macaca fascicularis*, Seacat et al., 2003). Also the serum ALT activity has been demonstrated to increase in PFOS-exposed rodents (Seacat et al., 2003) and carps (Hoff et al., 2003b). Decreases in serum triglyceride and cholesterol concentrations after PFOS exposure have been shown in rodents (Haughom and Spydevold, 1992; Seacat et al., 2003; Thibodeaux et al., 2003) and cynomolgus monkeys (Seacat et al., 2002).

Although the increase in serum ALT activity and the decreases in serum cholesterol and triglyceride concentration were observed in both tit species, the increases in relative liver weight and the hematocrite were only significantly related to the liver PFOS concentration in the great and blue tit nestlings, respectively. No significant differences were observed in liver PFOS concentration ranges in Blokkersdijk or Fort IV great and blue nestlings, however, suggesting that differences in PFOS liver concentration might not account for the observed species differences in effect profiles. Alternatively, the observed differences could be ascribed to differences in species sensitivity.

Seacat et al. (2003) showed that the lowest observed effect level (LOEL) for a significant increase in relative liver weight in PFOS-exposed male rats after 4 weeks of PFOS exposure corresponded with a liver PFOS concentration of 282000 ng/g ww. For serum ALT activity increase and cholesterol decrease, the LOEL in males was 568000 ng PFOS/g liver after 14 weeks of exposure. In females, the relative liver weight was

significantly increased at a mean liver PFOS concentration of 635000 ng/g ww after 14 weeks. In rats exposed to PFOS during gestation, the relative liver weight was shown to be significantly increased on day 2 and 9 after birth corresponding with a mean liver concentration of about 50000 ng PFOS/g ww at birth (Lau et al., 2003). In cynomolgus monkeys, increased relative liver weights were observed at mean liver PFOS concentrations of 395000 and 273000 ng/g ww for males and females after 183 days of PFOS exposure and decreased serum cholesterol concentrations at hepatic PFOS concentrations > 100000 ng/g ww (Seacat et al., 2002). The nestling PFOS concentration ranges for which significant correlations were found with the relative liver weight, serum ALT activity, cholesterol and triglyceride concentrations are well below these values (17-2788 and 69-3323 ng/g ww for great and blue tit, respectively). The reason for this discrepancy is not clear at present but might be due to differences in species sensitivity and differences in exposure conditions between the laboratory and the field. In order to account for laboratory-to-field extrapolation, for within- and between-species variability, and accounting for PFOS´ bioaccumulative capacities and persistence and extrapolation to chronic PFOS exposure conditions, a chronic PFOS exposure a correction factor of 1000 has been proposed for extrapolation of PFOS-induced effects in birds and mammals (Canadian Environmental Protection Act, 1999). If this correction factor is applied to the PFOS effect concentrations in rats and cynomolgus monkeys reported above, they come close to the PFOS concentrations measured in the Blokkersdijk tit nestlings (86-2788 and 317-3322 ng/g ww for great and blue tit, respectively) supporting the possibility that PFOS contamination might have affected some biochemical endpoints in the nestlings.

The total serum protein concentration was not significantly related to the presence of PFOS in tit nestling liver tissue. The hematocrite was significantly and positively related to the hepatic PFOS concentration in blue tit nestlings only suggesting PFOS-mediated erythrocyte enlargement or cell number or dehydration. This finding is concurrent with an observation in feral PFOS-polluted eels with similar hepatic PFOS concentrations (17-9031 ng/g ww, Hoff et al., 2005) but has not been investigated under laboratory conditions. Because the condition index was not shown to be significantly affected in our study, it was not suggested that the observed biological alterations could be indicative of effects on the condition of nestlings.

Because no significant differences were shown in the values of the biological endpoints investigated in males and females, neither for the Blokkersdijk, nor for the Fort IV nestlings, it is suggested that the values of these endpoints are sex-independent. Consequently, sex probably did not confound the studies relationships between the hepatic PFOS concentrations and the biological endpoints.

The present study does not suggest that the measured hepatic organochlorine or organobromine pollutants would have contributed to the modulation of the serum ALT activity, serum cholesterol, triglyceride concentration or hematocrite, endpoints that were significantly correlated with the liver PFOS concentration in both or one of both tit species under investigation. Overall, these results suggest that PFOS might be a relatively important determinant in the alteration of these endpoints.

6.6 Conclusion

In conclusion, this study shows for the first time that nestlings from two passerine bird species from Blokkersdijk, an area protected by a European Council directive on the conservation of wild birds, were severely PFOS contaminated. No differences in PFOS contamination were shown between sexes or species. The relative liver weight, serum ALT activity, cholesterol and triglyceride concentrations and hematocrite were suggested to be affected by PFOS contamination but not by any of the measured organochlorine and organobromine pollutants.

It is not clear at present whether these observed alterations could cause deleterious effects on a higher level of biologic organization. Chronic PFOS exposure reproductive studies in which adult mallard duck and northern bobwhite quails were exposed showed that the mean liver PFOS no observed adverse effect level values for 14-day survivability was 3.17 and 3.61 μg/g ww in male and female mallard chicks, respectively (United States Environmental Protection Agency, 2004c).

In quail chicks, the mean liver PFOS lowest observed adverse effect level values for 14-day survivability was 5.76 and 5.49 μg/g ww in male and female quail chicks, respectively (United States Environmental Protection Agency, 2004d). These latter values are in the same order of magnitude than the maximal hepatic PFOS concentrations measured in the Blokkersdijk tits (2788 and 3322 ng/g ww for great and blue tit, respectively) warranting further study on potential effects of PFOS exposure on survival in tit nestlings from Blokkersdijk.

Chapter 7

Conclusions and future perspective

Considering that "risk" is defined as "the probability of occurrence of adverse effects on man or environment resulting from exposure to a chemical or mixture" (van Leeuwen and Hermens, 1995), the studies presented in this thesis are a first contribution to the understanding of PFOS exposure associated risk in feral carp, eel, gibel carp, bib, plaice, blue and great tits and wood mice in Belgium and The Netherlands. This contribution was possible using information from the conducted laboratory PFOS exposure studies in carp (chapters 1 and 2) and information from the biomonitoring studies reporting on the hepatic PFOS concentrations in these species and describing the relationships between these tissue PFOS levels and a number of biological endpoints (chapters 3-6).

In order to perform an initial risk assessment, the measured liver PFOS concentrations in feral fishes, wood mice and tits reported in this thesis (chapters 3-6) can be compared with literature-based lowest observed effect concentrations (LOECs) based on liver tissue PFOS concentrations that were available prior to February 2005. These critical effect concentrations comprise LOECs for PFOS exposure in fishes (carp; LOEC fish = 0.016 μg PFOS/g liver; chapter 1; Hoff et al., 2003b), mammals (rat, mouse and cynomolgus monkey; LOEC mammal = 17.3 μg PFOS/g liver; Hu et al., 2002; Lau et al., 2003; Seacat et al., 2002, 2003; Thibodeaux et al., 2003) and birds (northern bobwhite quail and mallard duck; LOEC bird = 4.9 μg PFOS/g liver; United States Environmental Protection Agency, 2004a, b, c, d). Fig. 1a, 1b and 1c

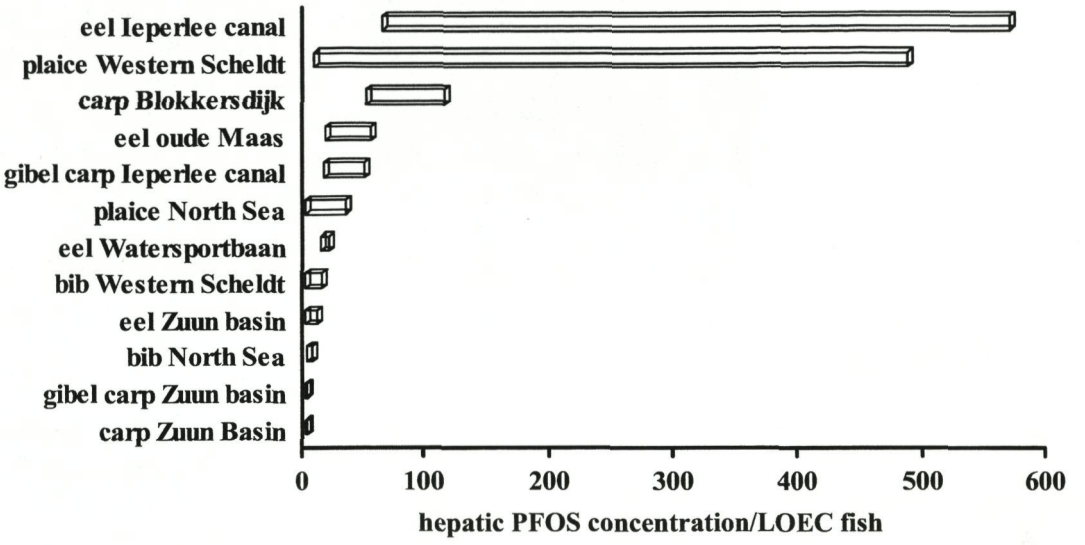
show the ratios of the minimal and maximal liver PFOS concentrations measured in the wildlife species studied in this thesis and literature-based minimal LOEC values.

It can be seen from Fig. 1a that the maximal ratios are found for eel from the Ieperlee canal, plaice from the Western Scheldt and carp from the Blokkersdijk pond. This suggests that the risk for fishes from these locations is very elevated although ratios vary considerably within one location. Maximal ratios for fish were the least elevated for the Zuun basin and the North Sea, suggesting a lower risk for individuals from those locations.

In Fig. 1b it is shown that in wood mice, the most elevated risk is found for mice from Blokkersdijk if compared to Galgenweel. Also in tits the most elevated risk is found in Blokkersdijk animals (Fig. 1c).

It is difficult to compare risks between fish, wood mice and tits because ratios were based on LOEC values for different effects.

a.


eel Ieperlee canal
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eel Zuun basin
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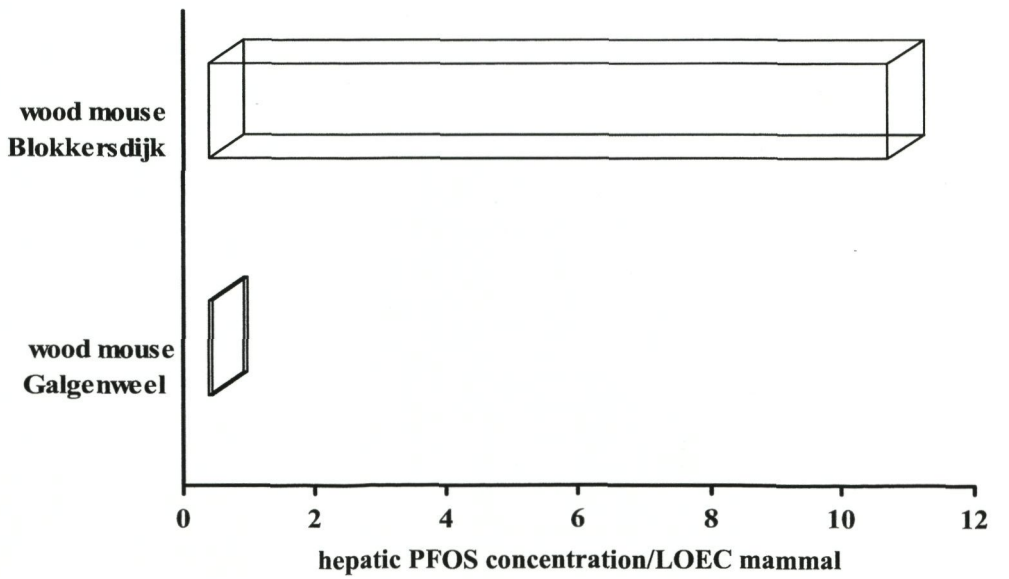
hepatic PFOS concentration/LOEC fish

b.


wood mouse
Blokkersdijk
wood mouse
Galgenweel
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6
8
10
12
hepatic PFOS concentration/LOEC mammal

c.

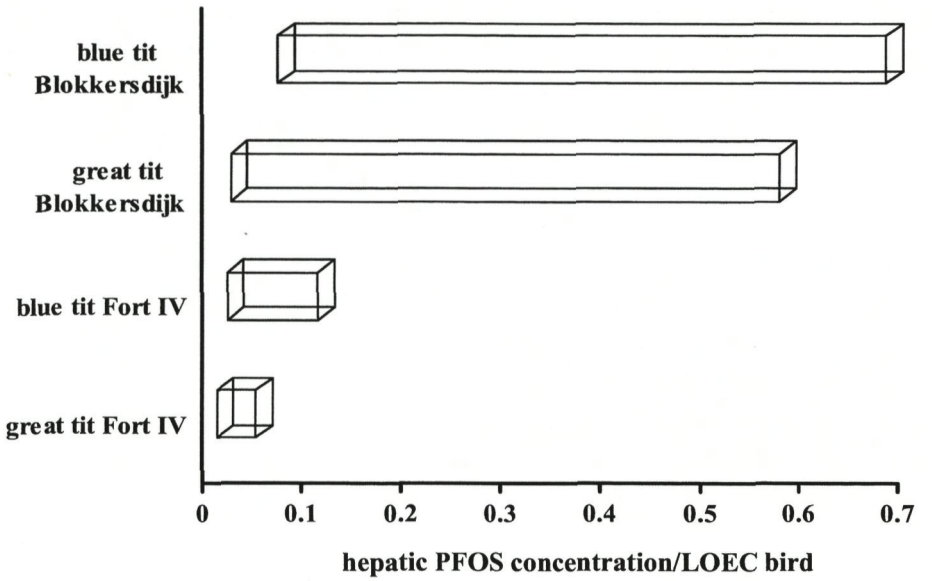

Fig. 1. Ratios of hepatic PFOS concentrations measured in wildlife and hepatic LOECs per species.

a: LOEC fish = 0.016 μg PFOS/g liver, average basepair length increase in carp (Hoff et al., 2003b).

b: LOEC mammal = 17.3 μg PFOS/g liver, decrease in serum cholesterol, high density lipoprotein decrease, total bilirubin in male cynomolgus monkey (Seacat et al., 2002).

c: LOEC bird = 4.9 μg PFOS/g liver, liver weight increase in female northern bobwhite quail (United States Environmental Protection Agency, 2004d).

In addition to the initial risk assessment of a number of feral species as described above, this thesis also provides additional information on PFOS exposure and effects in terrestrial and aquatic species by describing field-based relationships between hepatic PFOS concentrations and biological endpoints. An overview of these relationships is given in Tables 1, 2 and 3. These tables show that some of the investigated endpoints correlate significantly with the liver PFOS concentration in the different species that were analysed, suggesting that PFOS might affect biochemical endpoints in the studied wildlife species. Condition and growth were not suggested to be affected by PFOS exposure in the conducted studies.

Numerous factors such as nutrition, age, genotype or the presence of chemical pollutants other than PFOS might have affected the investigated biological endpoints in the field. Therefore, the observed significant relationships between the liver PFOS concentrations and the biochemical endpoints reported in Tables 1, 2 and 3 could be confounded by unknown factors. Interestingly, the need for identification and characterization of these confounding factors in the context of PFOS exposure related effect evaluation was demonstrated in this thesis by a study on feral eel (chapter 4). In that study, contamination with organochlorine compounds was suggested to be (co)responsible for the alteration of biochemical endpoints that were suggested to be modulated by PFOS exposure. Also the possibility of interactive effects of PFOS and other toxicants has been illustrated by *in vitro* experiments (Hu et al., 2003). In chapter 5 it was suggested that the observed significant positive relationship observed between the hepatic PFOS concentration and the serum triglyceride in wood mice could be ascribed to the positive relationships of both the hepatic PFOS concentration and the serum triglyceride concentration with age.

Table 1. Relationships between the hepatic PFOS concentration and liver endpoints.

	wood mouse	blue tit	great tit	eel	carp	gibel carp	plaice	bib
relative liver weight	**S**	NS	**S**	ND	ND	ND	ND	ND
liver weight	**S**	ND	ND	ND	ND	ND	ND	ND
protein content	ND	ND	ND	ND	ND	ND	NS	**S**
lipid content	ND	ND	ND	ND	ND	ND	NS	NS
carbohydrate content	ND	ND	ND	ND	ND	ND	NS	NS
microsomal lipid peroxidation	**S**	ND	ND	ND	ND	ND	ND	ND
peroxisomal β-oxidation	NS	ND	ND	ND	ND	ND	ND	ND
mitochondrial fraction protein content	ND	ND	ND	ND	ND	ND	ND	ND
mitochondrial fraction catalase activity	NS	ND	ND	ND	ND	ND	ND	ND

S = significant, NS = not significant, ND = not determined.

Table 2. Relationships between the hepatic PFOS concentration and blood endpoints.

	wood mouse	blue tit	great tit	eel	carp	gibel carp	plaice	bib
serum ALT activity	**S**[a,b]	**S**	**S**	**S**	**S**[c]	NS	NS	**S**
serum AST activity	ND	ND	ND	ND	ND	ND	NS	NS
serum cholesterol	NS	**S**	**S**	ND	ND	ND	ND	ND
serum triglyceride	**S**[a]	**S**	**S**	ND	ND	ND	ND	ND
hematocrite	ND	**S**	NS	**S**	NS	NS	ND	ND
serum Na^+	ND	ND	ND	NS	**S**	NS	ND	ND
serum Ca^{2+}	ND	ND	ND	NS	**S**	NS	ND	ND
serum Cl^-	ND	ND	ND	NS	**S**	NS	ND	ND
serum K^+	NS	ND	ND	NS	NS	NS	ND	ND
serum protein content	ND	ND	ND	**S**	**S**	NS	ND	ND

S = significant, NS = not significant, ND = not determined.

a: relationships opposite to what is reported in literature (triglyceride, Haughom and Spydevold, 1992; ALT activity, Goldenthal et al., 1978c).

b: only significant if the ALT activity is expressed in U/dl, not if the ALT activity is expressed in U/g protein.

c: only significant if the ALT activity is expressed in U/g protein, not if the ALT activity is expressed in U/l.

Table 3. Relationships between the hepatic PFOS concentration and organismal endpoints.

	wood mouse	blue tit	great tit	eel	carp	gibel carp	plaice	bib
condition factor	ND	NS	NS	ND	NS	NS	ND	ND
growth rate	ND	ND	ND	ND	NS	NS	ND	ND

NS = not significant, ND = not determined.

It should be noted that in contradiction to the conducted risk assessment, the latter correlations are based on the evaluation of effects under natural exposure conditions. This is especially important if little information is available on the extrapolation factors that need to be applied on laboratory derived exposure-effect relationships and critical effect concentrations obtained under "unnatural" laboratory conditions. Until now, it is not known how natural conditions influence PFOS-mediated effects.

It should also be noted that the liver PFOS concentration data and the biological endpoint data from the field studies originate from the same species in contradiction to the described risk assessment that does not consider LOECs for the same species (except for carp) in which the hepatic PFOS concentrations were measured. Consequently, possible differences in species sensitivity are not always taken into account in the initial risk assessment, except for carp. Although not investigated to a

great extent, laboratory based PFOS exposure studies in birds, for example (mallard duck and northern bobwhite quail) suggest that different species might have different sensitivities regarding PFOS-mediated effects (United States Environmental Protection Agency, 2004c, 2004d). These studies show that the mean lowest observed adverse effect concentration for 14-day survival in liver tissue from quail chicks was 5.5 µg/g wet weight (ww), while the mean no observed adverse effect concentration for this effect in mallard chicks was very similar (3.4 µg/g ww).

A difference between the risk analysis and the correlation analyses is also that the performed risk evaluation could reflect a "worst case scenario" because LOECs were considered while the correlation analyses more closely reflect observations from "the real world outside".

A common limitation of the risk assessment and correlation analyses described in this thesis is that most of the investigated effect endpoints do not necessarily reflect adverse effects such as effects on mortality, growth and reproduction. Also the link of the investigated effects with effects on the population, community or ecosystem level is unclear and should be an objective for further research.

In conclusion, future studies should particularly aim at the further evaluation of PFOS-mediated effects under controlled conditions and the further characterization of relationships between PFOS exposure concentrations and biological effects in the field. Scientific research should also focus on the identification and characterization of confounding factors and address the issues of environmental relevance, adversity of effects, species sensitivity, relevance of effects regarding higher levels of biological organization and laboratory to field

extrapolation in order to improve the present understanding of PFOS-associated risk in wildlife.

Summary

The conducted labour described in this thesis consisted out of two major parts.

The first part (chapters 1-2) dealt with the assessment of PFOS-related biochemical and histological effects in freshwater fishes, using the common carp as a laboratory test species.

The goal of the studies described in the second part (chapters 3-6) was twofold. Firstly, PFOS exposure levels of wildlife in Flanders (Belgium) and the Southern part of The Netherlands were assessed through the measurement of hepatic PFOS pollution levels in a number of freshwater, estuarine and marine fishes, in the wood mouse and two tit species. Secondly, the evaluation of relationships between the PFOS contamination levels on the one hand and a set of biochemical and organismal endpoints on the other hand, provided initial insights in the context of the PFOS-related biochemical and organismal effects in the feral species of interest.

1. Laboratory-based PFOS effect assessments

In chapter 1, the study of the biochemical and histological effects of PFOS exposed common carp revealed that PFOS can induce serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities indicative for liver damage after a short term PFOS exposure period. PFOS was also suggested to interfere with DNA homeostasis in liver tissue. No effects were observed on serum lipid levels or total antioxidant activity in serum. In liver tissue, no observations were made suggesting inflammation, induction of peroxisomal β-oxidation or

alterations in catalase activity. No clear histological effects were observed.

For the induction of serum ALT and AST activities, dose-effect relationships were established, allowing the calculation of EC_{10} values. The toxicity thresholds derived from these EC_{10} values suggested that PFOS exposure might increase ALT and AST activities in wildlife.

In chapter 2 was described that in addition to these observed effects, PFOS exposure of carp can induce the transcription of hepatic genes homologuous to chymotrypsinogen, toxin-1, basic fatty acid binding protein and lectin from other teleosts after two weeks of PFOS exposure, resulting in hepatic PFOS concentrations of 31.5 ± 13.3 μg/g wet weight (mean ± SD). These effects were not confirmed by real-time PCR analysis after normalization with actin.

2. PFOS biomonitoring assessments

In chapter 3, hepatic PFOS concentrations ranging between 11 and 217 ng/g wet weight (ww) in bib (*Trisopterus luscus*) and between 107 ng/g ww to 7760 ng/g in plaice (*Pleuronectes platessa*) from the North Sea and in the Western Scheldt (The Netherlands). The respective muscle concentrations ranged between < 10 and 111 ng/g ww for bib and < 10 ng/g ww and 87 ng/g ww for plaice. Both the PFOS liver and muscle concentrations demonstrated the existence of a PFOS pollution gradient along the Western Scheldt with higher levels at the upstream locations and a relatively low degree of PFOS pollution at the marine locations.

In bib, significant positive correlations were found between the liver PFOS concentration and the serum ALT activity and the liver protein content, indicative for the induction of hepatic damage and the induction

of compensatory mechanisms, detoxification or repair processes, respectively.

In chapter 4, hepatic PFOS concentrations were reported for feral gibel carp (*Carassius auratus gibelio*), carp (*Cyprinus carpio*), and eel (*Anguilla anguilla*) captured at different locations in Flanders (Belgium). The liver PFOS concentrations in fishes from the Ieperlee canal (Boezinge) and the Blokkersdijk pond (Antwerp), neighbouring a fluorochemical production plant, were most elevated (Ieperlee canal, 250-9031 ng/g ww; Blokkersdijk pond, 633-1822 ng/g ww). The PFOS concentrations were the lowest in fishes from the the Zuun basin (Sint-Pieters-Leeuw), ranging between 11.2 and 162 ng/g ww. In eel from the Oude Maas pond (Dilsen-Stokkem) and Watersportbaan basin (Ghent), PFOS concentrations between 212 and 857 ng/g ww were measured.
In eel and carp, the liver PFOS concentration was significantly and positively related with the serum ALT activity, and negatively with the serum protein content. The hepatic PFOS concentration correlated significantly and negatively with the serum Na^+, Ca^{2+} and Cl^- concentrations in carp whereas in eel, a significant positive relation was found with the hematocrite suggesting that PFOS might mediate a number of serological effects in freshwater fishes in Flanders.
The observed serological alterations in eel were also significantly correlated with the hepatic PCB 28, PCB 74, γ-hexachlorocyclohexane (γ-HCH) and hexachlorobenzene (HCB) concentrations, suggesting that these componds might be involved in the serological alterations in eel.

Chapter 5 described a biomonitoring study in wood mice (*Apodemus sylvaticus*) from Blokkersdijk (Antwerp), a nature reserve in the immediate vicinity of a fluorochemical plant in Antwerp (Belgium) and

from Galgenweel, three kilometers further away. PFOS concentrations in the Blokkersdijk mice ranged between 0.47 and 178.55 μg/g ww. Perfluorononanoic, perfluorodecanoic, perfluoroundecanoic and perfluorododecanoic acid were found sporadically in the liver tissue of the Blokkersdijk mice. At Galgenweel, the liver concentrations were significantly lower than at Blokkersdijk (0.14 to 1.11 μg/g ww). The liver PFOS levels were suggested to be sex-independent but they were shown to depend on age because increased levels of PFOS bioaccumulation in older mice were observed. The obtained results also suggested maternal PFOS transfer to the young.

Several liver endpoints were significantly elevated in the Blokkersdijk mice compared to mice from Galgenweel: the liver weight, relative liver weight, the peroxisomal β-oxidation activity, the microsomal lipid peroxidation level and the mitochondrial fraction protein content. For the mitochondrial fraction catalase activity no significant difference between locations was found. Only the liver weight, relative liver weight and the liver microsomal lipid peroxidation level increased significantly with the liver PFOS concentration. No indications for PFOS-mediated effects on the serum triglyceride, cholesterol or potassium levels were obtained. The liver PFOS concentration was negatively related to the serum alanine aminotransferase activity.

Next to the fish and mouse biomonitoring studies, a PFOS biomonitoring survey was also conducted on great tit (*Parus major*) and blue tit (*Parus caeruleus*) nestlings from Blokkersdijk and Fort IV (chapter 6). The hepatic PFOS concentrations in Blokkersdijk (86-2788 and 317-3322 ng/g ww for great and blue tit, respectively) were among the highest ever measured and were significantly higher than at the control area (17-206

and 69-514 ng/g ww for great and blue tit, respectively). The hepatic PFOS concentration was found to be species- and sex-independent.

In both tit species, the liver PFOS concentration correlated significantly and positively with the serum ALT activity and negatively with the serum cholesterol and triglyceride levels but not with condition or serum protein concentration. In the great tit, a significant positive correlation was observed between the liver PFOS concentration and the relative liver weight. In the blue tit, the hepatic PFOS concentration correlated positively and significantly with hematocrite values. Next to PFOS, 11 organochlorine pesticides, 20 polychlorinated biphenyl congeners and 7 polybrominated diphenyl ethers were measured in liver tissue but none of these latter compounds were suggested to be involved in the observed biological alterations.

Bibliography

1. 3M Company, 1978. The effect of continuous aqueous exposure to 14C-78.02 on hatchability of eggs and growth and survival of fry of fathead minnow (*Pimephales promelas*). 3M Company, St Paul, MN, USA.

2. 3M Company, 1979. 96h Acute toxicity test on bluegill sunfish. 3M Company, St Paul, MN, USA.

3. 3M Company, 1985a. Acute toxicity of PFOS to rainbow trout in freshwater. 3M Company, St Paul, MN, USA.

4. 3M Company, 1985b. Acute toxicity of PFOS to rainbow trout in saltwater. 3M Company, St Paul, MN, USA.

5. 3M Company, 1994. *Pimephales promelas* 96-hour toxicity test data summary. 3M Company, St Paul, MN, USA.

6. 3M Company, 1999. Reports "The science of organic fluorochemistry" and "Perfluorooctane sulfonate: current summary of human sera, health, and toxicological data. 3M Company, St Paul, MN, USA.

7. 3M Company, 2000a. Voluntary use and exposure information profile for perfluorooctanesulfonic acid and various salt forms. 3M Company submission to the US EPA, April 27, 2000. 3M Company, St Paul, MN, USA.

8. 3M Company, 2000b. Phase-out plan for POSF-based products. Submitted to US EPA July 7, 2000. 3M Company, St Paul, MN, USA.

9. 3M Company, 2000c. Verbal Comments by William Weppner (Director of Environmental, Health, Safety and Regulatory Affairs). Informal OECD meeting to discuss PFOS initial assessment report. 3M Company, St Paul, MN, USA.

10. 3M Company, 2000d. 96-Hour static acute toxicity test with the fathead minnow (*Pimephales promelas*). 3M Company, St Paul, MN, USA.

11. 3M Company, 2000e. Early life-stage toxicity test with the fathead minnow (*Pimephales promelas*). 3M Company, St Paul, MN, USA.

12. 3M Company, 2001a. Environmental Laboratory Project Number EOO-1716. Water solubility in natural seawater and 3.5 % sodium chloride solution-shake flask method. 3M Company, St Paul, MN, USA.

13. 3M Company, 2001b. Perfluorooctane sulfonate, potassium salt (PFOS): a flow-through bioconcentration test with the bluegill (*Lepomis macrochirus*). 3M Company, St Paul, MN, USA.

14. 3M Company, 2002a. 104-Week dietary chronic toxicity and carcinogenicity study with perfluorooctane sulfonic acid potassium salt in rats. 3M Company, St Paul, MN, USA.

15. 3M Company, 2002b. Perfluorooctanesulfonate, potassium salt (PFOS): 96-hour semi-static acute toxicity test with the sheepshead minnow (*Cyprinodon variegatus*) in saltwater. 3M Company, St Paul, MN, USA.

16. 3M Company, 2003. Environmental and health assessment of perfluorooctane sulfonic acid and its salts. 3M Company, St Paul, MN, USA.

17. Abdellatif, A.G., Preat, V., Vamecq, J., Nilsson, R., Roberfroid, M., 1990. Peroxisome proliferation and modulation of rat liver carcinogenesis by 2,4-D, 2,4,5-T, perfluorooctanoic acid and nafenopin. Carcinogenesis 11: 1899–1902.

18. Abe, T., Nagase, S., 1982. Preparation, properties, and industrial applications of organofluorine compounds. In: Banks, R.E. (Ed.), John Wiley & Sons, New York, USA, pp. 19-44.

19. Adams, J., 2003. The proteasome: structure, function, and role in the cell. Cancer Treat. Rev. 29: 3-9.

20. Aebi, H., 1984. Catalase *in vitro*. Method. Enzymol. 105: 121-126.

21. Ahmed, M.B., Khater, M.R., 2001. Evaluation of the protective potential of *Ambrosia maritima* extract on acetaminophen-induced liver damage. J. Ethnopharmacol. 75: 169-174.

22. Alexander, B.H., Olsen, G.W., Burris, J.M., Mandel, J.H., Mandel, J.S., 2003. Mortality of employees of a perfluorooctanesulfonyl fluoride manufacturing facility. Occup. Environ. Med. 60: 722-729.

23. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., Fu, P.C., 1974. Enzymatic determination of total serum cholesterol. Clin. Chem. 20: 470-475.

24. Almeida, M.G., Fanini, F., Davino, S.C., Aznar, A.E., Koch, O.R., Barros, S.B.M., 1997. Pro- and anti-oxidant parameters in rat liver after short term exposure to hexachlorobenzene. Hum. Exp. Toxicol. 16: 257-261.

25. Arason, G.J., 1996. Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immun. 6: 277-289.

26. Arnao, M.B., Cano, A., Acosta, M., 1999. Methods to measure the antioxidant activity in plant material. A comparative discussion. Free Rad. Res. 31: 589-596.

27. Ashby, J., Brady, A., Elcombe, C.R., Elliot, B.M., Ishmael, B., Odum, J., Tugwood, J.D., Kettle, S., Purchase, I.F.H., 1994. Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. Hum. Exp. Toxicol. 13 Suppl. 2: S1-S117.

28. Austin, M.E., Kasturi, B.S., Barber, M., Kannan, K., MohanKumar, P.S., MohanKumar, S.M.J., 2003. Neuroendocrine effects of perfluorooctane sulfonate in rats. Environ. Health Persp. 111: 1485-1489.

29. Bagenal, T.B., Tesch, F.W., 1978. Methods for assessment of fish production in fresh waters, Blackwell Scientific Publications, Oxford, UK.

30. Bergmeyer, H.U., Hørder, M., Rej., R., 1986a. Approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase. J. Clin. Chem. Clin. Biochem. 24: 481-495.

31. Bergmeyer, H.U., Hørder, M., Rej., R., 1986b. Approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 2. IFCC method for aspartate aminotransferase. J. Clin. Chem. Clin. Biochem. 24: 497-510.

32. Berthiaume, J., Wallace, K.B., 2002. Perfluorooctanoate, perfluorooctanesulfonate, and N-ethyl perfluorooctanesulfonamido ethanol; peroxisome proliferation and mitochondrial biogenesis. Toxicol. Lett. 19: 23-32.

33. Beyst B., Mees J., Cattrijsse, A., 1999. Early postlarval fish in the hyperbenthos of the Dutch Delta (south-west Netherlands). J. Mar. Biol. Ass. UK 79: 709-724.

34. Biesemeyer, J.A., Harris, D.L., 1974. Eye and skin irritation report on sample T-1117. Project No. 4102871. Warf Institute Inc.

35. Black, M.C., Ferrell, J.R., Horning, R.C., Martin, L.K.Jr, 1996. DNA strand breakage in freshwater mussels (*Anodonta grandis*) exposed to lead in the laboratory and field. Environ. Toxicol. Chem. 15: 802-808.

36. Boulanger, B., Vargo, J., Schnoor, J.L., Hornbuckle, K.C., 2004. Detection of perfluorooctane surfactants in Great Lakes water. Environ. Sci. Technol. 38: 4064-4070.

37. Braunbeck, T., Görge, G., Storch, V., Nagel, R., 1990. Hepatic steatosis in zebra fish (*Brachydanio rerio*) induced by long-term exposure to γ-hexachlorocyclohexane. Ecotox. Environ. Safe. 19: 355-374.

38. Brumley, C.M., Haritos, V.S., Ahokas, J.T., Holdway, D.A., 1995. Validation of biomarkers of marine pollution exposure in sand flathead using arochlor-1254. Aquat. Toxicol. 31: 249-262.

39. Burris, J.M., Lundberg, J.K., Olsen, G.W., Simpson, C., Mandel, J., 2002. Determination of serum half-lives of several fluorochemicals. 3M Company, St Paul, MN, USA.

40. Canadian Environmental Protection Act, 1999. Environmental screening assessment report on perfluorooctane sulfonate, its salts and its precursors that contain the $C_8F_{17}SO_2$ or $C_8F_{17}SO_3$ moiety. Available: http://www.ec.gc.ca/CEPARegistry/the_act/ [accessed February 2004].

41. Case, M.T., York, R.G., Christian, M.S., 2001. Rat and rabbit oral developmental toxicology studies with two perfluorinated compounds. Int. J. Toxicol. 20: 101-109.

42. Cattrijsse, A., Dankwa, H.R., Mees, J., 1997. Nursery function of an estuarine tidal marsh for the brown shrimp *Crangon crangon*. J. Sea Res. 38: 109-121.

43. Christian, M.S., Hoberman, A.M., York, R.G., 1999. Oral (gavage) cross-fostering study of PFOS in rats. Study Number T-6295. Argus Research Laboratories.

44. Concha, M.I., Smith, V.J., Castro, K., Bastias, A., Romero, A., Amthauer, R.J., 2004. Apolipoproteins A-I and A-II are potentially important effectors of innate immunity in the teleost fish *Cyprinus carpio*. Eur. J. Biochem. 271: 2984-2990.

45. Corbet, G.B., Harris, S., 1991. The Handbook of British Mammals. Blackwell Science, London, UK.

46. Corton, J.C., Anderson, S.P., Stauber, A., 2000. Central role of peroxisome proliferator-activated receptors in the actions of peroxisome proliferators. Annu. Rev. Pharmacol. Toxicol. 40: 491-518.

47. Cramp, S., Perrins, C.M., 1993. Birds of Europe, the Middle East and North Africa. The birds of the Western Palearctic. Volume VII. Flycatchers to Shrikes. Oxford University Press, Oxford, UK.

48. Dauwe, T., Chu, S.G., Covaci, A., Schepens, P., Eens, M., 2003. Great tit (*Parus major*) nestlings as biomonitors of organochlorine pollution. Arch. Environ. Con. Tox. 44: 89-96.

49. Dean, W.P., Jessup, D.C., Thompson, G., Romig, G., Powell, D., 1978. Fluorad fluorochemical surfactant FC-95 acute oral toxicity (LD50) study in rats. Study No. 137-083. International Research and Development Corporation.

50. De Coen, W., Janssen, C., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new biomarker to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquat. Ecosyst. Stress Recovery 6: 43-55.

51. De Coen, W.M., 1999. PhD dissertation, Ghent University.

52. De Coen, W.M., Vlaeminck, A., Hoff, P., Willems, K., Giesy, J., Blust, R., 2001. Evaluation of the toxicological mode of action of perfluorinated chemicals using cellular reporter assays. Proceedings, Abstracts of the 11th Annual Meeting of SETAC Europe, Madrid, Spain, 6-10 May 2001, p 62.

53. Denovan-Wright, E.M., Pierce, M., Sharma, M.K., Wright, J.M., 2000. cDNA sequence and tissue-specific expression of a basic liver-type fatty acid binding protein in adult zebrafish (*Danio rerio*). Biochim. Biophys. Acta 1492: 227-232.

54. Derbel, M., Hosokawa, M., Satoh, T., 1996. Differences in the induction of carboxylesterase RL4 in rat liver microsomes by various perfluorinated fatty acids, metabolically inert derivatives of fatty acids. Biol. Pharm. Bull. 19: 765-767.

55. De Wolf, H., Backeljau, T., Blust, R., 2000. Heavy metal accumulation in the periwinkle *Littorina littorea*, along a pollution gradient in the Scheldt estuary. Sci. Total Env. 262: 111-121.

56. De Wolf, H., Jordaens, K., Reusens, E., Blust, R., Backeljau, T., 2001. Esterase variation in the periwinkle *Littorina littorea*, along the western and eastern Scheldt estuarium. Mar. Environ. Res. 52: 373-382.

57. Dewulf, J., Van Langenhove, H., Everaert, M., Vanthournhout, H., 1998. Volatile organic compounds in the Scheldt estuary along the trajectory Antwerp-Vlissingen: concentration profiles, modelling and estimation of emissions into the atmosphere. Water Res. 32: 2941-2950.

58. Dimitrov, S., Kamenska, V., Walker, J.D., Windle, W., Purdy, R., Lewis, M., Mekanyan, O., 2004. Predicting the biodegradation products of perfluorinated chemicals using CATABOL. SAR and QSAR Environ. Res. 15: 69-82.

59. El-Demerdash, F.M., 2001. Effects of selenium and mercury on the enzymatic activities and lipid peroxidation in brain, liver and blood of rats. J. Environ. Sci. Heal. B 36: 489-499.

60. Elliott, B.M., Elcombe, C.R., 1987. Lack of DNA damage or lipid peroxidation measured *in vivo* in the rat liver following treatment with peroxisomal proliferators. Carcinogenesis 8: 1213-1218.

61. European Council directive of 2 April 1979 on the conservation of wild birds (79/409/EEC), 1979. Available: http://europa.eu.int/eur-lex/en/consleg/pdf/1979/en_1979L0409_do_001.pdf.

62. Gadelhak, G.G., 1992. PhD dissertation, Michigan State University.

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

64. Giesy, J.P., Hu, W., Jones, P.D., Newsted, J.L., Lau, C., 2003. Identification of genes reponsive to perfluorooctane sulfonic acid exposure using gene expression profiling. Proceedings, Abstracts of the 13th Annual Meeting of SETAC Europe, Hamburg, Germany, 27 April-1 May, p 143.

65. Goldenthal, E.I., Jessup, D.C., Geil, R.G., Mehring, J.S., 1978a. Ninety-day subacute rhesus monkey toxicity study no. 137–092. International Research Development Corporation, Mattawan, MI, USA.

66. Goldenthal, E.I., Jessup, D.C., Geil, R.G., Jefferson, N.D., Arceo, R.J., Ruecker, F.A., 1978b. Ninety-day subacute rhesus monkey toxicity study no. 137-085. International Research Development, Mattawan, MI, USA.

67. Goldenthal, E.I., Jessup, D.C., Geil, R.G., Jefferson, N.D., Arceo, R.J., Ruecker, F.A., 1978c. Ninety-day subacute rat toxicity study no. 137-085. Technical Report. International Research Development, Mattawan, MI, USA.

68. Goldenthal, E.I., Jessup, D.C., Geil, R.G., Mehring, J.S., 1979. Ninety-day subacute rhesus monkey toxicity study no. 137–087. International Research Development Corporation, Mattawan, MI, USA.

69. Grasty, R.C., Grey, B.E., Lau, C.S., Rogers, J.M., 2003. Prenatal window of susceptibility to perfluorooctane sulfonate-induced neonatal mortality in the Sprague-Dawley rat. Birth Defects Res. B 68: 465-471.

70. Griffiths, R., Double, M.C., Orr, K., Dawson, R.J.G., 1998. A DNA test to sex most birds. Mol. Ecol. 7: 1071-1075.

71. Hamerlynck O., Hostens K., 1993. Growth, feeding, production, and consumption in o-group bib (*Trisopterus luscus* L.) and whiting (*Merlangius merlangus* L.) in a shallow coastal area of the south-west Netherlands. ICES J. Mar. Sci. 50: 81-91.

72. Hansen, K.J., Clemen, L.A., Ellefson, M.E., Johnson, H.O., 2001. Compound-specific, quantitative characterization of organic fluorochemicals in biological matrices. Environ. Sci. Technol. 35: 766-770.

73. Hansen, K.J., Johnson, H.O., Eldridge, J.S., Butenhoff, J.L., Dick, L.A., 2002. Quantitative characterization of trace levels of PFOS and PFOA in the Tennessee river. Environ. Sci. Technol. 36: 1681-1685.

74. Harada, K., Saito, N., Inoue, K., Yoshiniga, T., Watanabe, T., Sasaki, S., Kamiyama, S., Koizumi, A., 2004. The influence of time, sex and geographic factors on levels of perfluorooctane sulfonate and perfluorooctanoate in human serum over the last 25 years. J. Occup. Health 46: 141-147.

75. Hatfield, T., 2001. Screening studies on the aqueous photolytic degradation of potassium perfluorooctane sulfonate (PFOS). 3M Company, St Paul, MN, USA.

76. Haughom, B., Spydevold, Ø., 1992. The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid. Biochim. Biophys. Acta 1128: 65-72.

77. Hoare, K., Beaumont, A.R., Davenport, J., 1995. Variation among populations in the resistence of *Mytilus edulis* embryos to copper: adaptation to pollution? Mar. Ecol.-Prog. Ser. 120: 155-161.

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

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

80. Hoff, P.T., Scheirs, J., Van de Vijver, K., Van Dongen, W., Esmans, E.L., Blust, R., De Coen, W., 2004. Biochemical effect evaluation of perfluorooctane sulfonic acid-contaminated wood mice (*Apodemus sylvaticus*). Environ. Health Persp. 112: 681-686.

81. Hoff, P.T., Van Campenhout, K., Van de Vijver, K., Covaci, A., Bervoets, L., Moens, L., Huyskens, G., Goemans, G., Belpaire, C., Blust, R., 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. Environ. Pollut. 137: 324-333.

82. Hoff, P.T., Van de Vijver, K., Dauwe, T., Covaci, A., Maervoet, J., Eens, M., Blust, R., De Coen, W. Evaluation of biochemical effects related to perfluorooctane sulfonic acid exposure in organohalogen-contaminated great tit (*Parus major*) and blue tit (*Parus caeruleus*) nestlings. Chemosphere in press.

83. Hoffman, D.J., Melancon, M.J., Klein, P.N., Rice, C.P., Eisemann, J.D., Hines, R.K., Spann, J.W., Pendleton, G.W., 1996. Developmental toxicity of PCB 126 (3,3′,4,4′,5-pentachlorobiphenyl) in nestling American kestrels (*Falco sparverius*). Fund. Appl. Toxicol. 34: 188-200.

84. Hostens, K., Mees, J., 1999. The mysid-feeding guild of demersal fishes in the brackish zone of the Western Scheldt estuary. J. Fish Biol. 55: 704-719.

85. Hostens, K., 2000. Spatial patterns and seasonality in the epibenthic communities of the Westerschelde (southern bight of the North Sea). J. Mar. Biol. Ass. UK 80: 27-36.

86. Hu, W., King, L.E., Jones, P.D., Giesy, J.P., 2000. Measurement of effects of perfluorinated compounds on cell membrane fluidity by flow cytometry. Proceedings, Abstracts of the 21th Annual Meeting of SETAC North America, Nashville, Tennessee, USA, 12-16 November 2000, p 291.

87. Hu, W.Y., Jones, P.D., Upham, B.L., Giesy, J.P., Trosko, J.E., 2001. Comparisons among perfluorinated compounds of effects on gap junction

intercellular communication. Proceedings, Abstracts of the 11th Annual Meeting of SETAC Europe, Madrid, Spain, 6-10 May 2001, p 161.

88. Hu, W., Jones, P.D., Upham, B.L., Trosko, J.E., Lau, C., Giesy, J.P., 2002. Inhibition of gap-junction intercellular communication by perfluorinated compounds in rat liver and dolphin kidney epithelial cell lines *in vitro* and Sprague-Dawley rats *in vivo*. Toxicol. Sci. 68: 429-436.

89. Hu, W.Y., Jones, P.D., De Coen, W., King, L., Fraker, P., Newsted, J., Giesy, J.P., 2003. Alterations in cell membrane properties caused by perfluorinated compounds. Comp. Biochem. Phys. C 135: 77-88.

90. Ikeda, T., Aiba, K., Fukuda, K., Tanaka, M., 1985. The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids. J. Biochem. 98: 475–482.

91. Ikeda, T., Fukuda, K., Mori, I., Enomoto, M., Komai, T., Suga, T., 1987. Induction of cytochrome P-450 and peroxisome proliferation in rat liver by perfluorinated octane sulphonic acid (PFOS). In: Fahimi, H.D., Sies, H. (Eds.), Peroxisomes in Biology and Medicine. Springer-Verlag, Heidelberg, Germany, pp. 304-308.

92. Inaba, K., Morisawa, M., 1991. A chymotrypsin-like protease involved in motility of sperm in salmonid fish. Biomed. Res.-Tokyo 12: 435-437.

93. Inoue, K., Okada, F., Ito, R., Kato, S., Sasaki, S., Nakajima, S., Uno, A., Saijo, Y., Sata, F., Yoshimura, Y., Kishi, R., Nakazawah, H., 2004. Perfluorooctane sulfonate (PFOS) and related perfluorinated compounds in human maternal and cord blood samples: assessment of PFOS exposure in a susceptible population during pregnancy. Environ. Health Persp. 112: 1204-1207.

94. Jacobs, M., Covaci, A., Schepens, P., 2002. Investigation of selected persistent organic pollutants in farmed Atlantic salmon (*Salmo salar*), salmon aquaculture feed, and fish oil components of the feed. Environ. Sci. Technol. 36: 2797-2805.

95. Johnson, J.D., Gibson, S.J., Ober, R.F., 1979a. Absorption of FC-95-14C in rats after a single oral dose. Riker laboratories, Inc., Subsidiary of 3M Company, St Paul, MN, USA.

96. Johnson, J.D., Gibson, S.J., Ober, R.F., 1979b. Extent and route of excretion and tissue distribution of total carbon-14 in rats after a single intravenous dose of FC-95-14C. Riker laboratories, Inc., Subsidiary of 3M Company, St Paul, MN, USA.

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

98. Jones, P.D., Hu, W., De Coen, W.M., Newsted, J.L., Giesy, J.P., 2003. Binding of perfluorinated fatty acids to serum proteins. Environ. Toxicol. Chem. 22: 2639-2649.

99. Junge, B., Carrion, Y., Bosco, C., Galleano, M., Puntarulo, S., Tapia, G., Videla, L.A., 2001. Effects of iron overload and lindane intoxication in relation to oxidative stress, Kupffer cell function, and liver injury in the rat. Toxicol. Appl. Pharm. 170: 23-28.

100. Kaikaus, R.M., Chan, W.K., Ortiz de Montellano, P.R., Bass, N.M., 1993. Mechanisms of regulation of liver fatty acid-binding protein. Mol. Cell. Biochem. 123: 93-100.

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

102. Kannan, K., Koistinen, J., Beckmen, K., Evans, T., Gorzelany, J.F., Hansen, K.J., Jones, P.D., Helle, E., Nyman, M., Giesy, J.P., 2001b. Accumulation of perfluorooctane sulfonate in marine mammals. Environ. Sci. Technol. 35: 1593-1598.

103. Kannan, K., Choi, J.-W., Iseki, N., Senthilkumar, K., Kim, D.H., Masunaga, S., Giesy, J.P., 2002a. Concentrations of perfluorinated acids in livers of birds from Japan and Korea. Chemosphere 49: 225–231.

104. Kannan, K., Corsolini, S., Falandysz, J., Oehme, G., Focardi, S., Giesy, J.P., 2002b. Perfluorooctanesulfonate and related fluorinated hydrocarbons in marine mammals, fishes, and birds from coasts of the Baltic and the Mediterranean Seas. Environ. Sci. Technol. 36: 3210–3216.

105. Kannan, K., Hansen, K.J., Wade, T.L., Giesy, J.P., 2002c. Perfluorooctane sulfonate in oysters, *Crassostrea virginica*, from the Gulf of Mexico and the Chesapeake Bay, USA. Arch. Environ. Contam. Toxicol. 42: 313-318.

106. Kannan, K., Newsted, J., Halbrook, R.S., Giesy, J.P., 2002d. Perfluorooctanesulfonate and related fluorinated hydrocarbons in mink and river otters from the United States. Environ. Sci.Technol 36: 2566-2571.

107. Kannan, K., Corsolini, S., Falandysz, J., Fillmann, G., Senthil Kumar, K., Loganathan, B.G., Mohd, M.A., Oliveiro, J., Van Wouwe, N., Yang, J.H., Aldous, K.M., 2004. Perfluorooctane sulfonate and related fluorochemicals in human blood from several countries. Environ. Sci. Technol. 38: 4489-4495.

108. Kawashima, Y., Suzuki, S., Kozuka, H., Sato, M., Suzuki, Y., 1994. Effects of prolonged administration of perfluorooctanoic acid on hepatic activities of enzymes which detoxify peroxide and xenobiotic in the rat. Toxicology 93: 85–97.

109. Key, B.D., Howell, R.D., Criddle, C.S., 1997. Fluorinated organics in the biosphere. Environ. Sci. Technol. 31: 2445-2454.

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

111. Khasawinah, A.M., Grutsch, J.F., 1989. Chlordane: 24-month tumorigenicity and chronic toxicity test in mice. Regul. Toxicol. Pharm. 10: 244-254.

112. Kosutzky, J., Kosutzka, E., Lencuchova, L., 1993. Long-range exposure of chickens to PCB: metabolic and immunotoxic effects. Vet. Med.-Czech 38: 287-296.

113. Kosutzky, J., Scrobanek, P., 1994. Long-range exposure of broiler chicks to PCB: clinical and biochemical changes. Vet. Med.-Czech 39: 397-405.

114. Kozuka, H., Yamada, J., Horie, S., Watanabe, T., Suga, T., Ikeda, T., 1991. Characteristics of induction of peroxisomal fatty acid oxidation-related enzymes in rat liver by drugs. Biochem. Pharmacol. 41: 617-623.

115. Kubwabo, C., Vais, N., Benoit, F.M., 2004. A pilot study on the determination of perfluorooctanesulfonate and other perfluorinated compounds in blood of Canadians. J. Environ. Monit. 6: 540-545.

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

117. Kudo, N., Kawashima, Y., 2003. Induction of triglyceride accumulation in the liver of rats by perfluorinated fatty acids with different carbon chain lengths: comparison with induction of peroxisomal beta-oxidation. Biol. Pharm. Bull. 26: 47–51.

118. Kurume Laboratory, 2002. Final report, biodegradation test of salt (Na, K, Li) of perfluoroalkyl (C = 4-12) sulfonic acid, test substance number K-1520 (test number 21520). Kurume Laboratory, Chemicals Evaluation and Research Institute, Japan.

119. Kvannes, J., Flatmark, T., 1991. A fluorimetric assay of acyl-CoA oxidase activity by a coupled peroxidatic reaction: elimination of interfering side reactions. J. Biochem. Biophys. Methods 23: 135–149.

120. Laden, F., Neas, L.M., Spiegelman, D., Hankinson, S.E., Willett, W.C., Ireland, K., Wolff, M.S., Hunter, D.J., 1999. Predictors of plasma concentrations of DDE and PCBs in a group of U.S. women. Environ. Health Persp. 107: 75-81.

121. Lake, B.G., Kozlen, S.L., Evans, J.G., Gray, T.J., Young, P.J., Gangolli, S.D., 1987. Effect of prolonged administration of clofibric acid and di-(2-

ethylhexyl)phtalate on hepatic enzyme activities and lipid peroxidation in the rat. Toxicology 44: 213-228.

122. Langston, W.J., Chesman, B.S., Burt, G.R., Pope, N.D., McEvoy, J., 2002. Metallothionein in liver of eels *Anguilla anguilla* from the Thames Estuary: an indicator of environmental quality? Mar. Environ. Res. 53: 263-293.

123. Lau, C., Rogers, J.M., Hanson, R.G., Barbee, B.D., Narotsky, M.G., Schmid, J.E., Richards, J.H., 2001. Developmental toxicity of perfluorooctane sulfonate (PFOS) in the rat and the mouse. Teratology 63: 290.

124. Lau, C., Thibodeaux, J.R., Hanson, R.G., Rogers, J.M., Grey, B.E., Stanton, M.E., Butenhoff, J.L., Stevenson, L.A., 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. Toxicol. Sci. 74: 382-392.

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

126. Leung, K.M.Y., Furness, R.W., 2001. Survival, growth, metallothionein and glycogen levels of *Nucella lapillus* (L.) exposed to subchronic cadmium stress: the influence of nutritional state and prey type. Mar. Environ. Res. 52: 173-194.

127. Liu, F.-T., 1993. S-type mammalian lectins in allergic inflammation. Immunol. Today 14: 486-490.

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

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

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

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

132. Martin, J.W., Whittle, D.M., Muir, D.C.G., Mabury, S.A., 2004b. Perfluoroalkyl contaminants in a food web from Lake Ontario. Environ. Sci. Technol. 38: 5379-5385.

133. Mathieson, S., George, S.G., McLusky, D.S., 1996. Temporal variation of total mercury concentrations and burdens in the liver of eelpout *Zoarces viviparus* from the Forth Estuary, Scotland: implications for mercury biomonitoring. Mar. Ecol.- Prog. Ser. 138: 41-49.

134. Mayes, B.A., McConnell, E.E., Neal, B.H., Brunner, M.J., Hamilton, S.B., Sullivan, T.M., Peters, A.C., Ryan, M.J., Toft, J.D., Singer, A.W., Brown, J.F.Jr., Menton, R.G., Moore, J.A., 1998. Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclor 1016, 1242, 1254, and 1260. Toxicol. Sci. 41: 62-76.

135. McEver, R.P., Moore, K.L., Cummings, R.D., 1995. Leukocyte trafficking mediated by selectin-carbohydrate interactions. J. Biol. Chem. 270: 11025-11028.

136. Mees, J., Reijnders, P.J.H., 1994. The harbour seal, *Phoca vitulina*, in the Oosterschelde: decline and possibilities for recovery. Hydrobiologia 282/283: 547-555.

137. Meijer, J., Bergstrand, A., DePierre, J.W., 1987. Preparation and characterization of subcellular fractions from the liver of C57B1/6 mice, with special emphasis on their suitability for use in studies of epoxide hydrolase activities. Biochem. Pharmacol. 36: 1139-1151.

138. Merilä, J., 1997. Expression of genetic variation in body size of collared flycatcher under different environmental conditions. Evolution 51: 526-536.

139. Moody, C.A., Martin, J.W., Kwan, W.C., Muir, D.C.G., Mabury, S.A., 2002. Monitoring perfluorinated surfactants in biota and surface water samples following an accidental release of fire-fighting foam into Etobicoke Creek. Environ. Sci. Technol. 36: 545-551.

140. Moody, C.A., Hebert, G.N., Strauss, S.H., 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, Michigan, USA. J. Environ. Monit. 5: 341-345.

141. Morgan, K., French, S.W., Morgan, T.R., 2002. Production of a cytochrome P450 2E1 transgenic mouse and initial evaluation of alcoholic liver damage. Hepatology 36: 122–134.

142. Moriwaki, H., Takata, Y., Arakawa, R., 2003. Concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in vacuum cleaner dust collected in Japanese homes. J. Environ. Monit. 5: 753-757.

143. OECD guidelines for the testing of chemicals, 1993. Volume 1, guideline 203.

144. OECD. Co-operation on existing chemicals. Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts (ENV/JM/RD(2002)17/FINAL), 2002. Available: http://www.oecd.org/dataoecd/23/18/2382880.pdf.

145. Olsen, G.W., Burris, J.M., Mandel, J.H., Zobel, L.R., 1999. Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. J. Occup. Environ. Med. 41: 799-806.

146. Olsen, G.W., Burris, J.M., Burlew, M.M., Mandel, J.H., 2003a. Epidemiologic assessment of worker serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations and medical surveillance examinations. J. Occup. Environ. Med. 45: 260-270.

147. Olsen, G.W., Logan, P.W., Hansen, K.J., Simpson, C.A., Burris, J.M., Burlew, M.M., Vorarath, P.P., Venkateswarlu, P., Schumpert, J.C., Mandel, J.H., 2003b. An occupational exposure assessment of a perfluorooctanesulfonyl fluoride production site: biomonitoring. AIHA J. 64: 651-659.

148. Olsen, G.W., Church, T.R., Miller, J.P., Burris, J.M., Hansen, K.J., Lundberg, J.K., Armitage, J.B., Herron, R.M., Medhdizadehkashi, Z., Nobiletti, J.B., O'Neill, E.M., Mandel, J.H., Zobel, L.R., 2003c. Perfluorooctanesulfonate and other fluorochemicals in the serum of American Red Cross adult blood donors. Environ. Health Persp. 111: 1892-1901.

149. Olsen, G.W., Hansen, K.J., Stevenson, L.A., Burris, J.M., Mandel, J.H., 2003d. Human donor liver and serum concentrations of perfluorooctanesulfonate and other perfluorochemicals. Environ. Sci. Technol. 37: 888-891.

150. Olsen, G.W., Church, T.R., Larson, E.B., van Belle, G., Lundberg, J.K., Hansen, K.J., Burris, J.M., Mandel, J.H., Zobel, L.R., 2004. Serum concentrations of perfluorooctanesulfonate and other fluorochemicals in an alderly population from Seattle, Washington. Chemosphere 54: 1599-1611.

151. Paucot, H., Wollast, R., 1997. Transport and transformation of trace metals in the Scheldt estuary. Mar. Chem. 58: 229-244.

152. Peakall, D., 1992. Animal biomarkers as pollution indicators. Chapman & Hall, London, UK.

153. Perillo, N.L., Pace, K.E., Seilhamer, J.J., Baum, L.G., 1995. Apoptosis of T-cells by galactin-1. Nature 378: 736-739.

154. Permadi, H., Lundgren, B., Andersson, K., De Pierre, J.W., 1992. Effects of perfluoro fatty acids on xenobiotic-metabolizing enzymes, enzymes which detoxify reactive forms of oxygen and lipid peroxidation in mouse liver. Biochem. Pharmacol. 44: 1183–1191.

155. Permadi, H., Lundgren, B., Andersson, K., Sundberg, C., De Pierre, J.W., 1993. Effects of perfluoro fatty acids on peroxisome proliferation and mitochondrial size in mouse liver: dose and time factors and effect of chain length. Xenobiotica 23: 761–770.

156. Peterson, J.S.K., Bain, L.J., 2004. Differential gene expression in anthracene-exposed mummichogs (*Fundulus heteroclitus*). Aquat. Toxicol. 66: 345-355.

157. Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: e45-50.

158. Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30: e36-45.

159. Pfaffl, M.W., Gerstmayer, B., Bosio, A., Windisch, W., 2003. Effect of zinc deficiency on the mRNA expression pattern in liver and jejunum of adult rats: monitoring gene expression using cDNA microarrays combined with real-time RT-PCR. J. Nutr. Biochem. 14: 691-702.

160. Poirier, H., Niot, I., Monnot, M.-C., Braissant, O., Meunier-Durmort C., Costet, P., Pineau, T., Wahli, W., Willson, T.M., Besnard, P., 2001. Differential involvement of peroxisome-proliferator-activated receptors α and δ in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. Biochem. J. 355: 481-488.

161. Pretti, C., Novi, S., Longo, V., Gervasi, P.G., 1999. Effect of clofibrate, a peroxisome proliferator, in sea bass (*Dicentrarchus labrax*), a marine fish. Environ. Res. 80: 294-296.

162. Queiroz, M.L.S., Bincoletto, C., Perlingeiro, R.C.R., Quadroz, M.R., Souza, C.A., 1998. Immunoglobulin levels in workers exposed to hexachlorobenzene. Hum. Exp. Toxicol. 17: 172-175.

163. Rattner, B.A., McGowan, P.C., Golden, N.H., Hatfield, J.S., Toschik, P.C., Lukei Jr, R.F., Hale, R.C., Schmitz-Alfonso, I., Rice, C.P., 2004. Contaminant exposure and reproductive success of ospreys (*Pandion haliaetus*) nesting in Chesapeake Bay regions of concern. Arch. Environ. Con. Tox. 47: 126-140.

164. Renner, R., 2001. Evidence of toxic effects and environmental impacts has sent researchers scrambling to obtain more data. Environ. Sci. Technol. 35: 154A-160A.

165. Roose, P., Cooreman, K., Vyncke, W., 1998. PCBs in cod (*Gadus morhua*), flounder (*Platichthys flesus*), blue mussel (*Mytilus edulis*) and brown shrimp *(Crangon crangon*) from the Belgian continental shelf: relation to biological endpoints and trend analysis. Chemosphere 37: 2199-2210.

166. Rusch, G.M., Rinehart, W.E., Bozak, C.A., 1979. An acute inhalation toxicity study of T-2306CoC in the rat. Project No. 78-7185. Biodynamics Inc.

167. Saito, N., Sasaki, K., Nakatome, K., Harada, K., Yoshinaga, T., Koizumi, A., 2003. Perfluorooctane sulfonate concentrations in surface water in Japan. Arch. Environ. Contam. Toxicol. 45: 149-158.

168. Saito, N., Harada, K., Inoue, K., Sasaki, K., Yoshinaga, T., Koizumi, A., 2004. Perfluorooctanoate and perfluorooctane sulfonate concentrations in surface water in Japan. J. Occup. Health 46: 49-59.

169. Sakai, T., Yasuda, T., Endo, M., 1987. Changes in myeloperoxidase and glucosephosphate isomerase activities in the neutrophil of turpentine-inflamed carp. Fish Pathol. 22: 159-162.

170. SAS Institute Inc., 1999. SAS/STAT Guide for Personal Computers, Version8. Cary, NC: SAS Institute, Inc.

171. Scarano, L.J., Calabrese, E.J., Kostecki, P.T., Baldwin, L.A., Leonard, D.A., 1994. Evaluation of a rodent peroxisome proliferator in two species of freshwater fish: rainbow trout (*Oncorynchus mykiss*) and Japanese medaka (*Oryzias latipes*). Ecotox. Environ. Safe. 29: 13-19.

172. Schreier, S., Malheiros, S.V.P., de Paula, E., 2000. Surface active drugs: self-association and interaction with membranes and surfactants. Physicochemical and biological aspects. Biochim. Biophys. Acta-Biomembr. 1508: 210-234.

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

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

175. Shabalina, I.G., Panaretakis, T., Bergstrand, A., DePierre, J.W., 1999. Effects of the rodent peroxisome proliferator and hepatocarcinogen, perfluorooctanoic acid, on apoptosis in human hepatoma HepG2 cells. Carcinogenesis 20: 2237-2246.

176. Shipley, J.M., Hurst, C.H., Tanaka, S.S., De Roos, F.L., Butenhoff, J.L., Seacat, A.M., Waxman, D.J., 2004. Trans-activation of PPAR alpha and induction of PPAR alpha target genes by perfluorooctane-based chemicals. Toxicol. Sci. 80: 151-160.

177. Simon, L.M., Laszlo, K., Kotorman, M., Vertesi, A., Bagi, K., Nemcsok, J., 1999. Effects of synthetic pyrethroids and methidation on activities of some digestive enzymes in carp (*Cyprinus carpio*). J. Environ. Sci. Heal. B 34: 819-828.

178. So, M.K., Taniyasu, S., Yamashita, N., Giesy, J.P., Zheng, J., Fang, Z., Im, S.H., Lam, P.K.S., 2004. Perfluorinated compounds in coastal waters of Hong Kong, South China, and Korea. Environ. Sci. Technol. 38: 4056-4063.

179. Sohlenius, A.-K., Messing Eriksson, A., Högström, C., Kimland, M., DePierre, J.W., 1993. Perfluorooctane sulfonic acid is a potent inducer of peroxisomal fatty acid β-oxidation and other activities known to be affected by peroxisome proliferators in mouse liver. Pharmacol. Toxicol. 72: 90-93.

180. Solé, M., Porte, C., Albaigés, J., 2001. Hydrocarbons, PCBs and DDT in the NW Mediterranean deep-sea fish *Mora moro*. Deep-sea Res. Pt. I 48: 495-513.

181. Spayd, R.W., Bruschi, B., Burdick, B.A., Dappen, G.M., Eikenberry, J.N., Esders, T.W., Figueras, J., Goodhue, C.T., LaRossa, D.D., Nelson, R.W., Rand, R.N., Wu, T.-W., 1978. Multilayer film elements for clinical analysis:

applications to representative chemical determinations. J. Clin. Chem. 24: 1343-1350.

182. Srivastava, A.S., Oohara, I., Suzuki, T., Singh, S.N., 1998. Activity and expression of glutamate oxaloacetate transaminase during the reproductive cycle of a fresh water fish *Labeo rohita*. Fisheries Sci. 64: 621-626.

183. Starkov, A.A., Wallace, K.B., 2002. Structural determinants of fluorochemical-induced mitochondrial dysfunction. Toxicol. Sci. 66: 244-252.

184. Steen, R.J.C.A., van der Vaart, J., Hiep, M., Van Hattum, B., Cofino, W.P., Brinkman, U.A.T., 2001. Gross fluxes and estuarine behaviour of pesticides in the Scheldt estuary (1995-1997). Environ. Pollut. 115: 65-79.

185. Stosik, M., Deptula, W., 1997. Resistance in carp (*Cyprinus carpio* L.) affected by a natural bacterial infection. Acta Vet.-Beograd 47: 121-128.

186. Tamura, H., Iida, T., Watanabe, T., Suga, T., 1991. Lack of induction of hepatic DNA damage on long-term administration of peroxisome proliferators in male F-344 rats. Toxicology 69: 55-62.

187. Taniyasu, S., Kannan, K., Horii, Y., Hanari, N., Yamashita, N., 2003. A survey of perfluorooctane sulfonate and related perfluorinated organic compounds in water, fish, birds, and humans from Japan. Environ. Sci. Technol. 37: 2634-2639.

188. Telford, W.G., King, L.E., Fraker, P.J., 1992. Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry. J. Cytometry 155: 1862-1872.

189. Thibodeaux, J.R., Hanson, R.G., Rogers, J.M., Grey, B.E., Barbee, B.D., Richards, J.H., Butenhoff, J.L., Stevenson, L.A., Lau, C., 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations. Toxicol. Sci. 74: 369-381.

190. Thomford, P.J., 1998. 4-Week capsule toxicity with perfluorooctane sulfonic acid potassium salt in cynomolgus monkeys. Unaudited draft. Study No. 6329-222 for 3M, St. Paul, MN, USA by Covance Laboratories Inc., Madison, WI, USA.

191. Thomford, P.J., 2000. 4-Week capsule toxicity with perfluorooctane sulfonic acid potassium salt in cynomolgus monkeys. Unaudited draft. Study No. 6329-223 for 3M, St. Paul, MN, USA by Covance Laboratories Inc., Madison, WI, USA.

192. Tomy, G.T., Tittlemier, S.A., Palace, V.P., Budakowski, W.R., Braekevelt, E., Brinkworth, L., Friesen, K., 2004. Biotransformation of N ethylperfluorooctanesulfonamide by rainbow trout (*Oncorynchus mykiss*) liver microsomes. Environ. Sci. Technol. 38, 758-762.

193. Toth, L., Juhasz, M., Varga, T., Csikkel-Szolnoki, A., Nemcsok, J., 1996. Some effects of $CuSO_4$ on carp. J. Environ. Sci. Heal. B 31: 627-635.

194. United States Environmental Protection Agency. Toxicological summary, PFOS – Dietary acute mallard study, 2004a. AR-226 docket no. 226-1734.

195. United States Environmental Protection Agency. Toxicological summary, PFOS – Dietary acute northern bobwhite study, 2004b. AR docket no. 226-1746.

196. United States Environmental Protection Agency. Toxicological summary, PFOS-dietary chronic definitive reproductive study: mallard, 2004c. AR-226 docket no. 226-1741.

197. United States Environmental Protection Agency. Toxicological Summary, PFOS-dietary chronic definitive reproductive study: northern bobwhite, 2004d. AR-226 docket no. 226-1752.

198. Vaglio, A., Landriscina, C., 1999. Changes in liver enzyme activity in the teleost *Sparus aurata* in response to cadmium intoxication. Ecotox. Environ. Safe. 43: 111-116.

199. Van Alsenoy, V., Bernard, P., Van Grieken, R., 1993. Elemental concentrations and heavy metal pollution in sediments and suspended matter from the Belgian North Sea and the Scheldt estuary. Sci. Total Environ. 133: 153-181.

200. Vandelannoote, A., Yseboodt, R., Bruylants, B., Verheyen, R., Coeck, J., Maes, J., Belpaire, C., Van Thuyne, G., Denayer, B., Beyens, J., De Charleroy, D., Vandenabeele, P., 1998. Atlas van de Vlaamse beek- en riviervissen. Water-Energik-Vlario, Wijnegem, Belgium.

201. van den Brink, N.W., Bosveld, A.T.C., 2001. PCB concentrations and metabolism in common terns (*Sterna hirundo*) from different breeding colonies in the Netherlands. Mar. Pollut. Bull. 42: 280-285.

202. van den Broek, W.L.F., 1978. Dietary habits of fish populations in the Lower Medway Estuary. J. Fish Biol. 13: 645-654.

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

204. Vanden Heuvel, J.P., Kuslikis, B.I., Van Rafelghem, M.J., Peterson, R.E., 1991b. Disposition of perfluorodecanoic acid in male and female rats. Toxicol. Appl. Pharmacol. 107: 450–459.

205. Van de Vijver, K.I., Hoff, P.T., Van Dongen, W., Esmans, E.L., Blust, R., De Coen, W.M., 2003a. Exposure patterns of perfluorooctane sulfonate in aquatic invertebrates from the Western Scheldt estuary and the southern North Sea. Environ. Toxicol. Chem. 22: 2037-2041.

206. Van de Vijver, K.I., Hoff, P.T., Das, K., Van Dongen, W., Esmans, E.L., Jauniaux, T., Bouquegneau, J.-M., Blust, R., De Coen, W.M., 2003b. Perfluorinated chemicals infiltrate ocean waters: link between exposure levels and stable isotope ratios in marine mammals. Environ. Sci. Technol. 37: 5545-5550.

207. Van de Vijver, K.I., Hoff, P.T., Das, K., Van Dongen, W., Esmans, E.L., Siebert, U., Bouquegneau, J.-M., Blust, R., De Coen, W.M., 2004. Baseline study of perfluorochemicals in harbour porpoises (*Phocoena phocoena*) from Northern Europe. Mar. Pollut. Bull. 48: 992-997.

208. Vandorpe, E., Verhagen, R., 1979. An age reference model for the wood mouse, *Apodemus sylvaticus* (Linnaeus, 1758), by use of the lens technique. Annales Soc. r. Zool. Belg. 109: 133–140.

209. van Leeuwen, C.J. and Hermens, J.L.M., 1995. Risk assessment of chemicals. An Introduction. Kluwer Academic Publishers, Dordrecht, The Netherlands.

210. Vannecke, D., Leclercq, G., Plum, J., Vandekerckhove, B., 1995. Characterization of distinct stages during the differentiation of human CD69+ CD3+ thymocytes and identification of thymic emigrants. J. Immunol. 155: 1862-1872.

211. Voorspoels, S., Covaci, A., Schepens, P., 2003. Polybrominated diphenyl ethers in marine species from the Belgian North Sea and the Western Scheldt Estuary: levels, profiles, and distribution. Environ. Sci. Technol. 37: 4348-4357.

212. Vricella, L.A., Debegona, J.A., Gundry, S.R., Vigesaa, R.E., Kawauchi, M., Bailey, L.L., 1992. Aggressive peritoneal dialysis for treatment of acute kidney failure after neonatal heart transplantation. J. Heart Lung Transplant. 11: 320–329.

213. Watanabe, M., Tanabe, S., Tatsukawa, R., Amano, M., Miyazaki, N., Petrov, E.A., Khuraskin, S.L., 1999. Contamination levels and specific accumulation of persistent organochlorines in Caspian seal (*Phoca caspica*) from the Caspian Sea, Russia. Arch. Environ. Contam. Toxicol. 37: 396-407.

214. Wendelaar Bonga, S.E., Lock, R.A.C., 1992. Toxicants and osmoregulation in fish. Neth. J. Zool. 42: 478-493.

215. Werle, E., Schneider, C., Renner, M., Volker, M., Fiehn, W., 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res. 22: 4354-4355.

216. Wilson, R.W., Wood, C.M., Houlihan, D.F., 1996. Growth and protein turnover during acclimation to acid and aluminium in juvenile rainbow trout (*Oncorynchus mykiss*). Can. J. Fish. Aquat. Sci. 53: 802-811.

217. Xu, L., Krenitsky, D.M., Seacat, A.M., Butenhoff, J.L., Anders, M.W., 2004. Biotransformation of N-ethyl-N-(2-hydroxyethyl)perfluorooctanesulfonamide by rat liver microsomes, cytosol, and slices and by expressed rat and human cytochromes P450. Chem. Res. Toxicol. 17: 767-775.

218. Yagi, K., 1976. A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 15: 212–216.

219. Yang, J.-H., Kostecki, P.T., Calabrese, E.J., Baldwin, L.A., 1990. Induction of peroxisome proliferation in rainbow trout exposed to ciprofibrate. Toxicol. Appl. Pharmacol. 104: 476-482.

220. Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., Speed, T.P., 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. 30: e15-e24.

221. York, R.G., Case, M.T., Christian, M.S., 2000. Rat and rabbit developmental toxicology studies with two perfluorinated compounds administered orally. Int. J. Toxicol. 19: 35 [Abstract].