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TISSUE DISTRIBUTION STUDIES AND RISK ASSESSMENT OF PERFLUOROALKYLATED AND POLYFLUOROALKYLATED SUBSTANCES (PFASS)

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Cover: A whole-body autoradiogram of a male mouse 48 hours after dosing with ^{35}S-labelled perfluorooctane sulfonate (PFOS).

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ABSTRACT

Perfluoroalkylated and polyfluoroalkylated substances (PFASs) represent a large class of man-made chemicals. These substances have emerged as environmental contaminants due to their extraordinary resistance to degradation, potential for bioaccumulation, toxicity and a global presence in humans, wildlife and the environment. In the Swedish population 17 PFASs have so far been analyzed in blood. In animal studies, PFASs cause liver toxicity and reproductive/developmental toxicity as well as a range of other toxic effects. Detailed data on the tissue distribution of PFASs, which could contribute to better understanding of their toxicity, are limited. Also, health risk assessment information has been lacking for all PFASs except the most studied, perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA).

The aims of this thesis were to 1) generate detailed tissue distribution data on PFOS in perinatal and adult mice and on its replacement chemical perfluorobutane sulfonate (PFBS) in adult mice; and 2) and assess potential risks to human health associated with exposure to the 17 PFASs analyzed in the general Swedish population and occupationally exposed ski waxers, for all PFASs individually and in combination.

The results of the experiments showed that following exposure of pregnant dams PFOS was readily transferred to mouse fetuses resulting in tissue levels similar to or higher than maternal blood levels. PFOS was markedly distributed to the perinatal and maternal lungs; showing the highest levels of the tissues analyzed in fetuses/pups on gestational day 20 and postnatal day 1. This finding may help to explain the respiratory distress seen in neonatal and adult rodents following exposure to PFOS. Further, in adult male mice after short-term dietary exposure to one environmentally relevant low dose and one experimentally relevant high dose, PFOS was recovered in all 19 examined tissues, with similar tissue distribution profiles at both doses, though with a higher tissue:blood ratio at the higher dose. The highest concentrations of PFOS were found in liver, lungs, blood, kidneys and whole bone and the major body compartments were liver, bone, blood, skin and muscle. Blood hemoglobin levels were markedly increased at the high dose which could be connected to the localization of PFOS in bone marrow. In a similar experiment PFBS was recovered in all 20 examined tissues in adult male mice after short-term dietary exposure to the same molar concentration as the high dose of PFOS. The distribution and compartment profiles were similar to those of PFOS with the exception of a remarkably high concentration in cartilage. Also, PFBS displayed significantly lower tissue concentrations and tissue: blood ratios than PFOS and a less marked erythropoietic effect.

The risk assessment of PFASs showed that hepatotoxicity and reproductive/ developmental toxicity may be of concern for high local exposure and occupational exposure but indicated no risk for the general population. Concern for the less studied endpoints immunotoxicity and altered mammary gland development was identified for the general population and the occupationally exposed. A need of additional toxicological data for all investigated toxicological endpoints was recognized.

Altogether, the work included in this thesis has generated experimental data that can be used to improve risk assessment of PFASs. It has also assessed the risks associated with current exposures to PFASs in Sweden and identified data needs.

LIST OF PUBLICATIONS

This thesis is based on the four papers listed below, referred to in the text by their roman numerals

I. **Borg D**, Bogdanska J, Sundström M, Nobel S, Håkansson H, Bergman Å, DePierre JW, Halldin K, Bergström U. Tissue distribution of ^{35}S-labelled perfluorooctane sulfonate (PFOS) in C57B1/6 mice following late gestational exposure. Reprod Toxicol, 2010, 30(4):558-565.

II. Bogdanska J, **Borg D**, Sundström M, Bergström U, Halldin K, Abedi-Valugerdi M, Bergman Å, Nelson B, DePierre JW, Nobel S. Tissue distribution of ^{35}S-labelled perfluorooctane sulfonate in adult mice after oral exposure to a low environmentally relevant dose or a high experimental dose. Toxicology, 2011, 284, 54-62.

III. Bogdanska J, Sundström M, Bergström U, **Borg D**, Abedi-Valugerdi M, Bergman Å, DePierre JW, Nobel S. Tissue distribution of ^{35}S-labelled perfluorobutane sulfonic acid in adult mice following dietary exposure for 1-5 days. (*Submitted for publication).*

IV. **Borg D**, Lund B-O, Lindquist N-G, Håkansson H.Cumulative health risk assessment of 17 perfluoroalkylated and polyfluoroalkylated substances (PFASs) in the Swedish population. *(Submitted for publication)*

PUBLICATIONS NOT INCLUDED IN THE THISIS

I. Goodwin RJA, Nilsson A, **Borg D,** Langridge-Smith PRR, Harrison DJ, Mackay CL, Iverson SL, Andrén PE. Conductive carbon tape used for support and mounting of both whole animal and fragile heat-treated tissue sections for MALDI MS imaging and quantitation. J Proteomics. 2012, 75(16):4912-20.

CONTENTS

LIST OF ABBREVIATIONS

6:2 FTS		6:2 Fluorotelomer sulfonate
8:2 FTOH		8:2 Fluorotelomer alcohol
AF		Assessment Factor
AFFFs		Aqueous film-forming foams
ANOVA		Analysis of variance
BCF		Bioconcentration factor
BMD		Benchmark dose
CA		Concentration addition
DPM		Decays per minute
GD		Gestational day
HI		Hazard Index
HQ		Hazard Quotient
LOAEL		Lowest-observed-adverse-effect-level
N-EtFOSE		N-ethyl perfluorooctane sulfonamidoethanol
NOAEL		No-observed-adverse-effect-level
PFAA		Perfluoroalkylated acid
PFASs		Perfluoroalkylated and polyfluoroalkylated substances
PFCA		Perfluoroalkylated carboxylic acid
	PFBA	Perfluorobutanoic acid
	PFPeA	Perfluoropentanoic acid
	PFHxA	Perfluorohexanoic acid
	PFHpA	Perfluoroheptanoic acid
	PFOA	Perfluorooctanoic acid
	PFNA	Perfluorononanoic acid
	PFDA	Perfluorodecanoic acid
	PFUnDA	Perfluoroundecanoic acid
	PFDoDA	Perfluorododecanoic acid
	PFTrDA	Perfluorotridecanoic acid
	PFTeDA	Perfluorotetradecanoic acid
PFOSA		Perfluorooctane sulfonamide
PFSA		Perfluoroalkylated sulfonic acid
	PFBS	Perfluorobutane sulfonate
	PFHxS	Perfluorohexane sulfonate
	PFOS	Perfluorooctane sulfonate
	PFDS	Perfluorodecane sulfonate
PND		Postnatal day
POD		Point of departure
POP		Persistent Organic Pollutant
PPAR		Peroxisome Proliferator Activated Receptor
TDI		Tolerable daily intake

1 INTRODUCTION

1.1 CHEMICALS AND ENVIRONMENTAL CONTAMINANTS

Our society is characterized by a broad use of chemicals and humans are exposed directly or indirectly to a variety of chemical substances on a daily basis. As a result of their intrinsic properties some chemicals end up in the environment and in human populations, even in remote areas where they have never been produced or used. An increasing number of experimental and epidemiological studies indicate that exposure to low doses of certain chemicals may give rise to a wide range of adverse health effects, including neurotoxicity, metabolic diseases, immune deficiency, impaired reproduction and cancer (reviewed in Hotchkiss et al. 2008; reviewed in Wigle et al. 2008). Fetuses are particularly sensitive due to critical developmental periods and the effects may manisfest first much later in life. The World Health Organization (WHO) estimates that more than 25% of the global disease burden is linked to environmental factors, including exposure to hazardous chemicals (IPCS/WHO 2010). In the view of a ten-fold worldwide increase in production and use of chemicals during the last 40 years, which is expected to increase even further (IPCS/WHO 2010), it is of great importance that we have sufficient knowledge about chemicals and how to use them in ways that are safe for human health and the environment.

A number of individual chemicals and groups of chemicals have during the past 50 years been classified as environmental contaminants or persistent organic pollutants (POPs) based on their persistent, bioaccumulative and toxic properties. In particular, many of these contaminants are halogenated hydrocarbons containing chlorine, bromine or fluorine. In the 1960s and 1970s, chlorinated compounds such as the insecticide DDT, the polychlorinated biphenyls (PCBs) used as electric insulators, and dioxins which are formed unintentionally in various industrial and combustion processes, were discovered and recognized as POPs (Naturvårdsverket 1998; UNEP 2013). In the 1970s and 1980s, brominated compounds, used as flame retardants in potentially flammable materials, were discovered in humans and wildlife far from local sources and identified as environmental contaminants (Law et al. 2003; Rahman et al. 2001; Sjödin et al. 2003). In the late 1990s and the early 2000s, the perfluorinated and polyfluorinated substances (PFASs) were found in wildlife and human blood, similarly as the chlorinated and brominated compounds.

1.2 PER- AND POLYFLUOROALKYLATED SUBSTANCES (PFASS)

Perfluoroalkylated and polyfluoroalkylated substances (PFASs) belong to a family of more than 800 man-made, highly fluorinated, organic chemicals that have been used since the 1950s as components of and precursors for surfactants and surface protectors in industrial and consumer applications (3M 1999; Kissa 2001; OECD 2007).

In 2001, it was reported that perfluorooctane sulfonate (PFOS) was present globally in a wide range of wildlife species (Giesy and Kannan 2001; Giesy et al. 2001; Kannan et al. 2001). Similarly in 2001, the presence of PFOS, perfluorooctanoic acid (PFOA) and other PFASs in the blood of the general population in the Unites States was reported

(Hansen et al. 2001). The authors also suggested that PFASs were responsible for a considerable fraction of the organic fluorine that has been detected in serum of non-occupationally exposed human populations since the 1960s, but where compound-specific analytical methods were not available at that time. Since 2001, a large number of biomonitoring and toxicological studies on PFASs have been published and PFASs have been recognized as highly persistent environmental contaminants and generated concern due to their global presence in humans, wildlife and the environment.

1.2.1 Physicochemical properties

PFASs are characterized by their fully (*per*-) or partly (*poly*-) fluorinated carbon chains, typically four to fifteen carbons long and with a functional group at one of their tails (Figure 1). Due to the strength of the carbon-fluorine bond, one of the strongest chemical bonds known, PFASs are extremely resistant towards thermal, chemical and biological degradation (Järnberg et al. 2006; Kissa 2001). In addition, the fluorinated carbon chain is both oil-and water repellent (Kissa 2001), making PFASs useful in many industrial and consumer applications. However, their resistance to degradation also makes them persistent in the environment. Perfluorinated sulfonates and carboxylates are considered stable end-stage products that will not degrade under any normal environmental circumstances (Järnberg et al. 2006). They can, however, be generated from the degradation of precursor molecules, e.g. the polyfluorinated 8:2 fluorotelomer alcohol (8:2 FTOH) that can generate PFOA with the same "backbone" structure (reviewed in Frömel and Knepper. 2010, Figure 1). Perfluorinated sulfonates and carboxylates are strong acids and mainly present in their non-volatile acid forms in the environment and in biota. Fluorotelomers, on the other hand, are volatile and can be transported in the atmosphere (Houde et al. 2006).

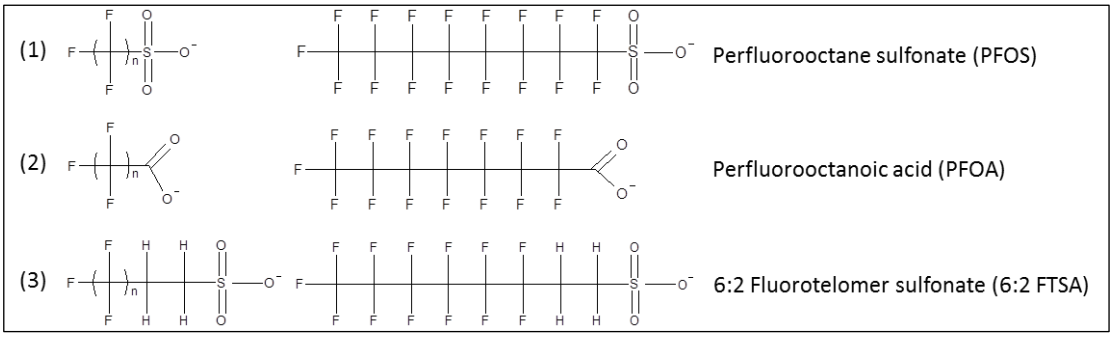

Figure 1. Schematic chemical structures of perfluorinated sulfonates (1), carboxylates (2) and fluorotelomer alcohols (3), including perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA) and 6:2 FTSA.

1.2.2 Nomenclature

PFASs are named according to the number of carbons on the alkyl chain and their respective functional group, e.g. the four carbon chain with a sulfonate group is named perfluorobutane sulfonate (PFBS). For many years there has been an inconsistent terminology used for PFASs, with different and sometimes overlapping abbreviations. Recently, however, Buck et al. (2011) proposed a terminology and classification scheme for PFASs which is also used herein:

- PFASs = Perfluoroalkylated and polyfluoroalkylated substances (singular PFAS).
- PFAA = Perfluoroalkylated acid.
- PFSA = Perfluoroalkylated sulfonic acid.
- PFCA = Perfluoroalkylated carboxylic acid.
- Long-chain PFASs = PFSAs with ≥ 6 carbons and PFCAs with ≥ 8 carbons.
- Homologues = Different PFASs sharing the same functional group.

Further, the term "congener" used herein represents individual PFASs.

1.2.3 Uses of PFASs

PFASs have been widely used as components of and precursors for surfactants and surface protectors in industrial applications and consumer products. Examples include impregnating agents for clothing and textiles, coatings for paper and packaging, waxes (including ski waxes) and cleaning agents, insecticides, fire-fighting foams, hydraulic fluids in airplanes and process chemicals in the manufacture of fluoropolymers such as Teflon® and Gore-Tex® (3M 1999; KemI 2009; Kissa 2001).

1.2.4 Sources to the environment

PFASs can be released to the environment during their entire life-cycle, at production and use, from products containing PFASs, and after their use as leakage from e.g. waste disposals and landfills. Aqueous film-forming foams (AFFFs) used for fire-fighting have been pointed out as a significant source of PFOS and other PFASs, as demonstrated by elevated levels around fire-fighting training areas (Weiss et al. 2012) and airports where AFFFs have been used (Moody et al. 2003; Nunes et al. 2011). Also, atmospheric degradation of volatile precursor molecules, e.g. fluorotelomers forming PFCAs is likely to occur (Ellis et al. 2004). There is and has not been any production of PFASs in Sweden (KemI 2006) and the PFASs detected in the Swedish environment is likely a result of release from industrial and consumer use of PFASs and PFASs-containing products, leakage from waste disposals, landfills and sewage treatment plants as well as from atmospheric import.

1.2.5 Biological behavior

In the environment, PFASs are associated mostly to aquatic ecosystems. This is likely due to their solubility in water (reviewed in Rayne and Forest. 2009) and their ability to bioaccumulate in fish, with the bioconcentration factor (BCF) being proportional to carbon chain length, at least up to a chain length of 11 carbons (Martin et al. 2003). PFASs can also biomagnify in food chains, as demonstrated by the highest levels being found in top predators such as the polar bear, mink, otter and seal (Giesy and Kannan 2001; Kannan et al. 2002, 2005; Kelly et al. 2009). In contrast to "classic" persistent organic contaminants, e.g. chlorinated and brominated compounds, PFASs does not distribute to and store up in fatty tissues in living organisms, but bind to proteins such as albumin in liver, plasma and eggs, and fatty acid binding proteins in cells due to their structural similarity to endogenous fatty acids (Kannan et al. 2005; Kerstner-Wood et al. 2004; Luebker et al. 2002).

1.2.6 Human exposure

1.2.6.1 Sources

Food, in particular fish and seafood, is proposed to be the main source of human exposure for several PFASs and may account for as much as 99% and 84% of the total PFOS and PFOA intake, respectively (Haug et al. 2010). However, dust may also represent a major source on an individual basis (Haug et al. 2011). Fetuses are exposed via placental transfer during pregnancy (Kim et al. 2011) and breast milk is the main source of PFASs for infants (Haug et al. 2011), through which their intake may equal the dietary intake in adults (Thomsen et al. 2010). The highest exposures occur in occupational settings, most likely through inhalation of PFASs-containing aerosols and dust (reviewed in ATDSR 2009; Vestergren and Cousins 2009). The highest PFASs levels have been detected in individuals in PFASs production facilities (reviewed in Lau et al. 2007). In Sweden, professional ski waxers have shown the highest serum concentrations of PFASs (Nilsson et al. 2010).

1.2.6.2 Measures of human exposure to PFASs

Serum concentrations of PFASs are commonly used as measure of human exposure. Blood/serum is a suitable matrix to analyze based on the relatively long half-lives of many PFASs in humans, spanning from months to years (reviewed in Lau 2012a). One advantage of using blood/serum concentrations is also that these represent an integrated measure of exposure for PFSAs and PFCAs irrespective of the source, e.g. precursor molecules that can be metabolized to e.g. PFOS and PFOA. Another advantage of using serum concentrations is that it enables easy comparisons to internal dose levels in animal studies and facilitates kinetic extrapolations from animals to humans.

1.2.6.3 Human exposure levels

In human populations of western countries such as Sweden, Germany and the United States, PFAS congeners are commonly found at low ng/ml serum concentrations, (Glynn et al. 2012; Olsen et al. 2012; Schroter-Kermani et al. 2012). Higher ng/ml serum concentrations of PFOS and PFOA have occasionally been detected, then associated with high local exposures due to contaminated food and ground water (Emmett et al. 2006; Hovgard et al. 2009). Temporal trend studies have shown that PFOS, the current and for a long time dominant PFAS congener in human serum in western countries (Glynn et al. 2012; Haug et al. 2009; Kato et al. 2011), now shows a decreasing trend together with PFOA in western countries (Haug et al. 2009; Kato et al. 2011; Olsen et al. 2012; Schroter-Kermani et al. 2012) including Sweden (Glynn et al. 2012; Figure 13). This is likely due to the phase-out of PFOS-related production in 2002 by the major manufacturer (3M, 2011) and an ongoing phase-out of PFOA by some manufacturers (U.S. EPA, 2010). In contrast, serum concentrations of PFBS, perfluorohexane sulfonate (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnDA) in individuals living in the Swedish city Uppsala have been shown to increase (Glynn et al. 2011). The increase in PFBS levels is likely due to its introduction as a replacement chemical for the six- and eight carbon homologues (Ehresman et al.

2007). The increase in PFHxS serum levels, different to the trend observed in other western countries (Olsen et al. 2012; Schroter-Kermani et al. 2012), could be due to the recent detection of PFHxS in Uppsala's municipal water (Glynn 2012).

Occupationally exposed individuals are highly exposed as compared to the general population. Studies on workers in PFASs manufacturing facilities have shown levels in the low µg/ml serum range (Olsen et al. 2003), i.e. up to a thousand-fold higher than in the general population. In Sweden, where no production of PFASs occur, the most highly exposed are likely professional ski waxers (Nilsson et al. 2010). The probable reason is that PFCAs are constituents of certain gliding waxes (Freberg et al. 2010) and a correlation between serum concentrations of PFCAs and the number of working years have been found, in contrast to serum concentrations of PFSAs where no such correlations have been found (Nilsson et al. 2010).

1.2.7 Kinetics and tissue distribution

1.2.7.1 Absorption

Studies in rodents have shown high oral absorption of PFASs. In rats, bioavailability of nearly 100% have been shown for PFOS, PFOA and perfluorohexanoic acid (PFHxA) (Gannon et al. 2011, Gibson and Johnson 1979; Johnson et al. 1979) and high absorption rates have also been demonstrated for perfluorobutanoic acid (PFBA) and PFBS (Chang et al. 2008; Olsen et al. 2009). Absorption for other congeners can also be assumed to be high based on their similar physicochemical properties. Quantitative studies on other exposure routes, i.e. inhalation or dermal absorption, are lacking. However, toxicity studies on PFOS and PFOA using these exposure routes demonstrate absorption based on observed toxic effects (Kennedy et al. 2004; OECD 2002). No human PFASs absorption data is available.

1.2.7.2 Distribution

PFASs tissue distribution data are limited. A few animal studies have until now investigated the tissue distribution of PFOS, PFOA and PFDA, showing that these PFASs are present at the highest levels in liver and serum, followed by kidneys and lungs (Hundley et al. 2006; Johnson et al. 1979; Kudo et al. 2006; Vanden Heuvel et al. 1991a, b). These findings have been confirmed in a human post-mortem study showing highest levels of PFOS in liver, lungs, kidneys and blood and highest levels of PFOA in lungs, kidneys, liver and blood (Maestri et al. 2006). However, detailed tissue distribution data have been essentially nonexistent for all PFASs, including different life-stages and doses, with the exception of one study using two doses of PFOA in rats, showing a larger proportion distributed to liver at the lower dose and a larger proportion distributed to serum and other tissues at the higher dose (Kudo et al. 2006). The characteristic distribution of PFASs to liver and serum is, at least partly, due to their high affinity to proteins. A large number of PFASs have been shown to be highly bound to serum albumin (Bischel et al. 2011; Kerstner-Wood et al. 2004) and PFOS and PFOA have been shown to bind to the liver-fatty acid binding protein (L-FABP), a hepatic intracellular fatty-acid binding protein (Luebker et al. 2002).

A 1:1 ratio for human serum and plasma levels of a number of PFAAs have been shown (Ehresman et al. 2007) and levels in these matrices can thus be considered comparable. Also, a 2:1 ratio for serum to whole blood was shown, equal to the volume displacement by the red blood cells in serum. Thus whole blood levels of these PFAAs can be doubled to obtain their corresponding serum levels. This could be assumed also for other PFAAs based on their similar physicochemical properties. In contrast, the non-charged perfluorooctane sulfonamide (PFOSA) was shown to deviate from this ratio and distributes to a larger extent to whole blood than plasma (Kärrman et al. 2006).

1.2.7.3 Metabolism

No metabolism has been shown for PFSAs or PFCAs. Studies on PFOS, PFOA and perfluorodecane sulfonate (PFDS) in rats have shown that they are excreted without forming any metabolites or conjugates (OECD 2002; U.S. EPA 2005; Vanden Heuvel et al. 1991). Thus, PFSAs and PFCAs are believed to represent metabolically inert and stable end-stage products. However, certain precursor PFASs have in rodents been shown to transform, to various extents, into their perfluorinated sulfonate or carboxylate "backbone structures", e.g. PFOSA and N-ethyl perfluorooctane sulfonamidoethanol (N-EtFOSE) into PFOS, and the polyfluorinated 8:2 FTOH into PFOA and PFNA (3M 2003; Henderson and Smith 2007; Seacat and Luebker 2000).

1.2.7.4 Excretion

One characteristic for PFASs is a marked difference in elimination kinetics depending on carbon chain length, species and sex (reviewed in Lau 2012a, Table 1). The major elimination route of PFASs is urinary excretion and to a smaller extent biliary and fecal excretion (Han et al. 2012). In general the rate of elimination from serum 1) increases with decreasing carbon chain length, 2) occurs more rapidly in rats > mice > non-human primates > humans, 3) is faster for carboxylates than for the corresponding sulfonates, and 4) show pronounced sex differences within certain species (e.g., faster elimination in female rodents). The reason for these differences in elimination is likely that PFASs are substrates to renal organic anion transporters (Han et al. 2012), regulating active renal reabsorption, and these transporters are differentially expressed between species and sex and have shown varying affinities for different PFASs carbon chain lengths (Han et al. 2012; Kudo et al. 2002; Weaver et al. 2010).

In both humans and animals, PFASs are transferred to the fetus via the placenta and to the offspring via breast milk (reviewed in ATDSR 2009). Studies in humans have shown varying rates of placental and breast milk transfer between congeners, with levels in fetal serum ranging from 30% to 200% of that in maternal serum, but with most congeners showing a lower concentration in fetal serum (reviewed in Fromme et al. 2010; Gutzkow et al. 2012; Kim et al. 2011; Liu et al. 2011). Levels in breast milk have been shown to range from 1% to 12% of that in maternal serum (Kärrman et al. 2007; Kim et al. 2011; Liu et al. 2011).

Table 1. Serum half-lives of PFAS congeners in different species (including humans).

Congener	Rat	Mouse	Monkey	Human	References
PFBS	0.6 - 4.0h (♀) 2.1 - 4.5h (♂)	NA	15h - 3.5d (♀) 8.1h - 4d (♂)	46d (♀) 24d (♂)	Chengelis et al. 2009; Olsen et al. 2009
PFHxS	1d (♀) 30d (♂)	25 - 27d (♀) 28 - 31d (♂)	87d (♀) 141d (♂)	8.5y	Olsen et al. 2007; Sundstrom et al. 2012a
PFOS	62 - 71d (♀) 38 - 41d (♂)	30 - 38d (♀) 36 - 43d (♂)	110d (♀) 132d (♂)	5.4y	Chang et al. 2012; Olsen et al. 2007
PFBA	1 - 2h (♀) 6 - 9h (♂)	3h (♀) 5 - 16h (♂)	1.7d (♂, ♀)	3.6d (♀) 3.0d (♂)	Chang et al. 2008
PFHxA	0.4 - 1.2h (♀) 1.0 - 2.4h (♂)	N.A.	2.4h (♀) 5.3h (♂)	N.A.	Chengelis et al. 2009; Gannon et al. 2011; Ohmori et al. 2003
PFOA	2 - 4h (♀) 4 - 6d (♂)	17d (♀) 19d (♂)	33d (♀) 21d (♂)	3.8y	Butenhoff et al. 2004; Kemper and Jepson 2003; reviewed in Lau et al. 2007; Ohmori et al. 2003; Olsen et al. 2007
PFNA	1 - 2d (♀) 30 - 31d (♂)	26 - 68d(♀) 34 - 69d (♂)	NA	NA	Ohmori et al. 2003; Tatum-Gibbs et al. 2011
PFDA	59d (♀) 40d (♂)	NA	NA	NA	Ohmori et al. 2003

h = hours, d = days, y = years, N.A. = not available

1.2.8 Toxicity

1.2.8.1 Hepatotoxicity

Different PFASs show relatively similar toxicological profiles. Repeated-dose studies in rodents and monkeys point out the liver as a main target organ (Lau et al. 2007). The PFASs-induced hepatotoxicity is on a cellular level, with increasing dose, manifested as hepatocellular hypertrophy → hepatocellular vacuolation → hepatocellular pigmentation → hepatocellular necrosis (reviewed in ATSDR 2009). The hepatocellular hypertrophy (liver cell enlargement) occurs rapidly following PFASs exposure, aggravating little with time, and is reversible upon cessation of exposure. The hepatocellular vacuolation (formation of cytoplasmic vacuoles), and pigmentation (accumulation of lipofuscin, believed to represent lysosomal accumulation of poorly digested lipid (Haschek et al. 2002)), observed at higher doses (Butenhoff et al. 2012), have also shown to be reversible after end of exposure. At the highest dose levels hepatocellular necrosis (cell death) occurs. In addition, increased liver weight occur following PFASs exposure in rodents as well as non-human primates, and PFOS and PFOA have been shown to cause liver tumors in rodents likely via non-genotoxic mechanisms (reviewed in Lau et al. 2007).

1.2.8.2 Reproductive and developmental toxicity

A number of PFASs have shown reproductive and developmental toxic properties following *in utero* exposure. The toxicity is manifested as reduced fetal, perinatal (the time period shortly before and after birth) and/or neonatal (newborn) body weight and viability as well as reduced pup body-weight gain and litter loss in the dams (reviewed in Lau et al. 2007). The most adverse of these toxic effects is a dose-dependent marked increase in neonatal mortality that has been observed for PFOS, PFOA and PFNA (reviewed in Lau et al. 2004; Wolf et al. 2007, 2010). On the basis of labored breathing and cyanosis, the neonatal mortality is proposed to be due to disrupted pulmonary function, but the exact reason has not been clarified. For PFOS, morphological indications of delayed or impaired lung maturation have been shown (Grasty et al. 2003, 2005) and a direct interaction of PFOS with components of the pulmonary surfactant has also been suggested as an underlying mechanism (Abbott et al. 2009; Lehmler et al. 2006). Cross-fostering studies have revealed that the effects occurring after PFOS and PFOA exposure are due to gestational exposure (Luebker et al. 2005a; Wolf et al. 2007) and a critical window of exposure has been identified with administration as late as gestational day (GD) 19 being sufficient to induce this toxicity (Grasty et al. 2003).

In addition, PFOS and PFOA have shown also other reproductive/developmental toxic effects in rodents following *in utero* exposure, such as developmental neurotoxicity (Johansson et al. 2008; Onishchenko et al. 2011; Viberg et al. 2013) delayed sexual maturation (Lau et al. 2004), impaired mammary gland development (White et al. 2007) and histopathological changes in the female reproductive tract (Dixon et al. 2012).

1.2.8.3 Other types of toxicity

In addition to hepatotoxicity and reproductive/developmental toxicity, other common toxic effects observed in rodents and primates following repeated PFASs exposure include decreased body weight, effects on lipid metabolism (decreased serum cholesterol and triglycerides) and thyroid hormone levels (decreased triiodothyronine (T_3) and thyroxine (T_4)), immunotoxicity (atrophy of thymus and spleen and reduced antigen response) (reviewed in Lau et al. 2007) and respiratory distress also in adult rodents (Cui et al. 2009). In addition, exposure of mice to a low dose of PFOA *in utero* has been shown to induce overweight and affect metabolic hormone levels in adult life (Hines et al. 2009).

1.2.9 Mode of action

The mode(s) of action for PFASs has not been clarified. PFASs show a structural analogy to endogenous fatty acids (reviewed in Lau 2012a) and are treated as fatty acids in the body, such as transport on albumin in blood (Bischel et al. 2011) and intracellular binding to fatty acid binding proteins (FABP) (Luebker et al. 2002). Also, as endogenous fatty acids, PFASs are ligands of the peroxisome-proliferator activated receptor alpha (PPAR-α) (Wolf et al. 2008), a nuclear receptor and regulator of lipid metabolism (Berger and Moller 2002). Compounds that bind PPAR-α induce proliferation of peroxisomes ("peroxisome proliferators") leading to catabolism of fatty acids and cholesterol, particularly in the liver which is the main organ for lipid storage and mobilization (Lee et al. 2003). Peroxisome proliferators induce hepatocellular hypertrophy and increased liver weight by increasing the number and size of peroxisomes (Berger and Moller 2002). Peroxisome proliferators are also associated with liver tumors in rodents, however this effect is not considered relevant for humans since it is not observed in humans or non-human primates (Peters et al. 2005). PFASs have shown increasing affinity for PPAR-α with increasing chain lengths *in vitro* and PFCAs were stronger activators of PPAR-α than PFSAs (Wolf et al. 2008). On the other hand, it has also been shown that increased liver weight and peroxisomal β-oxidation in rodents following PFASs exposure is not correlated to the length of the carbon chain, but to the hepatic concentration of the congener (Kudo and Kawashima 2003; Kudo et al. 2000, 2006). In humans the PPAR-α receptor is expressed to a lower extent than in rodents and appears to be less sensitive to the effects of PFASs (Albrecht et al. 2013; reviewed in Klaunig et al. 2003; Wolf et al. 2008, 2012). Though PFASs bind PPAR-α and induce effects similar to peroxisome proliferators in rodents and non-human primates PFOS have been shown to do so without affecting markers for peroxisome proliferation, indicating that other mechanisms of action are involved (Lau et al. 2007). This is supported by findings in PPAR-α knockout mice showing hepatotoxicity following exposure to PFOA, but not following exposure to the prototypic PPAR-α-ligand WY 14,643, suggesting that these effects are independent of PPAR-α (Wolf et al. 2008; Figure 2 and 3).

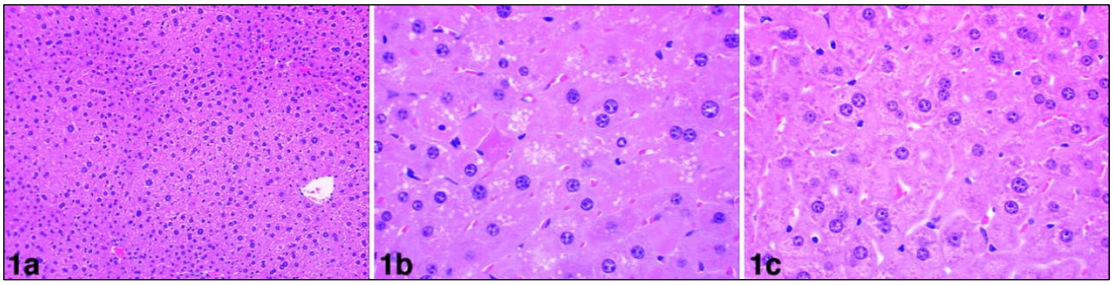

Figure 2. HE-stained livers from wild-type SV/129 mice. Control wild-type mice (1a, 200x original mag). Wild-type mice (1b, 600x original mag) treated with 10 mg/kg PFOA had diffuse hepatocyte hypertrophy with numerous eosinophilic cytoplasmic granules that were morphologically consistent with peroxisome proliferation. There was also accumulation of small, clear cytoplasmic vacuoles. Wild-type mice (1c, 600x original mag) treated with Wyeth 14,463 had similar hepatocyte alterations to the PFOA-treated mice. Reprinted from Wolf et al. (2008) with permission from SAGE Publications.

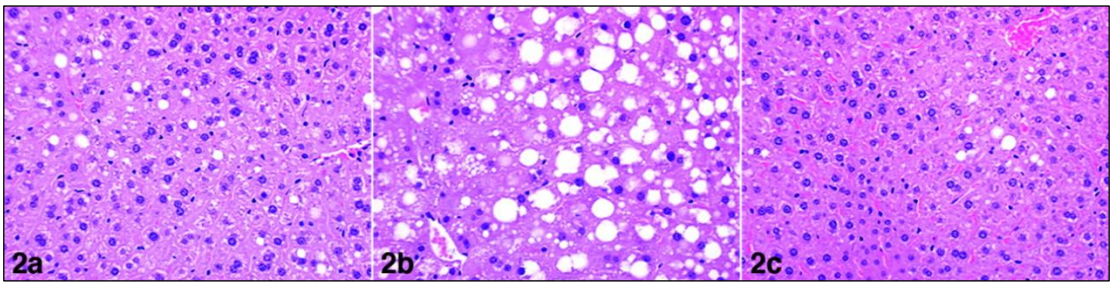

Figure 3. HE-stained livers from PPAR-α knockout mice. Control PPAR-α knockout mice (2a, 200x original mag) had scattered clear cytoplasmic vacuoles morphologically consistent with lipid accumulation. PPAR-α knockout mice treated with 10 mg/kg PFOA (2b, 400x original mag) had diffuse accumulation of clear, variably sized cytoplasmic vacuoles with fuzzy borders. PPAR-α knockout mice treated with Wyeth 14,463 (2c, 200x original mag) were not different from control mice. Reprinted from Wolf et al. (2008) with permission from SAGE Publications.

Regarding reproductive/developmental toxicity, studies in rodents have shown that the neonatal mortality following *in utero* exposure to PFOA and PFNA are PPAR-α-dependent (Abbott et al. 2007; Wolf et al. 2008), but PPAR-α independent for PFOS (Abbott et al. 2009) indicating other mechanisms of action. Direct chemical interactions with pulmonary surfactant have been proposed (Abbott et al. 2009; Lehmler et al. 2006; Xie et al. 2007). Also, activation of other nuclear receptors by PFASs have been revealed, such as other PPARs (β and γ) as well as the pregnenolone X receptor (PXR) and the constitutive androstane receptor (CAR) (Bjork et al. 2011; Elcombe et al. 2010, 2012), adding more complexity into the mechanisms of action of PFASs. Human and mouse PXR and CAR have been shown to respond similarly with regard to hepatocellular hypertrophy and increased liver weight following exposure to e.g. phenobarbital and chlordane (Ross et al. 2010), and it cannot be excluded that the response could be similar also for PFASs. In addition, other modes of action of PFASs have been proposed, such as oxidative stress, effects on cell-signaling pathways, epigenetic changes, interference with cell communication and alterations in mitochondrial bioenergetics (reviewed in Lau 2012a, b).

Though some effects by PFASs are mediated via PPAR-α, there are strong indications that other mechanisms are involved in e.g. hepatotoxicity and reproductive toxicity. Since the mechanism(s) of action of PFASs have not been clarified, and no evidence have been presented that would rule out these effects from occurring in humans, it is reasonable to consider these endpoints of human relevance.

1.2.10 Epidemiological data

Epidemiological studies on PFASs are available for general and highly exposed populations. Concerning effects of PFASs on the liver, studies have been performed in PFASs productions workers but with no associations being found between increasing levels of PFOA or PFOS/PFOA and changes in the measured biomarkers of hepatotoxicity (Costa et al. 2009; Olsen et al. 2003).

Concerning reproductive toxicity, some studies have reported associations between PFASs exposure and reduced birth weight whereas others have not. In the general population, one study observed an association between increased serum levels of PFOA and PFOS in umbilical cord blood and decreased birth weight (Apelberg et al. 2007). Two studies reported either a correlation between increased maternal plasma levels of PFOA, but not PFOS, and decreased birth weight, (Fei et al. 2007) or between increased maternal serum levels and decreased birth weight for PFOS but not for PFOA (Washino et al. 2009). Two other studies did not find any correlations between maternal serum levels of different PFASs and birth weight in the general population (Grice et al. 2007) or between serum levels of PFOS in production workers and pregnancy outcome (Monroy et al. 2008). In a population highly exposed to PFOA via contaminated drinking water, no clear associations were found between serum levels of PFOA and pregnancy outcomes or birth defects (Nolan et al. 2009, 2010, Savitz et al. 2012a, b); though weak and/or inconsistent associations with early preterm birth, fetal growth restriction and pregnancy induced hypertension were seen (Savitz et al. 2012a, b). These conclusions by Nolan et al. and Sawitz et al. were also shared by an independent scientific panel (C8 Science Panel 2011a, b, c, d).

Regarding other effects than hepatotoxicity and reproductive toxicity, some studies have found associations between increased serum levels of PFOA and serum levels of cholesterol and/or uric acid in PFASs production workers (Costa et al. 2009) and in a population highly exposed to PFOA via contaminated drinking water (Frisbee et al. 2010) whereas others have not found any associations (Emmett et al. 2006) or considered the data insufficient for firm conclusions (Steenland et al. 2010). The indications of an association between increasing serum levels of PFOA and cholesterol points in the opposite direction to the decreased levels of serum cholesterol often observed in animal studies following PFASs exposure. Studies on immunotoxicity, metabolic effects or mammary gland development are limited. One study reported an association between increasing levels of PFASs and decreased antibody response following vaccination in children (Grandjean et al. 2012) whereas another study did not find any correlation between prenatal exposure to PFOS and PFOA and increased risk of severe infectious diseases in early childhood (Fei et al. 2010). One study reported an association between increasing levels of PFOA during *in utero* exposure and overweight/obesity in females at 20 years of age in the general population whereas in another study in a population highly exposed to PFOA no clear associations were found between prenatal serum levels of PFOA and increased risk of metabolic syndrome, childhood obesity or infections (C8 Science Panel 2012).

1.2.11 Hazard/Risk assessment information

Hazard and/or risk assessment information on PFASs is primarily available for the two most studied congeners PFOS and PFOA. These have been assessed by national and international authorities and organizations such as the European Chemicals Agency (ECHA 2011), the European Food Safety Authority (EFSA 2008), the Swedish Chemicals agency (KemI 2004), the United States Environmental Protection Agency (U.S. EPA 2005, 2009) and the Organization for Economic Co-operation and Development (OECD 2002) as well as by the industry (3M 2003). Though hazard/risk assessment information is lacking for the vast majority of other congeners, some attention in this regard have been given to other PFASs such as PFBS (NICNAS 2005; MDH 2011a), PFBA (MDH 2011b) and a large number of PFASs that were addressed in a recent cumulative risk assessment of PFASs (Borg and Håkansson 2012, preceding work to Paper IV in this thesis).

1.2.12 Regulations

In 2002, the largest producer of PFOS and PFOS-related compounds (all precursors that can be degraded to PFOS) discontinued its production of these substances (OECD 2005). Since then, other risk-reducing measures have also been taken within e.g. the European Union (EU) and the United Nations to reduce the use of PFOS. PFOS and PFOS-related compounds were prohibited from use in chemical products and articles within the EU in 2008 (EU 2006) and were in 2009 included in the Stockholm Convention on Persistent Organic Pollutants (UNEP 2009) as well as in the Convention on Long-Range Transboundary Air Pollution (CLRTAP) (UNECE 2009) resulting in restrictions on their use. Although these measures led to a markedly decreased use of PFOS (KemI 2006), it is still produced elsewhere (UNEP 2008), particularly in China where the production of PFOS increased substantially after 2002 (Xie et al. 2013). To replace PFOS, several manufacturers have moved towards other per- or highly fluorinated compounds, such as fluorotelomers and shorter alkyl chain sulfonates, such as PFBS, sharing similar technical properties as PFOS (KemI 2006, 2009).

Regarding regulations of other PFASs than PFOS and PFOS-related compounds, PFUnDA, perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid (PFTrDA) and perfluorotetradecanoic acid (PFTeDA) have in the European Union's chemicals legislation REACH (Registration, Evaluation, Approval and restriction of CHemicals) been classified as Substances of Very High Concern (SVHCs) based on their very persistent and very bioaccumulative (vPvB) properties (ECHA 2012a, b, c, d). In addition, PFOA and its ammonium salt, ammonium perfluorooctanoate (APFO) have been proposed as SVHS substances based on their toxicity to reproduction (BAuA 2013a, b) and a similar proposal regarding PFNA is underway (ECHA 2013).

1.3 WHOLE-BODY AUTORADIOGRAPHY

Whole-body autoradiography (WBA) is a method to study the tissue distribution of a radiolabelled compound and/or its metabolites in an intact animal. Most commonly rats or mice are used, though also studies in e.g. guinea pigs, hamsters, rabbits, monkeys and non-mammalian species such as birds and fish have been performed (reviewed in Benard et al. 1985). The method, originally developed by Sven Ullberg in the 1950s (Ullberg 1954), has been used to investigate the tissue distribution of xenobiotics such as environmental contaminants, pesticides and metals (reviewed in Benard et al. 1985) though it is today mostly used by the pharmaceutical industry within preclinical drug development (Solon 2012).

The common procedure for WBA (described in Larsson and Ullberg 1981) entails a single dosing of a radiolabelled compound, with the route of administration depending on the compound and purpose of the study, and sacrifice of the animals by euthanasia at various time-intervals followed by deep-freezing at -75 °C in a carboxymethylcellulose (CMC) gel. Sections, commonly between 20-50µm thick, are then taken at different levels of the animal in a cryomacrotome and placed on x-ray film. After an appropriate exposure period, the section and film are separated and the compound and/or its metabolites (i.e. the radioactivity) will appear on the developed film (see example Figure 4). The section can then be stained or used unstained as reference for interpretation of the autoradiograms. The advantage of this technique is that it provides high resolution images of the qualitative distribution of the compound and/or its metabolites at the tissue level. To obtain quantitative data, this method can be combined with e.g. liquid scintillation counting.

Being a qualitative method for 25 years, quantitative whole-body autoradiography (QWBA) was developed in the 1970s. In QWBA, the whole-body sections are placed on phosphor imaging plates producing digital images of the radiation. Together with a radioactive scale this allows for digital image analyses and quantitation of the radioactivity and e.g. calculation of tissue specific half-lives of the compound.

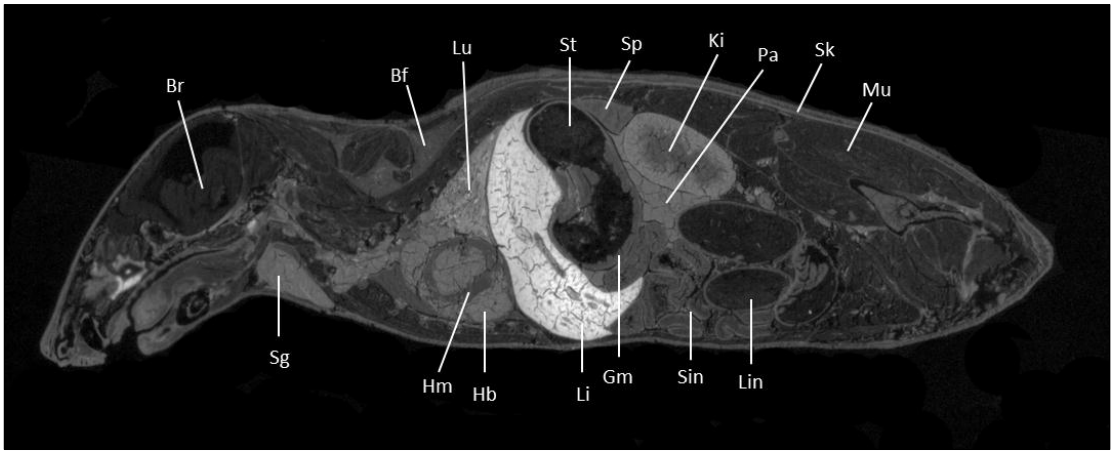

Figure 4. Example of a whole-body autoradiogram showing an adult male mouse 48 h after administration of a single oral dose of ^{35}S-labelled PFOS. The brighter areas correspond to higher levels of radioactivity. Bf = brown fat, Br = brain, Gm = gastric mucosa, Hb = heart blood, Hm = hears muscle, Ki = kidney, Li = liver, Lin = large intestine, Lu = lung, Mu = muscle, Pa = pancreas, Sg = salivary gland, Sin = small intestine, Sk = skin, Sp = spleen, St = Stomach.

1.4 HEALTH RISK ASSESSMENT OF CHEMICALS

1.4.1 Introduction

Health risk assessment of chemicals is the process to estimate the risk for a human (sub) population following exposure to a particular chemical compound, taking into account the inherent properties of the compound, the associated uncertainties as well as the characteristics of the specific target system. The process is often performed with the purpose to define "safe" exposure levels and provide guidance/limit values, such as Tolerable Daily Intake (TDI) for the particular compound.

1.4.2 Principles of health risk assessment

The risk assessment process of chemicals includes four steps: 1) hazard identification, 2) hazard characterization (dose–response assessment), 3) exposure assessment, and 4) risk characterization (IPCS/WHO 2004; U.S. EPA 2013; Figure 5). The hazard identification and hazard characterization are often together called hazard assessment.

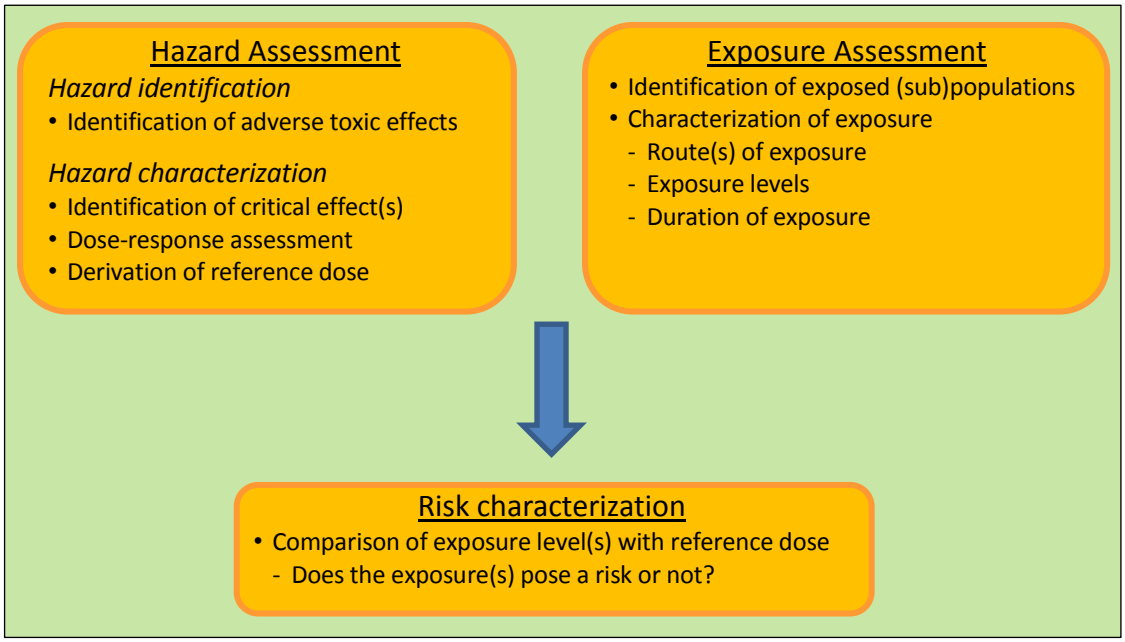

Figure 5. The different steps in chemical health risk assessment: Hazard Assessment, Exposure Assessment and Risk Characterization.

1.4.2.1 Hazard assessment

The hazard assessment step involves *hazard identification* and *hazard characterization* (often referred to as *dose-response assessment*).

The *hazard identification* identifies what adverse health effects that are or can be associated with exposure to the particular compound, employing toxicological, epidemiological, toxicokinetic and mode/mechanism of action data. In the subsequent *hazard characterization*, the "critical effect(s)" (the most sensitive adverse toxic effect(s) that is/are considered relevant for humans) are defined. Then, the dose-response relationship of the critical effect(s) are evaluated in order to define a "no effect" level, e.g. a No-Observed-Adverse-Effect-Level (NOAEL), a Lowest-

Observed-Adverse-Effect-Level (LOAEL) or a benchmark dose (BMD) that can be used as a "point of departure". To the point of departure, assessment factors for uncertainties in extrapolation of animal data to humans, for variability in susceptibility in human populations and for extrapolation of e.g. short-term toxicological studies into chronic exposure, are applied in order to derive a reference dose, i.e. a "safe" exposure level (Figure 6).

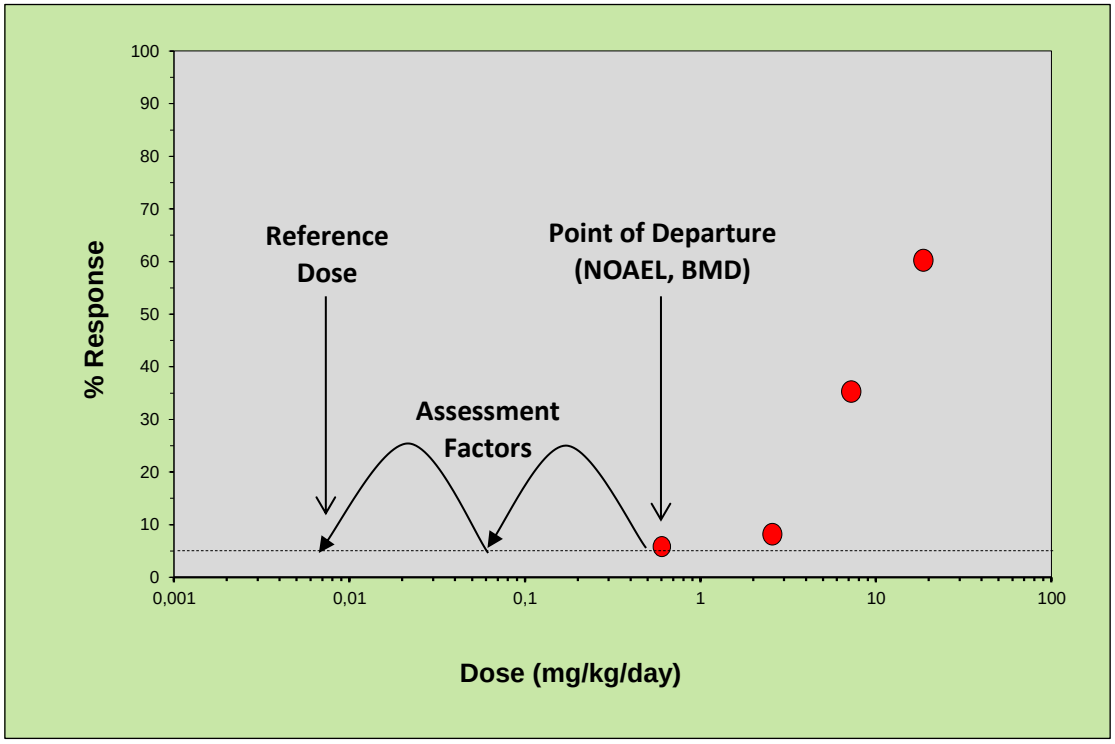

Figure 6. Schematic illustration of the derivation of a reference dose. To a "no effect" level of a critical effect, assessment factors are applied to derive a "safe" exposure level.

1.4.2.2 Exposure assessment

The exposure assessment step evaluates if any (sub) population groups are exposed to the particular compound and, if so, how, i.e. what are the exposure levels, the routes of exposure and the frequency and duration of the exposure.

1.4.2.3 Risk characterization

In the risk characterization, the exposure levels derived in the exposure assessment are compared with the derived ("safe") reference dose from the hazard assessment. If the exposure exceeds the reference dose, the (sub) population(s) of interest is considered at *risk*. It is, however, important to be aware of that the term "risk" represents that the margin of safety is too small from a regulatory perspective, and not an indication that adverse health effects have occurred.

1.4.3 Cumulative risk assessment

Health risk assessment of chemicals normally evaluates the effects of single compounds in isolation. However, compounds in a mixture may act together and induce a toxic effect that is larger than by the individual compounds themselves. Thus, assessing compounds individually may underestimate the total risk (Backhaus and

Faust 2012). During the last decade the area of mixture toxicology has developed and mixtures are being more commonly tested (Kortenkamp et al. 2009). However, due to the infinite number of possible chemical mixtures it is practically impossible to experimentally test for more than a very limited set of all chemical combinations (Backhaus et al. 2010). Thus, there is a need for reliable methods to assess the risk to combined exposure to multiple chemicals via all relevant routes and pathways, defined as *cumulative risk assessment* (WHO 2009).

Numerous methods have been developed to predict the toxicity and risk of mixtures based on their chemical composition and knowledge about the toxicities of the mixture components (Kortenkamp et al. 2009). Most of these methods are based on the concepts of Concentration Addition (CA) and Independent Action (Backhaus et al. 2010). CA assumes that the individual components act via a similar mode of action, only differing in their relative potency to elicit a toxic effect (Backhaus et al. 2010), whereas independent action assumes that the individual components act independently of each other (Backhaus et al. 2010). Both concepts assume that no interactions occur between the mixture components (SCHER 2011). CA, the most broadly used and often the default assumption of these two concepts (Kortenkamp et al. 2009) is generally defined by the formula:

$\sum_{i=1}^{n} \frac{C_i*}{ECx_i}$ = 1 where C_i* are the concentrations of individual substances 1 to n that elicits a fractional effect x (e.g. 50% mortality) and ECx_i denote the equivalent effect concentrations of the single substances (e.g. EC50*i*), i.e. those concentrations that alone would cause the same quantitative effect *x* as the mixture. In the CA formula a mixture component can be replaced totally or in part by an equal fraction of another component without changing the overall combined effect.

From CA a number of cumulative risk assessment methods have been developed, including the Hazard Index (HI), Point of Departure Index (PODI), Relative Potency Factors (RPF) and Toxic Equivalency Factors (TEF) (reviewed in Kortenkamp et al. 2009; reviewed in Sarigiannis and Hansen 2012):

The *Hazard Index* (HI) is defined as the sum of the respective Hazard Quotients (HQs) for individual mixture components, calculated as the ratio between exposure (e.g. daily intake) and a reference dose (e.g. tolerable daily intake (TDI)):

$HI_i = \sum_{i=1}^{n} HQ_i$; where $HQ_i = \frac{Exposure_i}{Reference\ dose_i}$. HI > 1 indicates a risk.

The Hazard Index has been proposed as the preferred approach when extensive mechanistic information of the mixture components is not available (SCHER 2011). It does not predict the overall health effect of the mixture, but provide a measure of the total risk based on the contributions by the individual components. Thus, the Hazard Index can be used to identify the largest contributors to a risk (Sarigiannis and Hansen, 2012) and, subsequently, as a prioritization tool for risk-reducing measures.

If this use of assessment factors is a problem, the *Point of Departure Index* (PODI) can be used instead. It represents the sum of the exposure of each compound divided by its respective point of departure, and therefore does not take into account uncertainties of the dataset. Instead it is compared to a default reference safety factor, often 100 (Sarigiannis and Hansen 2012).

$$PODI_i = \sum_{i=1}^{n} \frac{Exposure_i}{Point\ of\ departure_i}$$

The *relative potency factor* (RPF) approach is an application for compounds that are assumed to be toxicologically similar (U.S. EPA. 2000). The concentrations of the mixture components are scaled up relative to the concentration of an index compound and then summed up. The total toxicity of the mixture is then assessed in terms of the toxicity of the equivalent concentration of the index compound:

$$C_m = \sum_{i=1}^{n} (C_i * RPF_i)$$

The *toxic equivalency factor* (TEF) is a specific type of RPF that was first developed for dioxins, describing the total equivalent quantity (TEQ) of an index compound. It is based on assumptions of a similar mechanism of action and parallel dose-response curves. The total toxicity of the mixture is assessed in terms of the toxicity of an equivalent concentration of an index compound. The *total equivalent quantity, TEQ,* is estimated by summation of the concentrations of mixture components multiplied by the respective TEFs:

$$TEQ = \sum_{i=1}^{n} (C_i * TEF_i)$$

2 THE PRESENT INVESTIGATION

2.1 AIMS

The overall aims of the work in this thesis were to 1) generate new experimental data on the distribution of PFASs that can improve human health risk assessments of this class of compounds by using the mouse as a model for humans; and 2) assess human health risks associated with the current exposure levels of PFASs in Sweden.

More specifically, the project objectives in **part one** was to:

- In detail study the tissue distribution of PFOS in perinatal C57Bl/6 mice following *in utero* exposure in order to 1) from a distributional perspective contribute to clarify the mode of action for the PFOS-induced neonatal mortality, 2) provide data on the distribution of PFOS in perinatal rodents for use in human health risk assessment, and 3) provide information about potential new target organs for PFOS in perinatal rodents (**Paper I**).

- In detail study the tissue distribution of PFOS in adult male C57Bl/6 mice at a high dose commonly used in experimental studies and at a low dose of human relevance in order to 1) provide data on the distribution of PFOS in adult rodents for use in human health risk assessment, in particular with regard to improving extrapolation of high experimental doses to low doses of human relevance, 2) provide information about potential new target organs for PFOS in adult rodents, and 3) from a distributional perspective contribute to clarify the mode of action for general systemic toxicity of PFOS (**Paper II**).

- In detail study the tissue distribution of the replacement chemical for PFOS, PFBS, in male adult C57Bl/6 mice in order to 1) provide data on the distribution of PFBS in adult rodents for use in risk assessment, 2) provide information about potential new target organs for PFBS in adult rodents, and 3) compare the tissue distribution of PFBS with that of PFOS (**Paper III**).

More specifically, the project objectives in **part two** was to:

- Perform a cumulative risk assessment of 17 PFASs that have been analyzed in the Swedish population with the purpose to 1) perform the 1st cumulative risk assessment of PFASs, 2) provide risk assessment data that is lacking for the majority of the individual congeners, 3) provide a practical example on the use of a cumulative approach for risk assessment of a mixture of chemicals (**Paper IV**).

2.2 MATERIALS AND METHODS

The sections below provide a summary of the materials and methods used in this thesis. Detailed descriptions of the techniques, materials and methods used in the individual studies can be found in the associated publications and manuscripts.

2.2.1 Papers I-III, Tissue distribution studies on PFASs

2.2.1.1 Experimental animals and housing

In **Papers I-III**, C57Bl/6 mice were used as model species to investigate the perinatal and adult tissue distribution of PFOS as well as the adult tissue distribution of PFBS. The mouse is an extensively characterized experimental animal and the C57Bl/6 strain is a widely studied murine strain.

All animals were housed in the animal facilities of the Wenner-Gren Institute, Stockholm University, in polycarbonate cages with heat-treated pine-shavings for bedding, with access to a standard pellet diet and tap water *ad libitum* and with a 12h light/12h dark cycle, relative humidity of 40 - 60% and temperature of 22 ± 2 °C. The animals were allowed to acclimatize for at least 1 week prior to the experiments.

2.2.1.2 Chemicals

All solvents and other chemicals used in synthetic and analytical procedures in the experiments were of pro-analysis quality. The ^{35}S-sulfuric acid used for the synthesis of ^{35}S-perfluorooctane sulfonate and ^{35}S-perfluorobutane sulfonate (section 2.1.1.3) had a radiochemical purity of 100%.

2.2.1.3 ^{35}S-perfluorooctane sulfonate and ^{35}S-perfluorobutane sulfonate syntheses

The synthesis of ^{35}S-perfluorooctane sulfonate and ^{35}S-perfluorobutane sulfonate were performed as described in Sundström et al. (2012). The batch of ^{35}S-PFOS used in **Paper I** and the high daily dose exposures in **Paper II** had a specific radioactivity of 31.8 mCi/mmol, a chemical purity of 97% (the major impurity being PFOA) and a radiochemical purity of 97%. The batch of ^{35}S-PFOS used for low daily dose exposure in **Paper II**, designed to obtain a higher specific radioactivity, had a specific radioactivity of 59 mCi/mmol, a 90% chemical purity (10% being mainly PFOA) and a radiochemical purity of 95%. The batch of ^{35}S-PFBS in **Paper III** had a specific radioactivity of 29.6 mCi/mmol, a chemical purity of 95% and a radiochemical purity of 93% (5% being ^{35}S-perfluorobutanesulfinic acid).

2.2.1.4 Preparation of diet (Paper II and III)

For preparation of diet for the low daily dose exposure in **Paper II**, ^{35}S-PFOS was diluted with double-distilled water, mixed with powdered RMI (E) chow to obtain a concentration of 0.156 µg ^{35}S-PFOS/g food with a radioactivity of 0.018 µCi/g food. Based on an average food intake of 4 g/day and a body weight of 20 g this corresponds to an exposure of 0.031 mg ^{35}S-PFOS/kg/day. For preparation of diet for the high daily dose exposure in **Paper II**, ^{35}S-PFOS was supplemented with an appropriate amount of unlabeled PFOS and thereafter diluted with double-distilled

water to obtain a final concentration of 156 µg ^{35}S-PFOS/PFOS/g food with a radioactivity of 0.81 µCi/g food. Based on an average food intake of 3 g per day (the mice consumed less as an effect of PFOS in the diet) and a body weight of 20 g this corresponds to an exposure of 23 mg/kg/day. For preparation of the diet for exposure in **Paper III**, ^{35}S-PFBS was supplemented with unlabeled PFBS (95% purity), diluted in double-distilled water and mixed with powdered chow to obtain a final PFBS concentration of 95.3 µg ^{35}S-PFBS/PFBS/g food, corresponding to 0.32 µmol PFBS/g with a specific radioactivity of 3.5 µCi/g. This was the same molar dietary concentration as the high daily dosing of PFOS in **Paper II**.

2.2.1.5 Animal exposure and preparations for analyses (Paper I)

Six female C57Bl/6 mice received a single dose of ^{35}S-PFOS (0.8 µCi/g, 12.5 mg PFOS/kg bw) on GD16, five of these via oral gavage and the sixth by intravenous injection (to address possible differences in bioavailability between these two administration routes). The animals were thereafter monitored visually each day for possible signs of toxicity until the time of sacrifice by exposure to gaseous CO_2 on GD18 (two dams), GD20 (one dam) or PND1 (three dams and pups) for whole-body autoradiography and liquid scintillation counting of tissues. After sacrifice, the animals were placed in aqueous carboxymethyl cellulose (CMC) frozen in a bath of hexane cooled with dry ice.

2.2.1.6 Animal exposure and preparations for analyses (Paper II)

Male C57BL/6 mice were divided randomly into 6 groups of 3 mice. Three groups of mice were allowed to consume the low daily dose diet containing 0.156 µg PFOS/g for 1, 3 or 5 days and the 3 other groups received the high daily dose diet containing 156 µg PFOS/g for the same period of time. Body weights were measured at the beginning and end of each period and the food consumption was estimated by subtracting the weight of the remaining food from the weight of the food initially supplied to each mouse. At the end of the feeding period, the mice were bled under iso-flurane anesthesia and sacrificed by cervical dislocation for liquid scintillation counting of ^{35}S-PFOS in tissues. Blood samples were collected in capillary collection tubes containing anticoagulant and organs and tissues (liver, lungs, kidneys, heart, spleen, stomach, small and large intestine, epididymal fat, testes, inguinal fat pads, a muscle sample from m. *quadriceps femoris*, bone samples consisting of the whole femur and tibia, skin samples taken from the back between the two scapula, brain, thymus, thyroid gland and pancreas) were dissected out and washed in cold PBS. The stomach and intestines were emptied of their contents and washed carefully again with cold PBS. All tissues were weighed and stored frozen at -20 °C prior to liquid scintillation counting and determination of hemoglobin content. For whole body autoradiography, two male C57BL/6 mice received a single oral dose (0.8 µCi/g, 12.5 mg PFOS/kg) via gavage and were sacrificed 48h later by exposure to gaseous CO_2, mounted in aqueous CMC and frozen in a bath of hexane cooled with dry ice.

2.2.1.7 Animal exposure and preparations for analyses (Paper III)

Male C57BL/6 mice were divided randomly into 3 groups of 3 mice each for liquid scintillation counting of ^{35}S-PFBS in tissues, 2 additional mice were used for whole-body autoradiography and 3 untreated mice were used for determination of normal hemoglobin levels. For liquid scintillation counting, each group of mice was allowed to consume 95.3 μg PFBS/g food for 1, 3 or 5 days. For whole-body autoradiography following 5-day exposure, the dietary concentration was the same but with a level of radioactivity increased to 9 μCi/g food to enhance detection. With an average food intake of 3.7 g/day and a body weight of 22 g this exposure corresponded to a dose of 16 mg PFBS/kg/day (53 μmol PFBS/kg/day). At the end of the feeding period, the mice were bled under iso-flurane anesthesia and sacrificed by cervical dislocation for liquid scintillation counting of ^{35}S-PFBS in tissues or sacrificed for whole-body autoradiography in a CO_2 atmosphere and immediately frozen at -20 °C on a flat surface before being placed in aqueous CMC and frozen in a bath of hexane cooled with dry ice. For the liquid scintillation counting blood samples were collected in capillary collection tubes containing anticoagulant and organs and tissues (liver, lungs, kidneys, heart, spleen, stomach, small/large intestine, epididymal fat, testes, inguinal fat pads, a muscle sample from *m. quadriceps femoris*, the whole femur and tibia bones, skin samples (taken from the back between the two scapula), brain, thymus, thyroid gland, pancreas and cartilage from the thoracic bone) were dissected out. The stomach and intestines were emptied of their contents and carefully cleaned by washing with cold PBS. All tissues were then weighed and stored frozen at -20 ºC prior to liquid scintillation counting and determination of hemoglobin content.

2.2.1.8 Whole body autoradiography

For the whole-body autoradiography in **Papers I-III**, series of sagittal whole-body sections (20- and 40-μm thick) were taken at 8–15 different levels, collected onto tape (Scotch 6890, 3M Ltd., St. Paul, MN, USA) and freeze-dried. The tape sections were then air-dried, opposed to X-ray film at -20 °C that were subsequently developed. In **Paper I and II**, samples of liver, lungs, kidneys, brain and blood from adult males, dams, pups and the remaining fetuses were dissected out of the remains in the CMC blocks for liquid scintillation counting.

2.2.1.9 Histology

In **Papers I** and **II**, certain whole-body sections were stained with hematoxylin and eosin for comparison to the associated autoradiogram. Also, in **Paper II**, certain whole-body sections were stained for calcified bone using Von Kossa's staining Further, In **Paper III**, certain whole-body sections were stained for cartilage and calcified bone using a combination of a modified protocol for Safranin O/Fast Green staining and Von Kossa's staining.

2.2.1.10 Tissue scintillation counting and quantification of hemoglobin

In **Paper I**, tissue samples from the remains of the dams, fetuses and pups utilized for whole-body autoradiography were weighed, processed using the Solvable kit and analyzed in a liquid scintillation counter. The Solvable solubilization reagent was not found to quench or affect the counting efficiency of ^{35}S-PFOS. The process from chemical synthesis to completion of liquid scintillation results took up to 91 days and measured decays per minute (dpm) levels that were at least 6 times higher than background level. The radioactivity measured was subsequently converted into µg ^{35}S-PFOS/g tissue (wet weight) using the original specific radioactivity of this compound and compensation for radioactive decay.

In **Papers II** and **III**, tissue contents of ^{35}S-PFOS and ^{35}S-PFBS, respectively, were similarly as in **Paper I** determined in a liquid scintillation counter. **In Paper II**, entire organs/tissues following low daily dosing were subjected to liquid scintillation counting and determination of hemoglobin content, except for skin, whole bone and liver, from which portions were taken. Following high daily dosing, portions of the tissues were used. In **Paper III**, entire organs/tissues were used for liquid scintillation counting and determination of hemoglobin content, with the exception of liver, kidneys, brain, skin, cartilage and bone, from which portions were taken. First, blood and soft tissue samples were weighed and solubilized in Solvable and then divided into two parts: one for scintillation counting and the other for determination of hemoglobin. The samples were bleached with hydrogen peroxide. In this manner hemoglobin and PFOS or PFBS could be determined in the same solubilized sample. For solubilization of whole bone, a mixture of perchloric acid and hydrogen peroxide was utilized which did not allow for determination of hemoglobin. Two solubilization reagents did not influence the efficiency of liquid scintillation counting. Correction for the decay of ^{35}S was made in all cases. When only a portion of an organ/tissue was assayed, the total amount of PFOS or PFBS present in the organ/tissue was obtained by adjusting for the total weight. However, in the case of the skin, muscle, whole bone and blood, the total weights were not determined, and instead literature values for the relative contributions of these tissues to total body weight were utilized (16.5% for skin, 38.4% for muscle and 10.7% for whole bone, and 8% for blood).

In **Papers II** and **III**, hemoglobin contents in tissues were quantified using a hemoglobin assay kit. In this assay, hemoglobin is converted into a colored end-product by utilization of a detergent and the end-product is directly proportional to the hemoglobin concentration in the original sample. The resulting contents are expressed as mg hemoglobin per g tissue or blood. To correct the PFOS or PFBS concentrations in each tissue for PFOS or PFBS derived from the blood present in the same tissue, the radioactivity per mg hemoglobin in blood was calculated; this value was multiplied by the mg hemoglobin in the specific tissue; and the value obtained was subtracted from the total amount of PFOS or PFBS present.

2.2.1.11 Statistical analyses

In **Paper I**, the data set was found to be normally distributed and demonstrated equal variances. For statistical comparisons between all groups, one-way analysis of variance (ANOVA) was performed followed by Bonferroni's Multiple Comparison Test for group-wise comparisons.

In **Paper II**, the data sets was also normally distributed and with similar variance in all cases. One-way ANOVA was performed followed by an independent two-tailed t-test.

In **Paper III**, one-way ANOVA was performed followed by Tukey's post-test for multiple comparisons. To test for statistically significant trends, one-way ANOVA followed by post-test for linear trends was performed. An unpaired two-tailed t-test was utilized to test for statistically significant differences between two mean group values alone.

2.2.1.12 Ethical permits

The in-life phase of the work in **Papers I-III** was carried out at Stockholm University and was ethically approved by Northern Stockholm Ethical Committee for Animal Research (approval numbers N405/08 and N/183-10).

2.2.2 Paper IV, Cumulative risk assessment of PFASs

2.2.2.1 Exposure assessment

Biomonitoring data (blood/serum concentrations) of all PFAS congeners analyzed in the Swedish population from 2006 and onwards, collected from reports within the Swedish Health-Related Monitoring Programme, other national reports and scientific publications were used in the exposure assessment. External (oral/inhalation/dermal) exposures were not included. Two population groups were identified: individuals exposed indirectly via the environment (i.e. the general population) and occupationally exposed professional ski waxers. Based on the low number of individuals in the biomonitoring studies the highest PFASs concentrations in samples from selected key studies were used. Congeners present at concentrations under the limit of detection were included and treated as being < limit of detection. To enable comparisons between blood and serum/plasma concentrations, whole blood concentrations were converted into serum/plasma concentrations using the 1:2 whole blood:serum/plasma ratio (Ehresman et al. 2007).

2.2.2.2 Hazard assessment

The toxicological endpoints evaluated in the hazard assessment were hepatotoxicity (hepatocellular hypertrophy, hepatocellular vacuolation, increased liver weight and increased liver-to-body ratio) and reproductive toxicity (reduced fetal/perinatal /neonatal viability, reduced body weight/body weight gain and litter loss in the dams). Also, other endpoints if observed at a lower dose level than hepatotoxicity and reproductive toxicity were included. Points of departure were PFASs serum/plasma

concentrations at the respective NOAELs, LOAELs or BMDs. The toxicological data and key studies/critical effects were collected from already existing hazard- and/or risk assessments and supplemented with additional published relevant data from literature searches in PubMed, i.e., studies on hepatotoxicity or reproductive toxicity published after the hazard/risk assessment reports as well as studies showing other effects at lower concentrations than for hepatotoxicity and reproductive toxicity. For congeners where data for hepatotoxicity and reproductive toxicity and/or corresponding internal doses were lacking, read-across extrapolation to the closest most potent congener for the respective endpoint was performed. The read-across was performed on an equivalent molar basis. From the points of departure, reference doses were derived by the use of appropriate assessment factors (AFs) (Reference dose = point of departure/AFs) in accordance with REACH guidelines (ECHA, 2010). The following AFs were applied:

- Exposure duration: An AF of 2 was used for extrapolations of subchronic-to-chronic and subacute-to-chronic exposure for hepatotoxicity. This rather low AF is motivated by the rapid onset of hepatotoxicity and a limited aggravation with time. For other effects, AFs of 3 and 6 were applied for subchronic-to-chronic and subacute-to-chronic exposure, respectively.
- PODs: An AF of 3 was used for extrapolations from LOAEL to NOAEL in studies where no NOAEL could be established.
- Interspecies differences: An AF of 2.5 was applied for extrapolations of data from animals to humans with regard to toxicodynamic differences. No AF for toxicokinetic differences between animals and humans was used since internal doses were directly compared between the two.
- Intraspecies differences: AFs 10 and 5 were applied for the general population and workers (that is considered a more homogenous and less sensitive group than the general population), respectively, for differences in sensitivity among humans.
- Read-across extrapolations: An AF of 3 was used for extrapolations from shorter to longer congeners based on differences in potency. Shorter congeners are generally less potent than their longer homologues and thus no AFs were used for read-across extrapolations from longer to shorter congeners.

2.2.2.3 Risk characterization

Hazard Quotients (HQs) were derived for all individual congeners by comparing their respective reference doses (points of departure/AFs) with the exposure to evaluate whether the exposure level is tolerable or not: HQ = $\frac{Exposure}{Reference\ dose}$, where a ratio < 1 indicates a tolerable exposure level and a ratio > 1 indicates a non-tolerable exposure level. In addition, a cumulative risk characterization was performed for all the congeners combined by the derivation of Hazard Indexes (U.S. EPA, 1989) for hepatotoxicity and reproductive toxicity: Hazard Index = ∑HQs. Toxicological data for other endpoints were only available for a few individual PFAS congeners and it is unclear whether other PFASs exert these effects, thus a Hazard Index could not be derived for these endpoints.

2.3 RESULTS AND DISCUSSION

2.3.1 Tissue distribution studies

2.3.1.1 Tissue distribution of PFOS in perinatal and adult mice

In **Papers I** and **II** the tissue distribution of ^{35}S-labelled PFOS was for the first time investigated in detail in perinatal and adult animals, using C57Bl/6 mice.

In **Paper I,** ^{35}S-PFOS was readily transferred to the fetuses after exposure on GD16. On GD18, 48h after exposure, ^{35}S-PFOS was found at highest levels in kidneys followed by liver and lungs, all at higher concentrations than in the dam's blood. In fetuses and pups on GD20 and PND1, a pronounced elevation of ^{35}S-PFOS concentrations were observed in the lungs, being highest of the perinatal tissues (Figure 7 and Figure 8). In perinatal brains, there were regional distributions of ^{35}S-PFOS and the average levels were higher than in maternal brain and similar to that of the maternal blood. ^{35}S-PFOS levels were in the dams highest in the liver and lungs followed by blood. Also, ^{35}S-PFOS was distributed to bone tissue in fetuses and pups.

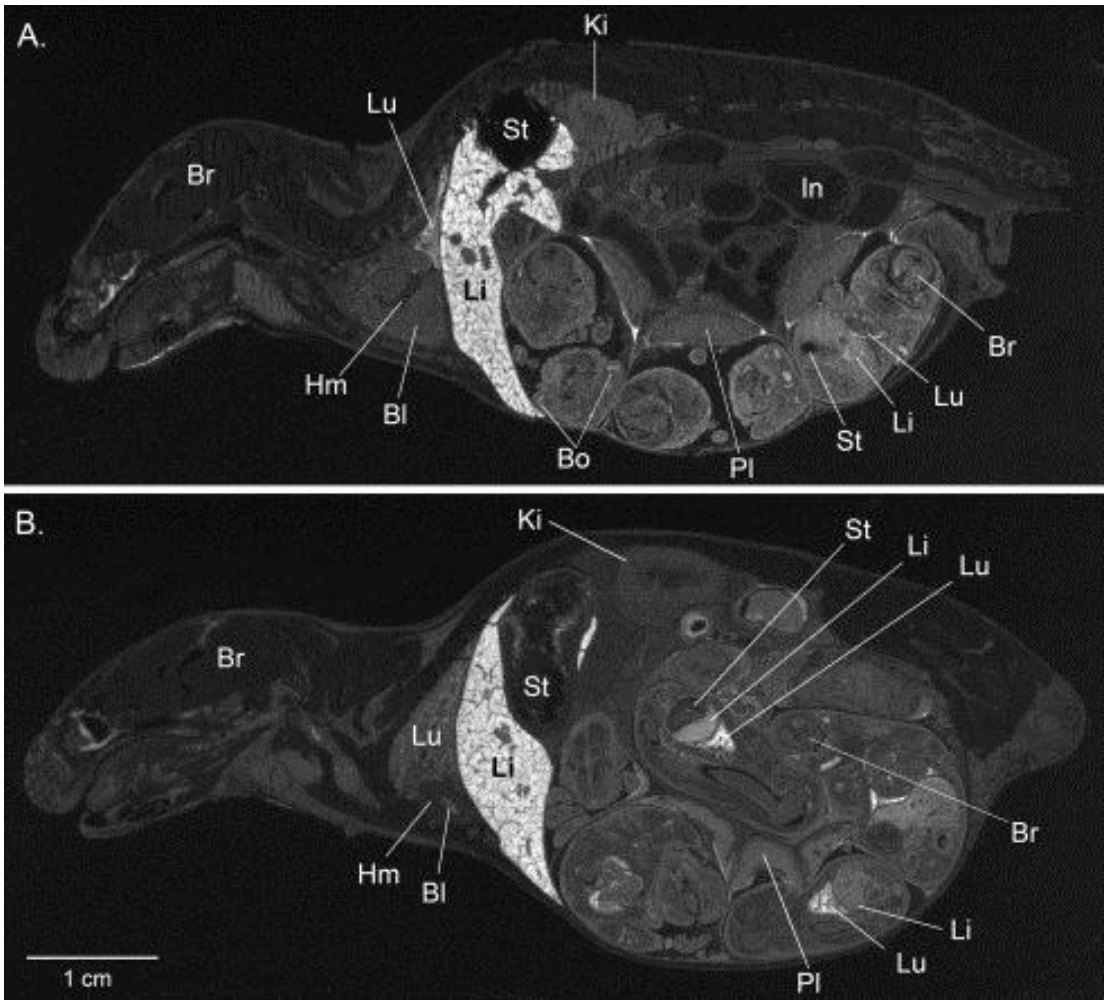

Figure 7. Autoradiograms of 40-µm sections of pregnant mice on gestational days (GD) 18 and GD20, 48 h and 96 h, respectively, after administration of a single oral dose of ^{35}S-labelled PFOS (12.5 mg/kg). Brighter areas correspond to higher levels of radioactivity. ^{35}S-PFOS was readily transferred to the fetuses which on GD18 (A) and GD20 (B) generally demonstrated tissue levels similar to or higher than in the blood of the dams. In the dams, the liver and the lungs contained the highest levels of ^{35}S-PFOS, and in the fetuses the kidneys (not observable) and the lungs contained the highest levels on GD18 and GD20, respectively. Bl = blood, Bo = bone, Br = brain, Hm = heart muscle, In = intestines, Ki = kidney, Li = liver, Lu = lung, Pl = placenta, St = stomach. Reprinted from Borg et al. (2010) with permission from Elsevier.

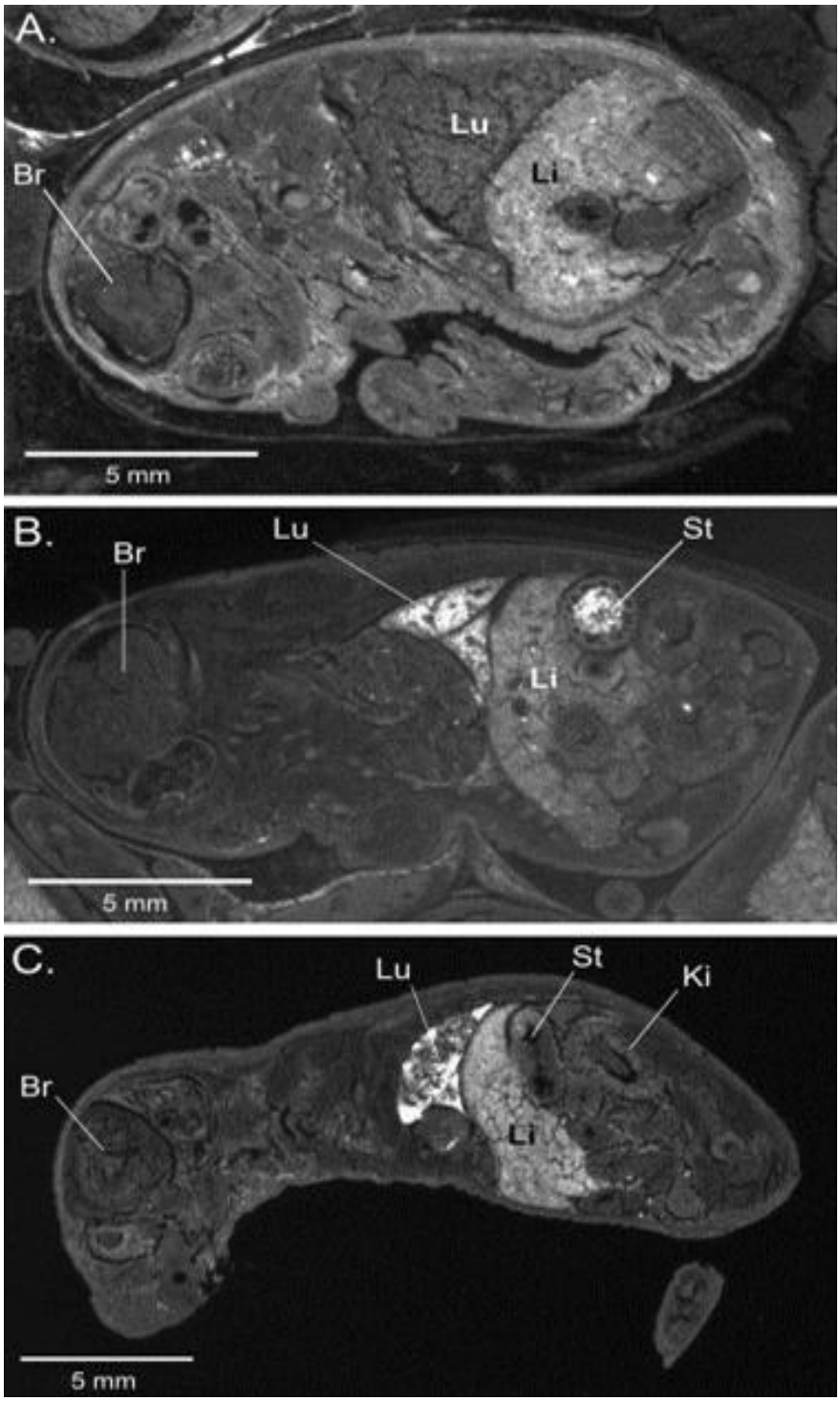

Figure 8. Autoradiograms of 40-µm thick sections of mouse fetuses on GD18 and GD20 and of pups on PND1 following exposure of the pregnant dams to a single dose of ^{35}S-labelled PFOS (12.5 mg/kg) orally or intravenously on GD16. The fetus on GD18 (A) originate from a intravenously exposed dam and the fetus on GD20 (B) and pup on PND1 (C) from an orally exposed dam, respectively. These autoradiograms were treated identically with respect to exposure time and image processing. The brighter areas correspond to higher local levels of radioactivity. On GD18 (A), the level of ^{35}S-PFOS was lower in fetal lungs than in the liver and with a relatively homogenous distribution. On GD20 (B), local levels in the lungs were higher than in the liver and the lungs demonstrated a somewhat heterogeneous distribution. On PND1 (C), local levels in the lungs was further increased displaying a more heterogeneous distribution. Br = brain, Ki = kidney, Li = liver, Lu = lung, St = stomach. Reprinted from Borg et al. (2010) with permission from Elsevier.

Paper I is the first study to demonstrate a selective localization of PFOS to perinatal lung tissue. The results are in line with toxicity data indicating that the lung is a target organ for the toxicity of PFOS in perinatal rodents (Grasty et al. 2003; Grasty et al. 2005; Lau et al. 2003; Luebker et al. 2005; Yahia et al. 2008) and that PFOS-induced neonatal mortality, at least partly, is due to respiratory distress. The exact cause of the respiratory distress observed in rodents after *in utero* exposure to PFOS has not been clarified. It has been proposed that PFOS could interfere with late stages of lung maturation, based on histological findings suggestive of immaturity of the neonatal lungs (Grasty et al. 2003, 2005). An alternative and/or complementary hypothesis is that PFOS, which has been shown to have a high tendency to partition into lipid bilayers (Lehmler et al. 2006), directly interacts with components of the pulmonary surfactant (Abbott et al. 2009; Lehmler et al. 2006) resulting in elevated surface tension and atelectasis, i.e. incomplete expansion of the lung, causing respiratory failure. Atelectasis has been observed in neonatal mice following *in utero* exposure to PFOS (Yahia et al. 2008) and PFOS has been shown to interact with and disturb the function of components of the pulmonary surfactant in vitro (Gordon et al. 2007; Lehmler et al. 2006; Xie et al. 2007). Our findings that PFOS is present in high levels in perinatal lungs following late gestational exposure is consistent with the hypothesis that PFOS directly impairs pulmonary function, possibly by interacting with pulmonary surfactant in the alveoli. PFOS was found at low levels in the amniotic fluid and is likely one source of the PFOS present in the lungs. Further, the localization of PFOS to adult lungs is consistent with findings that PFOS causes pulmonary congestion also in adult rats (Cui et al. 2009).

After the lungs the liver displayed on average the highest levels of ^{35}S-PFOS in the perinatal mice, approximately 2.5-fold higher than in maternal blood, though significantly lower than in the maternal liver that showed the highest ^{35}S-PFOS levels in the dams. These findings are similar to other studies (Chang et al. 2009; Lau et al. 2003; Luebker et al. 2005b; Thibodeaux et al. 2003) and confirm that the liver is a main target organ for PFOS.

^{35}S-PFOS was also present in the perinatal brain at a level close to that of the maternal blood and significantly higher than in the maternal brain. This high level in the perinatal brain is likely due to the incomplete development of the perinatal blood–brain barrier (Chang et al. 2009) and support the conclusion that PFOS can affect the central nervous system and cause behavioral defects in both mice (Johansson et al. 2008) and rats (Butenhoff et al. 2009).

Finally, bone tissue was in **Paper I** discovered as a potential toxicological target tissue for PFOS in perinatal mice. It may be that this presence of PFOS in the developing bones could contribute to the delayed ossification and cleft palate that has been observed in mice following exposure to PFOS in utero (Era et al. 2009; Thibodeaux et al. 2003).

2.3.1.2 Tissue distribution of PFOS in adult mice at different doses

In **Paper II**, the tissue distribution of ^{35}S-PFOS after dietary short-term exposure was determined in detail in adult male C57Bl/6 mice at two different doses, one similar to the high doses commonly used in toxicological studies and one 750-fold lower dose similar to human exposure levels. Also, the contribution of PFOS to the respective tissues by blood was adjusted for. The results showed that PFOS was recovered in a dose-dependent manner in all 19 tissues examined. The highest concentrations were detected in liver, lungs, blood and kidneys (Figure 9a, b), in line with previous findings (Johnson et al. 1979). The distribution profiles were qualitatively similar between the low and the high dose, however with a higher tissue:blood ratio at the higher dose. This shows that the tissue distribution profile can be qualitatively extrapolated from a high dose used in experimental studies to a low environmentally relevant dose. The lower tissue:blood ratios at the lower dose, were similar to those found in a study on human post-mortem material (Maestri et al. 2006)

The major body compartments for PFOS deposition in mice was calculated based on tissue concentrations multiplied by the weight of the organ/tissue. For larger tissues, skin, muscle, whole bone and blood, literature values for their weights were used. The result showed that after 5 days exposure to the low dose the major body compartments were the liver > (whole) bone > blood > skin > muscle. Correspondingly, the major body compartments at the high dose were the liver > skin > blood > (whole) bone > muscle.

In this experiment, bone was discovered as a possible toxicological target tissue for PFOS in adult male mice. When analyzed as a whole bone, the levels were similar to blood; however the autoradiograms revealed that PFOS in whole bone likely was localized to the bone marrow (Figure 9c, d).

One unexpected finding was that blood hemoglobin levels were markedly increased in the mice exposed to the high dose, up to 40%. The reason for this erythropoietic effect, which has not been observed in any other study, is not known but could possibly be connected to the localization of PFOS to the bone marrow.

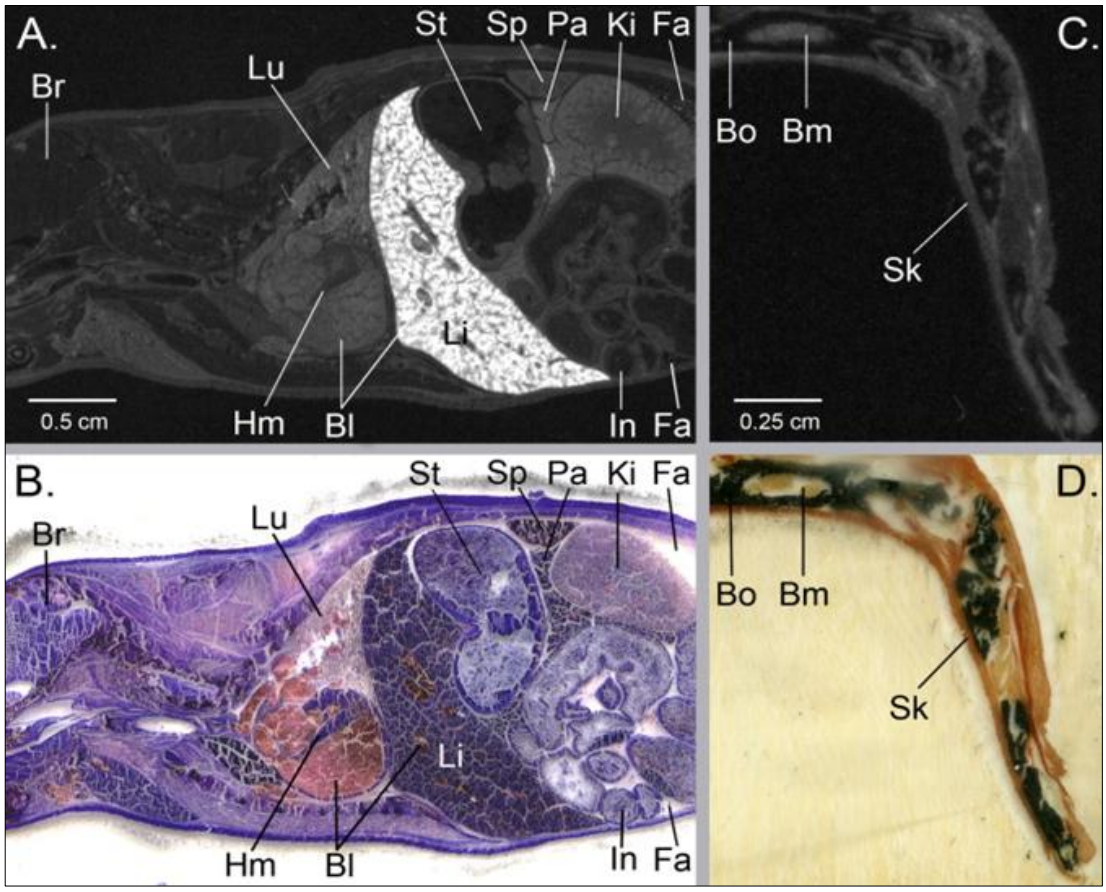

Figure 9. (A) Whole body autoradiogram of a 40-µm section of a mouse 48 h after administration of a single oral dose of ^{35}S-PFOS (12.5 mg/kg), and (B) the same section with hematoxylin/eosin staining. (C) Autoradiogram of a 40-µm section of a mouse femur 48 h after administration of a single oral dose of ^{35}S-PFOS (12.5 mg/kg) and (D) the same section with von Kossa's staining for calcified bone. In (A) and (C) the brighter areas correspond to higher levels of radioactivity and in (D) dark areas correspond to calcified bone. In (B) unstained areas correspond to fat (as caudal of kidney, surrounding the intestines and sub dermal), body cavities and freezing artifacts (cracks). Bl = blood, Bm = bone marrow, Bo = bone, Br = brain, Fa = fat, Hm = heart muscle, In = intestine, Ki = kidney, Li = liver, Lu = lung, Pa = pancreas, Sk = skin, Sp = spleen, and St = stomach. Von Kossa's staining revealed that the ^{35}S-PFOS was present only in the bone cavities, i.e. bone marrow, and not in the calcified bone. Reprinted (adapted) from Bogdanska et al. (2011) with permission from Elsevier.

2.3.1.3 Pattern of tissue distribution of PFBS and comparison to PFOS

In **Paper III**, the tissue distribution of PFBS, the replacement chemical for PFOS, was for the first time studied in detail. Adult male mice were used in the same manner and at the same molar concentration as the high dose of PFOS in **Paper II.** Similarly, the contribution of PFBS to tissues by the blood was adjusted for. The results revealed the presence of PFBS in all the 20 different tissues examined. The tissue levels increased from 1 to 3 days of exposure but appeared to level off thereafter. After 5 days of treatment the highest levels of PFBS were detected in liver, gastrointestinal tract, kidneys, cartilage, blood, whole bone, lungs and thyroid gland. In comparison to PFOS, the PFBS exposure resulted tissue levels in 5-40-fold lower than for the corresponding molar exposure to PFOS. This is most likely due to the rapid elimination of PFBS as compared to PFOS. Also, a slightly different tissue distribution pattern was shown, with lower organ concentrations for e.g. liver and lungs relative to the concentrations in the blood.

The estimated major body compartments for PFBS were whole-bone, liver, skin, blood and muscle. This is similar as for PFOS in **Paper II**, but with the exception that PFBS was estimated to be localized more to whole bone than to the liver, as was the case for PFOS. Similarly, as for PFOS, the autoradiograms revealed that PFBS in whole bone was more localized to the bone marrow than to the calcified bone itself.

In this experiment the autoradiograms also revealed a distinct localization of PFBS to cartilage in the adult male mice, a finding that has not been previously reported. In addition, the autoradiograms also showed relatively high levels of PFBS in male genital organs, with the exception of testes.

Also, as for PFOS in **Paper II**, the hemoglobin levels were increased following PFBS exposure, though being less pronounced. As for PFOS this erythropoietic effect could be connected to the localization of PFBS to the bone marrow.

2.3.2 Cumulative risk assessment of PFASs

Paper IV is the first study to evaluate the risks of cumulative exposure to 17 PFASs analyzed in the blood of the Swedish population. The cumulative approach selected was the Hazard Index approach. The study was performed as a risk assessment, including the different parts: hazard assessment, exposure assessment and risk characterization. The exposure data was derived from Swedish biomonitoring data (blood/serum levels of PFASs) in two populations; the general population and occupationally exposed professional ski waxers. The evaluated toxicity data consisted of publicly available data for hepatotoxicity and reproductive/developmental toxicity as well as other more sensitive toxic effects than hepatotoxicity and reproductive/developmental toxicity.

2.3.2.1 Exposure assessment

The exposure assessment showed that the different PFAS congeners generally were found at low ng/ml levels in the general population, though some were under the limit of detection (Table 2). However, PFOS was found at higher ng/ml levels in a small subpopulation consuming contaminated fish. In the occupationally exposed professional ski waxers the levels of some congeners were significantly higher than in the average population, i.e. PFNA and PFOA reaching high ng/ml and low µg/ml levels in serum, being approximately 125 and 200 times higher than in the general population (Table 2). Temporal trend studies in the general population showed that the levels of PFOS, PFDS, PFOSA and PFOA seem to decrease whereas the levels of PFBS, PFHxS, PFNA, PFDA and PFUnDA in serum seem to increase (Figure 10).

Table 2. Summary of Swedish human serum/plasma biomonitoring data on perfluoroalkylated and polyfluoroalkylated substances (PFASs) from key studies in the general population and occupationally exposed professional ski vaxers Selected concentrations represent the highest concentrations at the latest time-point in a temporal study or from a sample in a snapshot study taken no later than 2006.

Congener	General population	Occupationally exposed
	Serum concentration (ng/ml)	Serum concentration (ng/ml)
PFBS	0.10	N.A.[b]
PFHxS	8.0	8.6
PFOS	27.5/204[a]	54
PFOSA	< 0.040	N.A.[b]
PFDS	0.025	N.A.[b]
PFBA	N.A.	2.2
PFPeA	N.A.	0.28
PFHxA	< 0.22[b]	24
PFHpA	< 0.24[b]	40
PFOA	5.2	1070
PFNA	2.6	326
PFDA	0.70[b]	48
PFUnDA	0.83	5.6
PFDoDA	< 0.1	N.A[b]
PFTrDA	< 0.15	N.A[b]
PFTeDA	< 0.25	N.A[b]
6:2 FTS	< 3.6[b]	N.A[b]

N.A. = Not analyzed
[a] = Highly exposed subpopulation
[b] = Due to lack of exposure data the same value as for the general population will be used in the risk characterization.

2.3.2.2 Hazard assessment

The result of the hazard assessment showed that the different PFASs were relatively similar with regard to their potency for hepatotoxicity and reproductive toxicity with points of departure ranging from 4 - 89 and 4 - > 60 µg/ml serum, respectively (Table 3) However, toxicity data with internal doses were not available for all congeners, thus data for 12 of the 17 congeners had to be extrapolated. Some PFASs also showed effects at lower doses than hepatotoxicity and reproductive toxicity such as immunotoxicity and effects on mammary gland development that were observed at levels of human exposure. Epidemiological studies on PFASs did not provide any firm conclusions regarding the above mentioned endpoints.

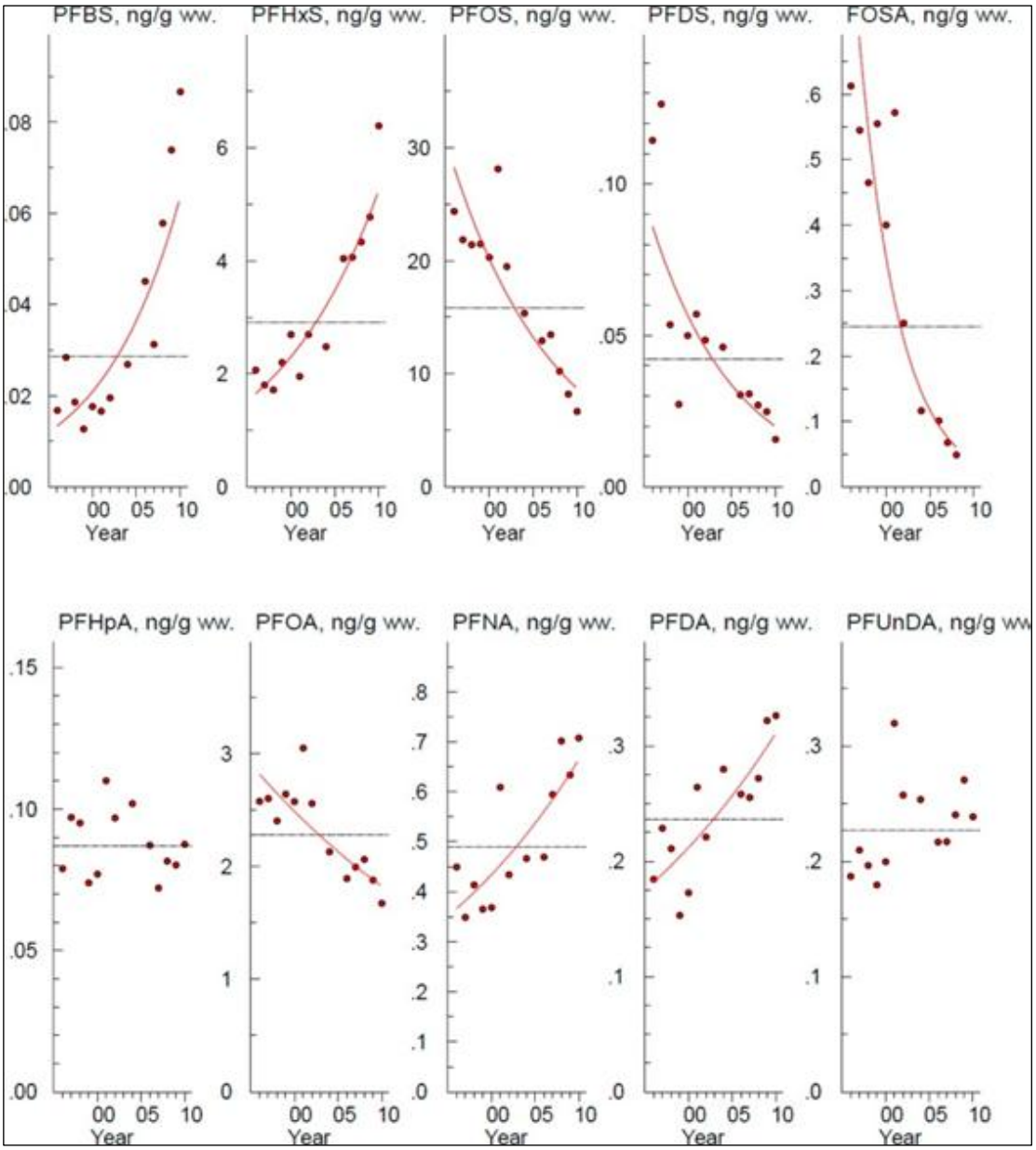

Figure 10. Temporal trends of PFAAs and FOSA in pooled blood serum samples from primiparous nursing women (*N* = 413), living in Sweden 1996–2010. Red dots are the geometric means for each year. The red line is the regression line obtained after linear regression analyses of log-normal PFAA levels between 1996 and 2010. The black horizontal line is the geometric mean concentration of the whole study period. Reprinted from Glynn et al. (2012). Copyright 2013 American Chemical Society.

Table 3. Summary of points of departure for hepatotoxicity and reproductive toxicity. Doses represent NOAELs if not stated other. For congeners lacking data, read-across from the closest most conservative congener on a molar basis has been performed. Original congener-specific data is marked in bold.

Congener	Point of Departure			
	Hepatotoxicity		Reproductive toxicity	
	External dose (mg/kg bw/day)	Internal dose (µg/ml serum)	External dose (mg/kg bw/day)	Internal dose (µg/ml serum)
PFBS	**100**	67[a]	**300**	> 45[a]
PFHxS	**1.0**	**89**	**> 10.0**	**> 60**
PFOS	**0.025**	**4.04**	**0.1**	**4.9**
PFOSA	0.024[b]	4.03[b]	0.1[b]	4.9[b]
PFDS	0.029[b]	4.85[b]	0.1[b]	5.9[b]
PFBA	**6.0**	**14**	**175**	**4.4**
PFPeA	0.04[c]	4.5[c]	0.55[c]	10.0[c]
PFHxA	**20**	5.4[c]	**100**	11.9[c]
PFHpA	**20**	6.2[c]	0.76[c]	13.8[c]
PFOA	**0.06**	**7.1**	**0.86**[d]	**15.7**[d]
PFNA	**0.83**[e]	**28.5**	**0.83**	**8.9**
PFDA	**1.2**	31.6[f]	**3.0**	9.9[f]
PFUnDA	1.01[f]	34.6[f]	1.01[f]	10.8[f]
PFDoDA	**0.02**[c]	37.7[f]	1.10[f]	11.8[f]
PFTriDA	1.19[f]	40.8[f]	1.19[f]	12.7[f]
PFTeDA	1.28[f]	43.9[f]	1.28[f]	13.7[f]
6:2 FTS	0.020[b]	3.45[b]	0.085[b]	4.2[b]

[a] = Read-across on a molar basis from PFHxS
[b] = Read-across on a molar basis from PFOS
[c] = Read-across on a molar basis from PFOA
[d] = BMDL/BMCL
[e] = LOAEL
[f] = Read across on a molar basis from PFNA

Table 4. Summary of points of departure for PFAS congeners and effects observed at a lower effect concentration than for hepatotoxicity and reproductive toxicity.

Congener	Point of Departure		
	Effect	External dose (mg/kg bw/day, µg/l)	Internal dose (µg/ml serum)
PFBS	Hematology (↓ hemoglobin and hematocrit)	60 [a]	N.A.[b]
PFHxS	Hematology (↓ hemoglobin)	0.3 [a,c]	44 [c]
PFOS	Immunotoxicity (↓ IgM response)	0.000166 [a]	0.0178
PFBA	↓ serum cholesterol	3.0 [a]	N.A.[b]
PFOA	Mammary gland development	0.005[c,d]	0.021[c]
	↑ adult body weight, serum leptin and insulin	0.01 [a,c]	N.A.[b]
6:2 FTS[e]	Nephrotoxicity	15[a]	N.A.[b]

N.A. = Not available
[a] = mg/kg bw/day
[b] = will not be used in the risk characterization based on the lack of serum concentration
[c] = LOAEL
[d] = µg/l water
[e] = No effect level for hepatotoxicity or reproductive toxicity identified

2.3.2.3 Risk characterization and conclusions

The outcome of the risk characterization did not indicate any risk for hepatotoxicity or reproductive toxicity associated with PFASs exposure in the general population, neither for congeners assessed individually nor in combination, based on the individual Hazard Quotients and the cumulative Hazard Indexes that were all < 1 (Table 5). The subpopulation that had consumed PFOS-contaminated fish, however, showed Hazard Quotients of 1.3 and 1.0, respectively, indicating concern. Also, regarding the more sensitive toxicological endpoints, immunotoxicity and disrupted mammary gland development, a risk was identified based on exposure to PFOS and PFOA, respectively, showing high Hazard Quotients of 229 and 18, respectively (Table 7).

For the occupationally exposed professional ski waxers a risk was identified for hepatotoxicity by PFOA and by all PFASs in combination (Table 6). For reproductive toxicity, a risk was identified by all PFASs in combination (Table 6). Also, as for the general population, a risk was identified for immunotoxicity and disrupted mammary gland development by PFOS and PFOA, respectively, showing very high Hazard Quotients of 228 and 1884, respectively (Table 7).

Overall, this first attempt of a cumulative risk assessment of PFASs showed that the Hazard Index approach is a suitable method to apply for this class of compounds. In addition to the above presented conclusions, the study also identified the following data gaps that, if filled, would improve future risk assessments:

- Additional hepatotoxicity and reproductive toxicity data for other congeners with internal dose measurements to reduce the number of extrapolations.
- Additional immunotoxicity data and data on effects on mammary gland development for other congeners.
- More knowledge on the mode- and mechanism of action on PFASs in order to clarify the human relevance of these effects.

Table 5. Individual Hazard Quotients (HQs) and Hazard Index for hepatotoxicity and reproductive toxicity in individuals exposed indirectly via the environment.

Congener	Exposure (ng/ml serum)	Hepatotoxicity					Reproductive Toxicity				
		Reference dose (ng/ml serum)	Hazard Quotient[a]	% of Hazard Index	Concern?		Reference dose (ng/ml serum)	Hazard Quotient	% of Hazard Index	Concern?	
					Yes	No				Yes	No
PFBS	0.108	1 335	0.000081	0.03		√	> 2 400	< 0.000060	< 0.03		√
PFHxS	8.50	1 780	0.0048	1.8		√	> 2 400	< 0.0035	< 1.9		√
PFOS	27.5/(204)[b]	162	0.17/(1.3)[b]	64.0	(√)	√	196	0.14/(1.0)	76.2	(√)	√
PFOSA	< 0.040	161	< 0.00025	< 0.09		√	196	< 0.0002	< 0.11		√
PFDS	0.035	65	0.00054	0.2		√	65	0.0004	0.24		√
PFHxA	< 0.22	108	0.0020	0.8		√	628	0.00046	< 0.25		√
PFHpA	0.135	125	0.0011	0.4		√	628	0.00024	0.13		√
PFOA	5.24	142	0.037	13.8		√	628	0.0083	4.5		√
PFNA	2.6	190	0.014	5.1		√	356	0.0073	4.0		√
PFDA	0.70	70	0.010	3.8		√	119	0.0053	2.9		√
PFUnDA	0.83	77	0.011	4.1		√	119	0.0058	3.3		√
PFDoDA	< 0.03	84	< 0.00036	< 0.1		√	119	< 0.00019	< 0.10		√
PFTrDA	< 0.15	91	< 0.0017	< 0.6		√	119	< 0.00088	< 0.48		√
PFTeDA	< 0.04	97	< 0.00041	< 0.15		√	119	< 0.00022	< 0.12		√
6:2 FTS	< 1.82	138	< 0.013	< 5.0		√	196	< 0.011	< 5.9		√
		Hazard Index (HI)	0.25 - 0.27 (1.3 - 1.4)		(√)	√		0.17 - 0.18 (1.1)		(√)	√

N.A. = Not available/not applicable

[a] - RCR = Exposure/DNEL, ratio < 1 = risk is considered controlled, ratio of > 1 = risk is considered not controlled

[b] = Highly exposed population

Table 6. Individual Hazard Quotients (HQs) and Hazard Index for hepatotoxicity and reproductive toxicity in occupationally exposed individuals.

Congener	Exposure (ng/ml serum)	Hepatotoxicity					Reproductive Toxicity				
		Reference dose (ng/ml serum)	Hazard Quotient[a]	% of Hazard Index	Concern?		Reference dose (ng/ml serum)	Hazard Quotient	% of Hazard Index	Concern?	
					Yes	No				Yes	No
PFBS	5.6	3 560	0.002	0.04		√	> 4 800	< 0.0016	< 0.09		√
PFHxS	8.6	3 560	0.002	0.04		√	> 4 800	< 0.0018	< 0.1		√
PFOS	54	323	0.17	3.1		√	392	0.14	8.0		√
PFOSA	< 0.040	323	< 0.00012	< 0.002		√	392	< 0.00010	< 0.006		√
PFDS	0.035	108	0.00027	0.005		√	131	0.00022	0.013		√
PFBA	2.2	560	0.0039	0.07		√	352	0.0063	0.36		√
PFPeA	0.28	284	0.0015	0.03		√	1 256	0.00035	0.02		√
PFHxA	24	284	0.11	2.0		√	1 256	0.025	1.5		√
PFHpA	40	284	0.16	2.9		√	1 256	0.036	2.1		√
PFOA	1070	284	3.8	69.0	√		1 256	0.85	49.3		√
PFNA	326	380	0.86	15.7		√	712	0.46	26.5		√
PFDA	48	127	0.34	6.3		√	237	0.18	10.6		√
PFUnDA	5.6	127	0.036	0.67		√	237	0.019	1.1		√
PFDoDA	< 0.03	127	< 0.00018	< 0.003		√	237	< 0.000096	< 0.006		√
PFTrDA	< 0.15	127	< 0.00082	< 0.015		√	237	< 0.00044	< 0.03		√
PFTeDA	< 0.04	127	< 0.00021	< 0.004		√	237	< 0.00011	< 0.006		√
6:2 FTS	< 1.82	323	< 0.0066	< 0.12		√	392	< 0.0054	< 0.32		√
		Hazard Index (HI)	5.5		√			1.7		√	

[a] - RCR = Exposure/DNEL, ratio < 1 = risk is considered controlled, ratio of > 1 = risk is considered not controlled

Table 7. Individual Hazard Quotients (HQs) for other endpoints in individuals exposed indirectly via the environment and in occupationally exposed individuals.

Congener	Effect	Indirect exposure					Occupational exposure				
		Exposure (ng/ml serum)	Reference dose (ng/ml serum)	Hazard Quotient[a]	Concern?		Exposure (ng/ml serum)	Reference dose (ng/ml serum)	Hazard Quotient[a]	Concern?	
					Yes	No				Yes	No
PFHxS	Hematology	8.6	98	0.08		√	8.6	196	0.04		√
PFOS	Immunotoxicity	27.5	0.12	229	√		54	0.24	228	√	
PFOA	Mammary gland development	5.24	0.28	18	√		1070	0.57	1884	√	

2.4 CONCLUSIONS

In summary, the results generated in **Papers I-IV** showed that:

- PFOS was readily transferred to mouse fetuses after exposure of pregnant dams generating tissue levels that were similar to or higher than the levels in maternal blood. The distribution of PFOS to perinatal and adult lungs was substantial; being highest of all tissues analyzed in fetuses and pups on GD20 and PND1. These findings may, at least partly, explain the respiratory distress seen in neonatal and adult rodents following exposure to PFOS.

- PFOS was recovered in all 19 examined tissues in adult male mice after short-term dietary exposure showing a similar tissue distribution profile between the high and the low dose but with a higher proportion of PFOS distributed to tissues as compared to blood at the higher dose. The highest PFOS concentrations were found in liver, lungs, blood, kidneys and whole bone and the major body compartments were liver, bone, blood, skin and muscle. Blood hemoglobin levels were markedly increased at the high dose which could be connected to the finding of significant localization of PFOS to bone marrow.

- PFBS was recovered in all 20 examined tissues in adult male mice after short-term dietary exposure at the same molar concentration as the high dose of PFOS in Paper II. The distribution and compartment profiles were similar to those of PFOS but PFBS displayed significantly lower tissue concentrations and tissue:blood ratios than PFOS. An erythropoietic effect was seen also for PFBS, though less marked than for PFOS, and a pronounced distribution to cartilage was observed

- The cumulative risk assessment of 17 PFASs analyzed in the Swedish population and in professional ski waxers showed that high local exposures and occupational exposure may be of concern for hepatotoxicity or reproductive/developmental toxicity but indicated no risk for the general population. Concern for immunotoxicity and altered mammary gland development was also identified for both the general population and the occupationally exposed. A need of additional toxicological data for all the assessed endpoints was noted.

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4 SVENSK SAMMANFATTNING

I vårt moderna samhälle exponeras människor för en mängd olika kemikalier dagligen. Beroende på kemikaliernas inneboende egenskaper kan vissa av dessa ansamlas i miljön, även i områden långt från där de har tillverkats eller använts. Ett ökande antal studier pekar på att exponering för låga halter av vissa kemikalier kan ge upphov till ett brett spektrum av negativa hälsoeffekter såsom neurologiska och metabola sjukdomar, försämrat immunförsvar, försämrad reproduktionsförmåga och cancer.

Perfluorerade och polyfluorerade ämnen (PFAS) utgör en stor grupp av mer än 800 industriellt framställda högfluorerade kemikalier. De har på grund av sina ytaktiva egenskaper använts sedan 1950-talet för bland annat impregnering av material som papper och textiler, i rengöringsmedel och vaxer (inklusive skidvallor), i brandsläckningsskum och för tillverkning av fluorpolymerer som Teflon® och Gore-Tex®. I slutet av 1990-talet och början av 2000-talet upptäcktes att PFAS förekom i ett stort antal prover från vilda djur över hela världen och i prover från blodbanker i USA. Även om man tidigare vetat att det funnit fluor i människors blod så har det förrän vid denna tidpunkt funnits metoder för att analysera PFAS. Sedan dessa upptäckter gjordes har ett stort antal studier visat på förekomsten av PFAS i människor, djur och miljön över hela världen. Detta har lett till att PFAS har blivit klassade som extremt svårnedbrytbara miljöföroreningar och gett upphov till oro på grund av deras omfattande globala spridning. I den svenska befolkningen har hittills 17 stycken PFAS analyserats i blodprover. I djurstudier har PFAS visats ge upphov till en rad toxiska effekter varav effekter på levern och fosterutvecklingen är vanligt förekommande, men även påverkan på blodfetter och hormoner, kroppsvikt, immunförsvar och andning har setts. Detaljerad information om hur PFAS fördelas i kroppen är väldigt begränsad och kunskap om fördelningen i kroppen kan öka förståelsen om hur deras toxicitet uppstår. Hälsoriskbedömningsinformation har också saknats för de flesta PFAS förutom de mest studerade, perfluoroktansulfonat (PFOS) och perfluoroktanoat (PFOA).

Syftet med mina studier har varit att 1) ta fram detaljerade data på distributionen av PFOS till olika organ och vävnader i perinatala (tiden före, vid och efter födseln) och vuxna möss samt för den kemikalie som numera används istället för PFOS - perfluorbutansulfonat (PFBS) i vuxna möss, och 2) bedöma potentiella risker för människors hälsa kopplad till exponeringen för de 17 PFAS som analyserats i blodet på den svenska befolkningen, var för sig och alla tillsammans.

Resultaten av mina studier visade att PFOS snabbt överfördes till musfoster efter dosering av moderdjuren. Deras vävnadsnivåer av PFOS var lika höga eller högre än nivåerna i moderdjurets blod. PFOS ansamlades markant i de perinatala lungorna där de förekom i högst halter av alla vävnader på dräktighetsdag 20 (dagen före födseln) och efter födseln. PFOS ansamlades även i hög grad i moderdjurens lungor. Sammantaget kan dessa resultat hjälpa till att förklara de effekter på andningen som tidigare setts i nyfödda och vuxna djur efter exponering för PFOS. En tänkbar verkningsmekanism är att PFOS, när det hamnar i lungorna, stör funktionen av den så

kallade surfaktanten. Denna fungerar som ett ytspänningsnedsättande ytskikt i lungorna och möjliggör en normal andning.

Vidare kunde PFOS mätas i alla 19 undersökta organ/vävnader i vuxna möss som exponerats under kort tid för PFOS via maten antingen för en låg dos, liknande den som människor utsätts för, eller en högre dos liknande den som ofta används i djurstudier. Distributionen såg likadan ut för de båda doserna men med skillnaden att mer PFOS fördelades till vävnaderna istället för i blodet vid den högre dosen. Detta visar att man kan överföra distributionsdata från höga doser av PFOS till låga doser. De högsta koncentrationerna av PFOS fanns i lever, lungor, njurar, ben (inklusive benmärg) och de största totala mängderna av PFOS återfanns i lever, ben (inklusive benmärg), blod, skinn och muskler. En vävnad som för första gången upptäcktes som ”målvävnad” för PFOS var benmärg. Detta kan möjligen förklara de höga halterna av PFOS som återfanns när man mätte på hela skelettbenet. Ett annat anmärkningsvärt resultat var att hemoglobinnivåerna var tydligt förhöjda i högdosgruppen vilket kan indikera att PFOS utövar en hematopoetisk (blodcellsbildande) effekt i benmärgen.

I ett ytterligare försök där PFBS gavs till vuxna möss i maten, på samma sätt och i samma dos som den högre dosen i det tidigare PFOS-försöket, kunde PFBS mätas i alla 20 undersökta organ/vävnader. Distributionsprofilen var lik den för PFOS, men med skillnaden att nivåerna var 5-40 gånger lägre och att mindre PFBS återfanns i vävnader jämfört med i blodet. Höga halter av PFBS kunde också uppmätas i brosk. Även här kunde en hematopoetisk effekt ses, men i lägre grad än för PFOS.

I det sista delarbetet gjordes en hälsoriskbedömning av exponeringen för de 17 PFAS som mätts i blodet hos den svenska befolkningen samt för högexponerade yrkesverksamma skidvallare. I studien undersöktes dels riskerna med varje PFAS-ämne individuellt (som oftast görs i en riskbedömning) samt för alla PFAS tillsammans vilket är första gången det görs för denna grupp av ämnen. Resultaten visade inte på någon risk[1] för effekter på levern eller på utvecklingstoxiska effekter i den allmänna befolkningen associerade med PFAS, varken individuellt eller i kombination. Däremot så kunde en risk förknippas med lokalt höga exponeringar, t ex hos personer som ätit kontaminerad fisk. För de yrkesexponerade skidvallarna kunde en risk för dessa effekter associeras med exponering för enskilda PFAS-ämnen och/eller alla PFAS tillsammans. För både allmänbefolkningen och den yrkesexponerade gruppen kunde en risk för effekter på immunsystemet och utvecklingen av bröstkörtlar påvisas. Ett behov av ytterligare toxikologiska data för alla toxikologiska effekter som ingått i bedömningen identifierades också.

Sammantaget har arbetet i denna avhandling genererat nya distributionsdata som kan användas för riskbedömning av PFAS. Den har också bedömt riskerna associerade med exponeringen för PFAS i Sverige och identifierat behov av ytterligare data.

[1]Med risk avses inte att det idag nödvändigtvis finns hälsoproblem på grund av kemikalien men visar på att marginalen mellan nuvarande exponeringsnivåer och toxiska effektnivåer är otillräcklig. En ytterligare förfining av riskbedömningen och/eller förebyggande åtgärder för att reducera exponeringen kan vara nödvändigt.

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