Occurrence and maternal transfer of perfluoroalkyl substances (PFASs), and their associations with thyroid hormones in hooded seal (*Cystophora cristata*) mother-pup pairs

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MASTER THESIS IN TOXICOLOGY

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UNIVERSITY OF OSLO

29.05.2015

2015

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Trykk: Reprosentralen, Universitetet i Oslo

Acknowledgements

The work presented in this master thesis was accomplished at the Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, and at the Laboratory of Environmental Toxicology at the Norwegian University of Life Sciences (NMBU), Campus Adamstua, under the supervision of Katrine Borgå (prof.) at UiO, Gro Dehli Villanger (PhD) at the Norwegian Institute of Public Health (FHI), Anuschka Polder (prof.) at NMBU, and Ketil Hylland (prof.) at UiO.

First of all, I would like to thank my main supervisor, Katrine, for excellent guidance and motivation throughout this master project. Thank you for always being positive and for motivating me to work hard. I would also like to thank my co-supervisors, Gro and Anuschka for being supportive, and for all the constructive criticism and proof reading. It has been greatly appreciated! Thank you, Gro, for including me in this project!

Big thanks to Mahin Karimi, Katharina B. Løken and the rest of the staff at the NMBU for training and guidance during the contaminant analysis. I would also like to thank Christian Lydersen and Kit M. Kovacs from the Norwegian Polar Institute (NP), who were involved with the sampling in the West Ice together with Bjørn Munro Jenssen at the Norwegian University of Science and Technology (NTNU).

A special thanks to Karl Johan for excellent collaboration during the long days at the laboratory, and for many productive and unproductive coffee breaks. Thanks to everyone at study room 4604, including Ragna, for helpful comments and for making the study time a lot more fun.

Finally I would like to thank my family for always supporting me, and especially Hugo for forcing me to have some free time in between all the work, so I wouldn't lose my mind, and for always being there.

Abstract

Long-range atmospheric transport, ocean currents, and rivers transport environmental contaminants, such as perfluoroalkyl substances (PFASs), to the Arctic. Some of these PFASs may reach high concentrations in the upper trophic levels in the arctic food web due to bioaccumulation and biomagnification, and may disrupt physiological processes due to interference with endogenous compounds. In mammals, thyroid hormones are important for several biological processes, especially during the developmental stages, and studies have shown that PFASs may disrupt the thyroid hormone homeostasis. The hooded seal (*Cystophora cristata*) is a top predator in the arctic marine food web and is therefore susceptible to high exposure to PFASs, and the pups are suspected to be exposed both *in utero* and via the milk. The present study reports, for the first time, levels of PFASs in hooded seal mother-pup pairs. The aims of the study were to investigate levels and patterns of PFASs in plasma and milk, and to determine if the PFASs are subject to maternal transfer via milk and/or placenta. The study also investigated associations between concentrations of PFASs and levels of free and total thyroid hormones in plasma. PFOS was the most predominant PFAS, and the mean PFOS levels reported herein were 13.4, 30.4 and 1.24 ng/g w.w. for maternal plasma, pup plasma and maternal milk samples, respectively. Levels were within the range of levels reported in similar studies on pinnipeds. PFAS levels were generally higher in plasma than milk, supporting that binding to plasma proteins limits their incorporation into milk. There were higher levels of PFHxS, PFOS, PFDoA, PFUdA and PFTrDA in plasma of pups than mothers, while levels of PFNA and PFDA were higher in mothers than pups. The current study confirmed maternal transfer of PFASs from hooded seal mothers to pups and that this occurs via milk and probably also placenta. In fact, the multivariate analysis implied that placenta could be a more important transfer route than milk. There were different transfer ratios for PFASs with different carbon chain lengths, with the lowest transfer efficiency for the intermediate chained PFASs. There were negative associations between specific PFASs and free thyroid hormone levels in both maternal and pup plasma, and between total thyroid hormones and specific PFASs in mothers. This indicates effects of PFASs on the thyroid hormone homeostasis. Positive correlations were reported for TT3:FT3 and several PFASs in mothers and for TT4:FT4 and PFTrDA in pups. The study of endocrine disruption is complex; it was therefore difficult to draw any conclusions on possible mechanisms or effects of PFAS exposure on thyroid hormones in hooded seals.

Abbreviations

ANOVA	Analysis of variance
BFR	Brominated flame retardant
C	Carbon
CH_3OH	Methanol
DDT	Dichloro-diphenyl-trichloroethane
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
F	Fluorine
FDU	Animal Research Authority
FT3	Free triiodthyronine
FT4	Free thyroxine
GLM	General linear model
H_2O	Water
HCl	Hydrogen chloride
HPLC MS-MS	High pressure liquid chromatography tandem mass spectrometry
HPT	Hypothalamus-pituitary-thyroid
LOD	Limit of detection
Log	Logarithm
N_2	Nitrogen gas
n.d.	Not detected
NMBU	Norwegian University of Life Sciences
NP	Norwegian Polar Institute
n.r.	Not reported
NTNU	Norwegian University of Science and Technology

OCP	Organochlorine pesticide
OHC	Organohalogen contaminant
OH-PCB	Hydroxylated polychlorinated biphenyl
PBDE	Polybrominated diphenyl ether
PC	Principal component
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PFAS	Perfluoroalkyl substance
PFCA	Perfluoroalkyl carboxylate
PFDA	Perfluorodecanoic acid
PFDoA	Perfluorododecanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PFSA	Perfluoroalkyl sulfonate
PFTrDA	Perfluorotridecanoic acid
PFUdA	Perfluoroundecanoic acid
POPs	Persistent organic pollutant
RDA	Redundancy analysis
rpm	Rotations per minute
R_p	Pearson's correlation coefficient
SD	Standard deviation
SE	Standard error
SULT	Sulfotransferase

TBG	Thyroxine-binding globulin
T3	Triiodothyronine
T4	Tetraiodothyroine/thyroxine
TH	Thyroid hormone
TH*	Radiolabelled thyroid hormone
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TT3	Total triiodothyronine
TT4	Total tetraiodothyroine/thyroxine
TTR	Transthyretin
UDP-GT	Uridine-diphosphate glucuronolsyl transferase
UiO	University of Oslo
w.w.	Wet weight

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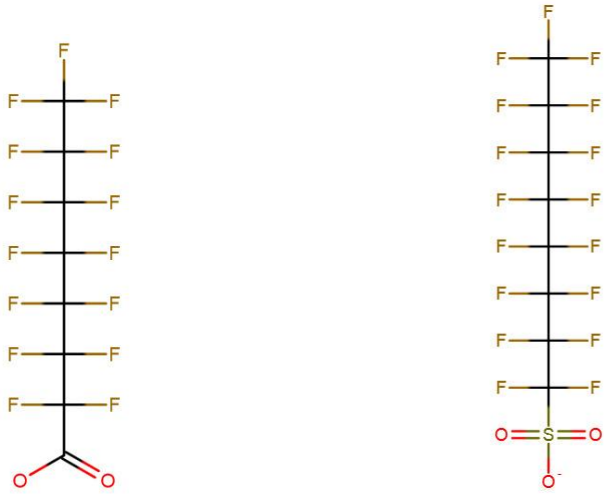
1. Introduction

Despite long distances from source regions, arctic environments and ecosystems are subjected to contamination by a complex mixture of industrial and agricultural chemicals and by-products known as persistent organic pollutants (POPs) (Macdonald *et al.* 2000). These are transported to the Arctic by long-range atmospheric transport, ocean currents, ice packs and rivers (AMAP 2004). The legacy POPs include the polychlorinated biphenyls (PCBs) and various organochlorine pesticides (OCPs). These chemicals have been regarded as an environmental concern for approximately the last five decades (McKinney *et al.* 2011). However, numerous recent- and current-use chemicals have also been newly known as environmental contaminants. These include perfluoroalkyl substances (PFASs) and their precursors, that are found to accumulate in the biota, including in the Arctic (Butt *et al.* 2010). Arctic ecosystems are vulnerable to contamination because of biomagnification which makes predatory organisms on top of the food web particularly at risk (Kannan *et al.* 2005), and because contaminants may add to the stress caused by the harsh, arctic environment (Jenssen *et al.* 2015).

1.1. Perfluoroalkyl substances (PFASs)

All PFASs that are found in the environment are anthropogenic (Giesy and Kannan 2002, Butt *et al.* 2010). The production of PFASs started in the 1950s, however, little attention has been devoted to these chemicals until recently, when new methods of detection became available (Hansen *et al.* 2001, AMAP 2004). Some PFASs are regarded as POPs, however, unlike legacy POPs which accumulate in lipid rich tissues, PFASs are known to bind to proteins and accumulate mainly in blood, liver, kidneys and bile secretions (Jones *et al.* 2003). PFASs were first reported as a contaminant in the Arctic by Giesy and Kannan (2001). PFASs have been incorporated into a wide range of industrial and consumer products (Prevedouros *et al.* 2005). They are molecules that consist of a carbon chain fully substituted with fluorine and a polar moiety in one end which may consist of a variety of groups (Figure 1). With the hydrophobic tail and the hydrophilic head, PFASs have common features with surfactants in general, meaning that they are soluble in water with a tendency to stay at surfaces or interfaces (3M 1999), making these chemicals key ingredients in non-stick cook wear, waterproof outdoor gear, fire fighting foams, etc. (Kissa 2001). PFASs can be divided into several classes, based on their molecular structure. Two groups of PFASs, the perfluoroalkyl acids (PFCAs) and perfluoroalkyl sulphonates (PFSAs; Figure 1) have in the past years

received attention because of their widespread presence in the environment, humans and wildlife (Houde *et al.* 2006a).



Perfluorooctanoic acid (PFOA) Perfluorooctanesulfonate (PFOS)

Figure 1. The chemical structure of an acid: perfluorooctanoic acid (PFOA), and a sulfonate: perfluorooctansulfonate (PFOS). (Figure: retrieved 10.05.2015, from http://www.chemicalize.org/)

The carbon-fluorine bond (C-F-bond) is one of the strongest in nature. This strong, high energy bond contributes to the stability of PFASs. PFASs are resistant against degradations by acids, bases, oxidants, reductants, photolytic processes, microbes, and metabolic processes. These properties are very useful for the industry, but the persistency is also the reason for their global distribution in the environment (3M 1999, Kissa 2001) and may lead to bioaccumulation and biomagnification in the marine food web (Haukås *et al.* 2007). In general terms, exposure studies have shown that PFASs may be teratogentic (i.e. induce developmental effects) (Lau *et al.* 2004, Lau *et al.* 2006), immunotoxic (Keil *et al.* 2008) and can act as endocrine disruptors (Thibodeaux *et al.* 2003, Jensen and Leffers 2008).

In 2000 the US Environmental Protection Agency (EPA) banned perfluorooctanesulfonate PFOS from the US market, and in May 2009 it was added to Annex B of the Stockholm Convention on POPs (www.pops.int). However, several PFASs continue to be manufactured as emulsifiers and additives in the polymerization process, as the industry has not yet found a suitable replacement for these compounds (Bossi *et al.* 2005a). The newly published Madrid

statement (Blum *et al.* 2015) argued to limit the production and use of PFASs and urged scientists to assemble a global inventory of all PFASs in use or present in the environment, investigate the mechanisms of toxicity and exposure, and to continue monitoring for legacy PFASs in different matrices and for environmental reservoirs of PFASs (Blum *et al.* 2015).

1.1.1 Bioaccumulation and biomagnification of PFASs

Because PFASs are nearly indestructible, they can persist in the environment for decades or more. This leads to higher levels in the environment, where they are prone to accumulate in the tissues of wildlife and humans. Through the process of biomagnification PFASs can be transferred up the food chain, where concentrations increase from one trophic level to the next via dietary accumulation (Gobas and Morrison 2000). In the Arctic, diet is the main source of exposure to most contaminants (AMAP 2004).

Field studies show varying degrees of bioaccumulation of the different PFASs in marine food webs (Tomy *et al.* 2004, Martin *et al.* 2004a, Houde *et al.* 2006b, Haukås *et al.* 2007, Powley *et al.* 2008). The current understanding is, however, that PFOS and longer chained PFASs (larger than C_8) bioaccumulate and persist in protein-rich compartments of fish, birds and marine mammals, and that the PFCAs with seven or fewer fluorinated carbons cannot be considered bioaccumulative according to current regulatory criteria used to identify bioaccumulative compounds (Conder *et al.* 2008).

PFASs have been reported to biomagnify in the arctic marine food web (Tomy *et al.* 2004, Bossi *et al.* 2005b, Powley *et al.* 2008, Kelly *et al.* 2009). The degree of trophic transfer of PFASs is similar to that of lipid soluble PCB, dichloro-diphenyl-trichloroethane (DDT) and polybrominated diphenyl ethers (PBDE) (Haukås *et al.* 2007). Retention in protein-rich compartments may be of toxicological importance, due to potential interference with basic cellular processes such as fatty acid metabolism (Goecke-Flora and Reo 1996) and transport of hormones (Gutshall *et al.* 1989, Zoeller *et al.* 2012).

1.2. Maternal transfer

During their prenatal and early postnatal development, mammals can be exposed to toxicants *in utero* from maternal accumulated toxicants, during the neonatal period via intake of mother's milk, or by direct ingestion or contact (Clutton-Brock 1991). During the neonatal period, mammalian reproduction includes a dependent phase in which offspring rely on milk as a primary source of energy (Clutton-Brock 1991). Due to the complexity of the

development of the mammalian brain, there are windows of susceptibility during prenatal and postnatal phases, where the interference with toxicants may be especially detrimental (Johansson *et al.* 2008).

Many PFASs are readily soluble in water, are reversibly bound to blood proteins such as albumin (Han *et al.* 2003), enter the enterohepatic circulation and appear to distribute in extracellular fluids. All this makes plausible the transfer to the embryo or foetus across the placenta or to the nursing pup via milk (Hinderliter *et al.* 2005). PFASs have been detected in cord blood, maternal milk and breastfeeding offspring of human, supporting both a prenatal and postnatal transfer of PFASs (Fromme *et al.* 2010), and several studies have reported maternal transfer of PFASs to the offspring via both milk and placenta in rats and humans (Inoue *et al.* 2004, Hinderliter *et al.* 2005, Midasch *et al.* 2007).

Maternal transfer of environmental contaminants can result in young neonates having high levels of exposure. This is of great concern, since developing mammals have a reduced ability to metabolise and excrete xenobiotics, and are generally considered to be more susceptible to toxic effects compared to adults (Grandjean and Landrigan 2006, Wolkers *et al.* 2009). However, there is little knowledge regarding maternal transfer of PFASs in highly exposed species such as marine mammals who have different lactation and energy transfer to offspring compared to humans and terrestrial animals.

1.3. Endocrine disruption: thyroid hormones

For the conservation of marine mammals and their ecosystems, it is important to know whether they are exposed to levels of environmental pollutants where biological effects might occur. Disturbances to the endocrine system seems to be a common denominator when investigating this (Reijnders 1994). According to the US EPA, an endocrine disruptor is defined as "any exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock *et al.* 1996). There is an increasing concern that exposure to environmental chemicals can disrupt hormone signalling during early development, thereby causing irreversible effects on health, reproduction and survival in later life-stages (Zoeller and Crofton 2000). Several studies have shown that PFASs disrupt the thyroid hormone homeostasis in animals (Lau *et al.* 2003, Thibodeaux *et al.* 2003, Yu *et al.* 2009).

Thyroid hormones, mainly thyroxine (T4) and triiodothyronine (T3) (Figure 2), are essential for normal development and maintenance of physiological functions. These hormones play important roles in regulating metabolism, growth and are key hormones for the development of the central nervous system and brain functions in mammals (Porterfield and Hendrich 1993, Zoeller *et al.* 2007). The adverse effects of PFASs on growth and neurological development are potentially related to thyroid hormone deficiency (Zoeller and Crofton 2000). However, the mammalian hypothalamus-pituitary-thyroid (HPT) axis is complex (depictured in Figure 3), and there are many overlapping target points for thyroid disrupting contaminants. Several physiological steps within this axis may be disturbed by xenobiotic exposure, resulting in thyroid hormone imbalance (Zoeller *et al.* 2007).

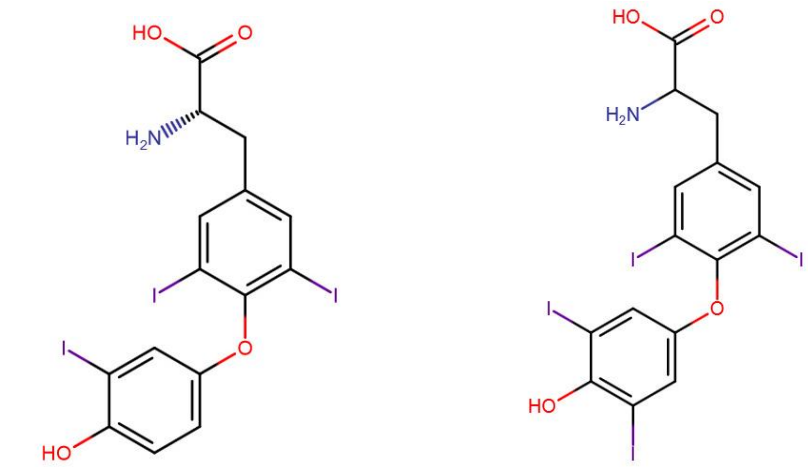


Figure 2. The chemical structure of the thyroid hormones triiodothyronine (T3) and thyroxine (T4). (Figure: retrieved 10.04.2015, from http://www.chemicalize.org/)

In young animals, neurodevelopmental deficits caused by hormonal imbalance may reduce learning, the ability to find and hunt prey, and change behaviour, ultimately effecting reproduction and survival (Derocher *et al.* 2003, Jenssen *et al.* 2015). Thyroid hormone disruption can also reduce an animal's ability to adjust the metabolic rate to factors like ice-cover, temperature, food availability and energy requirements, and thus their ability to adapt to environmental changes, which may be particularly adverse in the fast changing, harsh, arctic environment (Jenssen 2006, Jenssen *et al.* 2015). This highlights the importance of thyroid hormones, homeostasis, and the adverse effects PFASs may have on offspring if they are interfering with this hormone system.

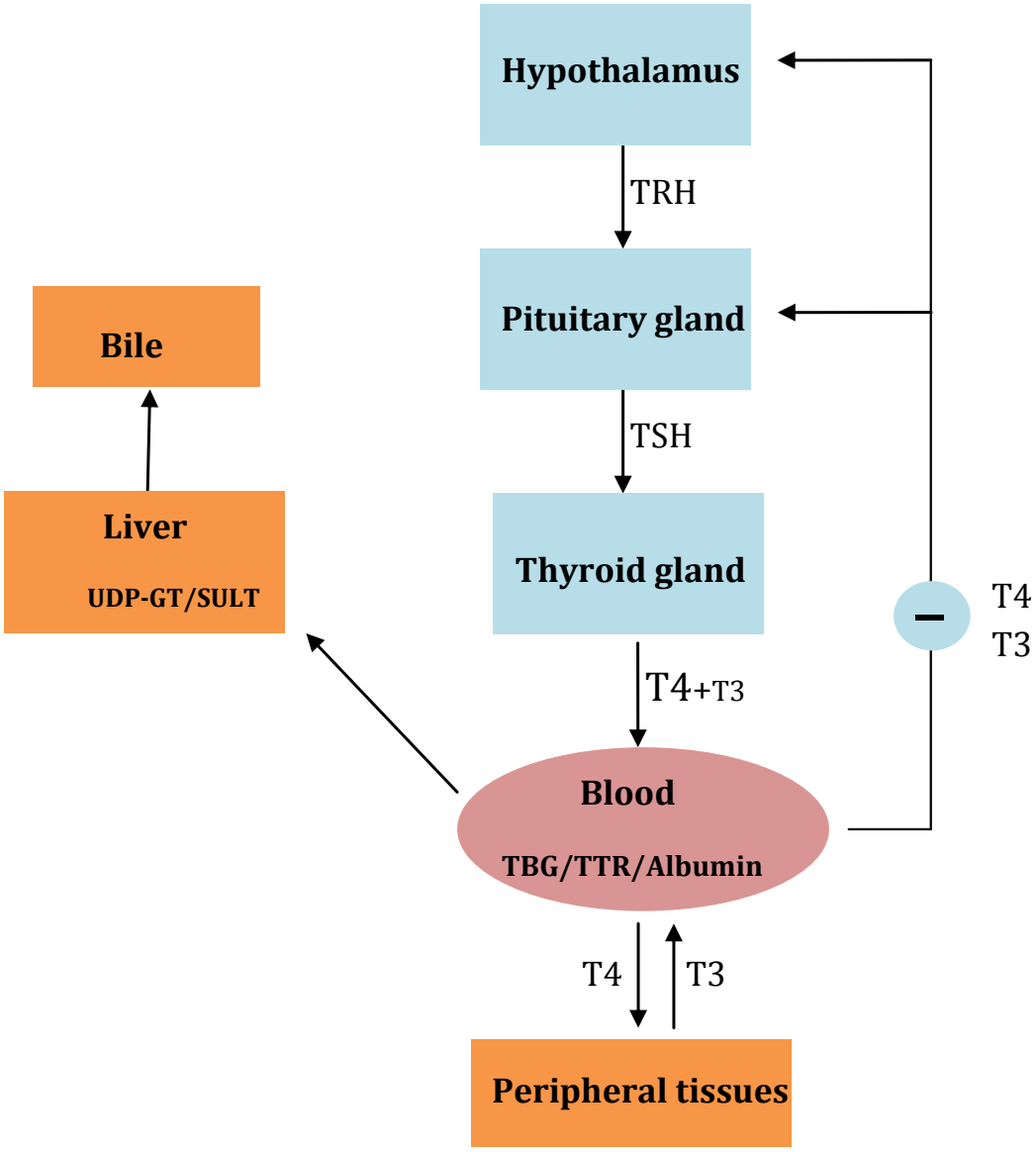


Figure 3. The mammalian HPT-axis. TRH: tripeptide thyrotropin-releasing hormone, TSH: thyroid-stimulating hormone, T4 and T3: Thyroid hormones, TBG: thyroxine-binding globulin, TTR: transthyretin, UDP-GT: UDP-glucuronosyl transferase, SULT: sulfotransferases. (Figure: private).

1.4. Present study species: the hooded seal (*Cystophora cristata*)

The marine mammal, hooded seal (*Cystophora cristata*), was chosen as a model in the present study because they have the shortest nursing period of any marine mammal, in which the pups have extreme growth rates (Bowen *et al.* 1985). They are defined as capital breeders, meaning that they have a high investment in the offspring, where reproduction is financed using stored energy (Stephens *et al.* 2009).

The hooded seal belongs to the pinniped family *Phocidae*, and inhabits the North Atlantic and the Arctic Oceans (Kovacs *et al.* 2009). They occupy a relatively high trophic level in the arctic marine food web, and feed on larger deep water species like Greenland halibut and a variety of redfish species in addition to squid, polar cod and crustaceans (Haug *et al.* 2007, Kovacs 2009).

The peak season for breeding is in late March for hooded seals in the West Ice, and they give birth to only one offspring per breeding (Kovacs *et al.* 2009). Seal species that give birth on ice floats are characterized by a short and intensive lactation period (Bowen *et al.* 1985). The hooded seal lactation period has an average duration of only three to four days, during which the pups can grow at rates exceeding 7 kg a day and increase their body mass from about 23-25 kg at birth to 45-50 kg at weaning (Kovacs and Lavigne 1992, Lydersen *et al.* 1997). The extreme rate of growth is accomplished through the intake of the most energy-rich milk produced by any marine mammal (> 60% fat) (Oftedal *et al.* 1988). The mass transfer rate from maternal tissues to pup tissue during these three to four days is around 60% (Kovacs and Lavigne 1992, Lydersen and Kovacs 1999).

Since the hooded seal may be susceptible to high exposure to PFASs via biomagnification in the arctic marine food web, and as these chemicals may be present in both blood and milk, the pups are suspected to be exposed both *in utero* through transplacental transfer and via the milk. Since the hooded seal's lactation period is very short and intensive, and the pups have no other exposure source than maternal transfer, the investigation of the intensity of the maternal transfer of PFAS was particularly interesting. No studies have previously been conducted to investigate levels, patterns and maternal transfer of PFASs, or the possible associations between PFAS concentrations and thyroid hormones in hooded seals.

1.5. Aims and hypotheses

Aim 1: Quantify levels and patterns of PFASs in hooded seal mother-pup pairs.

H1: Because PFASs bind to blood proteins and mainly accumulates in blood, liver and kidneys, there will be higher levels of PFASs in the plasma samples compared to the milk samples.

H2: There will be higher levels of PFASs in maternal plasma compared to that of the pup plasma because of exposure and accumulation over a longer period of time in adult hooded seals.

H3: Studies show that in wildlife, perfluorooctane sulfonate (PFOS) is generally the PFAS with the highest relative contribution. PFOS will therefore be the most predominant PFAS in the hooded seal samples (both plasma and milk).

Aim 2: Determine if PFASs are transferred from mother to pup via milk and/or placenta.

H4: Studies show that PFASs can be transferred to the milk. PFASs will therefore be detected in the hooded seal milk samples, and milk will work as a route for transfer of PFASs from mother to pup.

H5: Because PFASs bind efficiently to blood proteins, which limits the excretion into milk, PFASs will mainly be transferred from mother to pup via the placenta (prenatal transfer).

H6: Because chemicals with different molecular structures (chain length, functional group, etc.) have different binding affinities to proteins, there will be different transfer ratios for different PFASs, depending on their chemical structure and carbon chain length.

Aim 3: Identify possible associations between concentrations of PFASs and thyroid hormones in blood of hooded seal mothers and pups.

H7: PFAS will interference with the thyroid hormone homeostasis and affect both concentrations and ratios of thyroid hormones measured in plasma of hooded seal mothers and pups. Mostly negative associations between individual PFASs and thyroid hormone variables are expected, but positive relationships are also likely.

2. Materials and methods

2.1. Sampling

The blood and milk samples used in this study were collected by the Norwegian Polar Institute (NP) in the West Ice, north of Jan Mayen (approximately 73.3°N, 14.5°W, Figure 4), Norway in March 2008. The seals were approached from the research vessel "Lance" belonging to NP. The mothers were sedated during the sampling, while the pups were handled without sedation. Blood samples were obtained from 15 mother-pup pairs, while milk samples were obtained from 9 of the mothers (Figure 5). The sex of the pups was noted, the age (days) of the pups was estimated based on the developmental stage, and the body mass of both mothers and pups was measured to the nearest half kg (Gabrielsen *et al.* 2011).

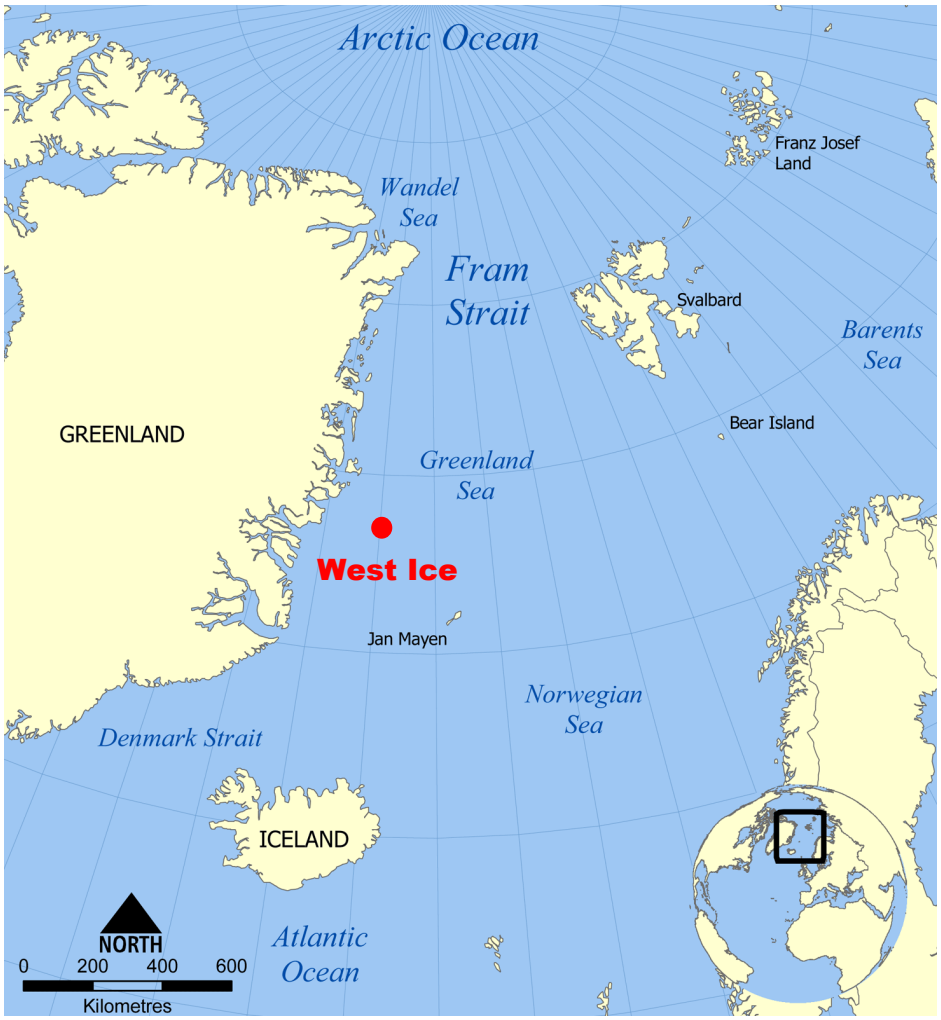


Figure 4. Sampling area. Red dot represents approximate coordinates. (Modified figure retrieved 15.05.2015, from http://en.wikipedia.org/wiki/Greenland_Sea).

The blood was collected from the caudal vein by injecting a syringe between the vertebrae, and collected in heparinised and ethylenediaminetetraacetic acid (EDTA) coated Venjoct® tubes (10 mL, Terumo Corporation, Belgium). The blood samples were centrifuged and plasma and serum were transferred to, and stored at, -70°C at the Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway (Gabrielsen *et al.* 2011). The milk samples (approximately 10 mL) were collected from adult females 10 minutes after an intramuscular injection of 20 IU oxytocin. All milk samples were stored and frozen at -20°C until analyses (Lydersen *et al.* 1997). Both blood and milk were later transferred to the Norwegian University of Life Sciences (NMBU), Campus Adamstua, Oslo, Norway, where they were stored at -20°C. All animal handling was performed after the principles and guidelines and by permit from the National Animal Research Authority (FDU) (Mattilsynet 2005).

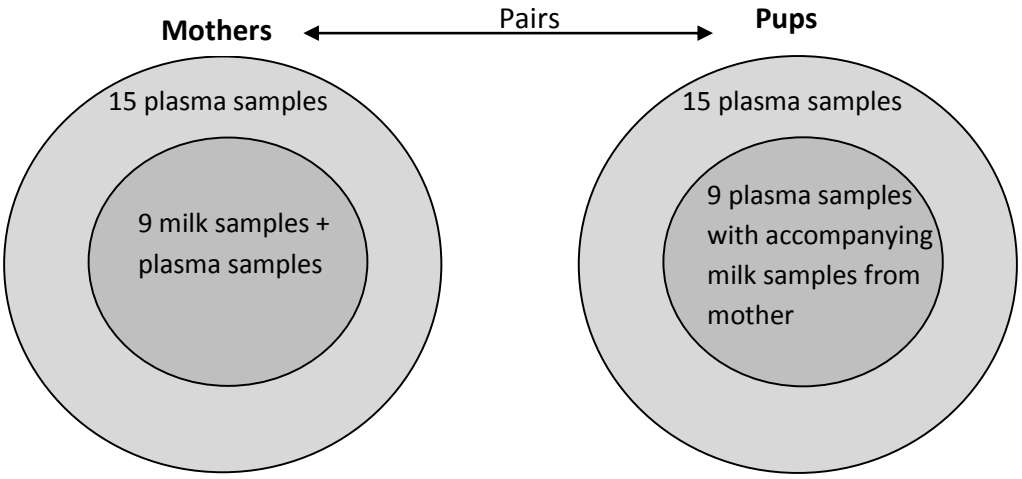


Figure 5. Schematic overview of the hooded seal samples collected from the West Ice (2008). (Figure: private)

2.2. Contaminant analysis

The PFASs were analysed at the Laboratory of Environmental Toxicology at NMBU. The method includes extraction with solvent, clean-up of fat and other pollutants with active coal and separation and detection with high-performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS). In addition, a multi-method (multimetode M-MT.2.2) was conducted to analyse for organochlorines (OCs) and brominated flame retardants (BFRs) in the milk samples. This was done in cooperation with Karl Johan Ullavik Bakken for his master's project. The results from the OC and BFR analyses were not used in the present study.

The hooded seal samples analysed in this study consisted of 30 plasma samples with 15 mother-pup pairs, and of these 15 pairs, nine had milk samples available. The method for PFAS determination had not previously been conducted on milk matrix at NMBU. Therefore, a validation of the method for the milk matrix was conducted, using milk of domesticated cows. The method proved successful, and the analysis could proceed with the hooded seal samples. Table 1 shows an overview of the PFAS contaminants analysed for.

Table 1. PFAS contaminants subject for analysis in hooded seal plasma- and milk samples from the West Ice (2009).

PFAS group	**PFAS contaminants**	**Chain length (Nr. of fluorinated carbons)**
PFSAs (sulfonates)	Perfluorohexanesulfonate (PFHxS)	C_6 (C_6)
	Perfluorooctanesulfonate (PFOS)	C_8 (C_8)
PFCAs (acids)	Perfluorooctanoic acid (PFOA)	C_8 (C_7)
	Perfluorononanoic acid (PFNA)	C_9 (C_8)
	Perfluorodecanoic acid (PFDA)	C_{10} (C_9)
	Perfluoroundecanoic acid (PFUdA)	C_{11} (C_{10})
	Perfluorododecanoic acid (PFDoA)	C_{12} (C_{11})
	Perfluorotridecanoic acid (PFTrDA)	C_{13} (C_{12})

2.2.1. Addition of internal standards

1 mL of the hooded seal plasma, and 0.5 mL x 2 of the hooded seal milk (milk was separated into A and B tubes because of higher lipid content) was weighed precisely in Falcon centrifuge tubes (VWR International, LLC Radnor, USA). All tubes and pipettes used were made of plastic (PFASs adhere less to the surface of plastic than glass, and thus leads to more accurate results). The internal standards, containing a ^{13}C-labeled PFAS mix were added to the samples. For the acids (500 ng/mL: PFOA, PFDA, PFNA, PFUdA, PFDoA, PFTrDA), internal standards were added to the samples to a concentration of 20 ng/mL in the final extracts. The internal standards for the sulfonates (1µg/mL: PFHxS, PFOS) were added to the samples to the same concentration as the acids.

2.2.2. Extraction

The samples were added 5 mL methanol (CH_3OH) (Rathburn chemicals, Walkerburn, Scotland) and shaken for 10 seconds on a Whirlymixer (MS2 Minishaker, IKA®, MA, USA) followed by 30 minutes of mixing in a Vibrax machine (Vibrax VXR, IKA®, MA, USA). The samples were then centrifuged at 3000 rpm for 10 minutes (Allegra® X-12R, Beckman Coulter, CA, USA). The supernatant was extracted and added to new Falcon tubes. The remaining deposits were then extracted a second time with 5 mL methanol added to the plasma samples and 3 mL added to the milk samples. This was first mixed with a spatula, then mixed on the whirlymixer (10 seconds) and then mixed on the Vibrax machine (30 minutes). This was followed by a second centrifugation (3000 rpm, 10 minutes). The supernatant was extracted and pooled with the supernatant from the first extraction. The supernatants were then evaporated to a volume of 2 mL using heat blocks (37°C) with a gentle flow of nitrogen gas (N_2) (Purity: 99.6%, Aga AS, Oslo, Norway).

2.2.3. Clean-up

Approximately 0.2-0.3 g active coal (ENVI-Carb™, Sigma-Aldrich, Oslo, Norway) was added to each sample (about 0.2 g added to the plasma samples and 0.3 g added to the milk because of higher lipid content in the milk matrix). The samples were mixed on the Whirlymixer (10 seconds) and then centrifuged (3500 rpm, 15 minutes). The supernatant was added to new plastic test tubes. 1 mL methanol was then added to the remaining deposits and the previous step (clean-up with active coal) was repeated. The supernatant was evaporated to dryness on heat blocks (37°C) with a flow of nitrogen gas. 1 mL of a methanol and water solution (1:1) was added to each sample (0.5 mL to each of the A and B milk samples). This

was rapidly mixed on the Whirlymixer. The A and B milk samples where then pooled and rapidly mixed again. The samples were then centrifuged (3000 rpm, 10 minutes) and transferred to vials with plastic inlets (200 µL).

2.2.4. Quantification of PFASs

The samples were analysed by separation of compounds on a high-performance liquid chromatographer (HPLC) with a Discovery C18 column: 15 cm x 2.1 mm x, 5 µm (Supelco, Sigma-Aldrich, Oslo, Norway) connected to a pre-column Supelguard Discovery C18 column: 2 cm x 2.1 mm x, 5 µm (Supelco, Sigma-Aldrich, Oslo, Norway) and detection with liquid chromatography tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS System). The injected volume was 5 µL. For more detailed HPLC-MS-MS settings, see Appendix A.

Native ^{12}C and ^{13}C-labeled equivalents were analyzed, representing all the groups of PFCAs and PFSAs. The labelled standards were used to produce a standard curve from which concentrations were calculated. Concentrations were calculated from the chromatographic data using the instrument control and data processing program Analyst® Software Version 1.6. The limits of detection (LOD) of the PFAS compounds were calculated by using three times signal to noise ratio found in the samples, unless a higher signal was recorded in the blank samples. The LOD for PFASs in milk ranged from 0.026 to 0.427 ng/g w.w. and the LOD in plasma ranged from 0.013 to 0.130 ng/g w.w. The specific LOD for each compound is listed in Table 3 (results).

2.2.5. Analytical quality assurance

The Laboratory of Environmental Toxicology, NMBU, is accredited by the Norwegian Accreditation for testing the analyzed chemicals in biological material according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). Although the quantification of PFASs in milk and plasma is not an accredited method, it was validated after the same criteria as the accredited methods for POPs.

For each sample series, a relative recovery rate was calculated from samples of low-contaminated material (mixed serum from dog and cat, R1-R4) spiked with known standards. The relative recovery rate for PFASs ranged from 88% to 109% in the milk samples and from 80% to 132% in the plasma samples.

For each series, there were three blanks (only solvents) and tree controls of serum (AMAP ringtest) to control for contamination.

2.3. Quantification of lipids

The lipid content was determined gravimetrically as a step in the multi method (multimetode M-MT.2.2) when analyzing for OCs and BFRs. The lipid concentration in the 9 milk samples was determined by adding 1 mL of the fat extracts to pre-weighed glasses and then placed on sand bath (40°C). After about 24 hours, when evaporated to dryness, the glasses were weighed again, until stable weight (± 0.002 g). The lipid content of the 30 plasma samples was determined gravimetrically in Gabrielsen *et al.* (2011). The whole extract was placed in pre-weighed glasses on a sand bath (40˚C) and evaporated using a gentle flow of N_2 gas until dryness (constant weight ± 0.002 g).

For the milk samples, the fat percentage was calculated using the following equation:

$$\frac{(\text{Weight of glass with fat - weight of empty glass}) * \text{glass volume} * 100}{\text{Weight of initial sample} * \text{removed volume}}$$

For the plasma samples, the fat percentage was calculated using the following equation:

$$\frac{(\text{Weight of glass with fat - weight of empty glass}) * 100}{\text{Weighed sample}}$$

2.4. Quantification of protein

A modified Lowey's method for quantification of protein (Lowry *et al.* 1951) was used to determine the protein concentration in the hooded seal milk and plasma samples. This procedure was conducted at the University of Oslo, Department of Biosciences, Norway. The protein assay is a 2-step colorimetric assay based on the reaction of proteins with an alkaline copper tartrate solution and a Folin reagent. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine (Lowry *et al.* 1951). The blue end product of the reaction is measured at 750 nm on a plate reader and the protein concentration is calculated using a protein standard to make a standard curve.

The protein standard, consisting of four dilutions (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL) were made of a bovine gamma albumin protein standard (Protein standard, 200 mg/mL BSA, Sigma-Aldrich, Oslo, Norway) and a 0.1 M Trizma Buffer (pH 8 at 4°C, made out of 6.35 g Trizma HCl and 1.18 g Trizma Base (Sigma-Aldrich, Oslo, Norway) in 500 mL

distilled H_2O). A dilution series was made of the hooded seal milk (9 samples), consisting of 20x, 100x and 200x dilutions and the hooded seal plasma (30 samples) consisting of 40x, 80x and 100x dilutions. The dilutions were made with Trizma Buffer, and the blanks consisted of only Trizma Buffer.

10 µL of protein standard/blank/samples were added to a clean 96-well microtitter plate (Nunclon™, VWR, Oslo, Norway) in triplicates for each concentration dilution. 25 µL of reagent A (DC™ Protein Assay, Bio-Rad laboratories, Inc, USA) was added to each well and the plate was gently shaken. This was followed by addition of 200 µL of reagent B (DC™ Protein Assay, Bio-Rad laboratories, Inc, USA) to each well. The plate was incubated for a minimum of 15 minutes and then the absorbance at 750 nm was read on a plate reader (Synergy MX, BioTek) with Gen5 software for measurements of absorbance and fluorescence. The results from the plate reader were used to construct a standard curve (y = a + bx) on the basis of the diluted protein standards and to calculate the protein concentration of unknown samples from the linear part of the standard curve. The protein concentrations were given in mg/mL.

2.5. Thyroid hormone data

Results on thyroid hormone levels in the plasma of mothers and pups in the present study were obtained from previous studies (Gabrielsen *et al.* 2011, Villanger *et al.* 2013). In brief, the method includes a radioimmunoassay, which is based on competitive binding to immobilized antibodies between an unknown amount of natural thyroid hormone from the samples and a known amount of thyroid hormone analogues labelled with ^{125}I (*TH). Binding of the thyroid hormone to the antibody inhibits binding of the TH*, which leads to more free TH* as the concentration of the unlabelled hormone increases. The antibody-bound TH*:free TH* ratio is measured in terms of radioactivity emitted from the antigen-antibody complex using a gamma counter, and expressed as % binding of TH*. Concentrations of hormones will be inversely proportional to the amount of radioactivity measured (Berson and Yalow 2006).

2.6. Statistical analyses

The statistical program, R (version 3.1.2, the R project for statistical computing) was used for the statistical analysis. Wet weight data (ng/g w.w.) of the PFASs were used in the statistical analysis when investigating individual matrices and when comparing plasma of mothers and pups, while protein normalised data (ng/g protein) were used when comparing plasma and milk samples because of differences in protein concentrations (see Appendix C). Data were

log transformed prior to the data analysis to reduce deviations from normality and homogeneity of variance. Normal distribution was tested for with Shapiro Wilk's test, and homogeneity of variance was tested for with Levene's test with the R package "lawstat". The level of significance was set to p = 0.05, and all p-values were two tailed.

One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test (Tykey HSD) was used to test for significant differences in biological variables (lipid%, protein concentration, body mass) between maternal plasma, pup plasma and milk samples. Tukey HSD is appropriate to use for multiple comparisons of means, because it adjusts the p-value, and reduces the chance of a type I error (rejecting a true null hypothesis) (Tukey 1949).

Pearson's product-moment correlation tests (parametric) were conducted to examine correlations between PFAS concentrations in the different matrices. Correlations were expressed using the Pearson's product-moment correlation (r_p) and the p-value of significance.

Because mother-pup pairs are not independent samples, paired t-tests were used in addition to multivariate analysis to investigate differences in PFAS levels in mother-pup pairs.

2.6.1. Multivariate analysis

There were 15 mother-pup pairs while milk was only sampled from 9 of the 15 mothers (Figure 5). A Hotelling's T2 test (R package "hotelling") was conducted to test for overall differences in PFAS levels between the group of 9 plasma samples with accompanying milk samples, and the whole group of plasma samples (n = 15) for both mothers and pups (Figure 5). Hotelling's T2 is the multivariate equivalent of the univariate Student's t-test for the difference between the means of two groups. This statistic is used to determine whether there exists a difference in *p* dimensions, rather than a single dimension, between two groups. With this test the probability of a type I error remains constant, in contrast to multiple t-tests, where the probability of making a type I error is greater than the significance level α, and increases with the number of tests run (Sparks *et al.* 1999). The Hotelling's T2 test showed no difference between the smaller and the larger groups of plasma (mothers: p = 0.9; pups: p = 1.0). Since the smallest group of plasma samples (n = 9) was a representative selection of the larger group of plasma samples (n = 15), one could assume that the group of milk samples (n = 9) could be a representative selection of a larger sample size of milk. All the plasma samples were used in the further analysis of PFASs when comparing levels in plasma of

mothers and pups (i.e. when milk samples were not included), to achieve a higher statistical power.

Principal component analyses (PCAs) were performed to explore similarities and differences between samples (concentrations and patterns) and relationships between the measured variables (PFASs or thyroid hormones). Redundancy analyses (RDAs) were carried out to relate this structure in concentrations and patterns to the explanatory variables. PCAs and RDAs for the PFASs were performed with the following response variables: PFHxS, PFOS, PFOA, PFNA, PFDA, PFUdA, PFDoA, PFTrDA and with the following explanatory variables: lipid% (lipid), protein concentration (protein), lactation period, body mass, ΣPFAS concentration in mothers (PFAS mother) and ΣPFAS concentration in milk (PFAS milk). For the thyroid hormone analysis the following response variables were used: TT4, FT4, TT3, FT3, TT4:TT3, FT4:FT3, TT4:FT4, TT3:FT3, with the following explanatory variables: lipid, protein, lactation period, body mass, PFHxS, PFOS, PFOA, PFNA, PFDA, PFUdA, PFDoA and PFTrDA.

In a PCA plot, the important information from the variables are extracted and represented as a set of new, uncorrelated variables, called principal components (PCs). The first PC accounts for the maximum amount of variance possible in a single variable, and subsequent PCs explain successively smaller quantities of the original variance (Bro and Smilde 2014). Only the two components with the highest eigenvalues and explaining most of the variation were extracted to make PCA plots. It is common for PCAs and other ordinations to be represented by biplots, defined as the joint representation of the rows (samples) and columns (variables) of a data matrix. It is usual practice for samples to be represented as points and the continuous variables (present study: PFASs or thyroid hormones) to be represented by arrows. The lengths of the arrows indicate the variation associated with the variable, and the cosine of the angle between the arrows reflects the correlation between them (i.e. there is no correlation if the arrows are perpendicular to each other). The arrows point in the direction of increasing value, and values of individual points can be determined from their position relative to arrows (Sparks *et al.* 1999, Bro and Smilde 2014).

The PCAs were conducted in the R package "vegan". When analysing variance in concentrations, log transformed PFAS concentrations (w.w. or protein normalised) were used. When analysing the structures in patterns, the data were transformed to percentage of total

PFAS for each matrix. Variables were standardized to unit variance, so that their contribution to the final model was equal, independent of their absolute values.

RDA is a form of constrained ordination, and is the multivariate analogue of simple linear regression. Redundancy analysis is based on similar principles as PCA, and thus makes similar assumptions about the data (Palmer *et al.* 2008). The first PCA loading plot showed a grouping of the different samples (maternal plasma, pup plasma and maternal milk), and an RDA was run with the factorial variable "sample group" as an explanatory variable, and the PFASs as response variables to check for significant differences between the samples groups (see Appendix C). As this proved to be significant, the following RDAs were conducted on plasma of mothers, plasma of pups and milk samples separately (R package "vegan"). The significance of the continuous explanatory variables (lipid%, protein concentration, body mass, lactation period, PFAS mother, PFAS milk) in separating the samples in the multivariate ordination space was analyzed by forward permutation tests (p-value = 0.05). The results from the RDAs were projected on to the PCAs, with the explanatory variables as passive variables. Passive variables do not participate in the determination of ordination axes, but are projected on to the unconstrained axes, visualizing correlations between response- and explanatory variables. Hence in the PCA plots, the explanatory variables are not affecting the spread of samples and relationship to and among response variables.

Pup number 4 was an outlier in the PCA plots (lower PFAS concentrations than the other pups, Figure 6), and was therefore removed when analysing the pups alone. This was done to prevent singular extremes from affecting the results, as the sample size (n) was small.

2.6.2. Post hoc tests

Linear regressions (GLM) were conducted following the multivariate analysis to quantify amount of variance explained (R^2) by the respective single explanatory variables for the most important relationships. The linear regressions were based on the PCAs with passive explanatory variables, where there seemed to be a correlation.

3. Results

3.1. Biological variables

The mean body mass of mothers and pups were 149 ± 29 kg and 32.7 ± 7.9 kg, respectively. The 15 pups consisted of 9 females and 6 males. There were no significant differences in body mass between sexes of the pups (t-test, $p = 0.3$). The body mass of the pups correlated positively with age ($r_p = 0.81$, $p < 0.001$). The mean age of the pups was 2.73 ± 0.78 days, which is also the mean lactation period.

Biological variables are shown in Table 2. The mean lipid percent was higher in plasma of pups than mothers (Tukey HSD, $p < 0.001$). The lipid percent in the milk samples was higher than plasma samples of both mothers and pups (Tukey HSD, $p < 0.001$). There were no differences in protein levels between plasma of mothers and pups (Tukey HSD, $p = 0.8$), while protein levels in milk were lower than levels in maternal and pup plasma (Tukey HSD, $p < 0.001$ and $p = 0.001$).

Table 2. Biological variables (minimum - maximum, median and mean ± SD) in maternal plasma, pup plasma and milk samples of hooded seals from the West Ice (2008).

	Plasma mothers (n = 15)			**Plasma pups (n = 15)**			**Milk mothers (n = 9)**		
	Min - max	Median	Mean ± SD	Min - max	Median	Mean ± SD	Min - max	Median	Mean ± SD
Body mass (kg)	93.0-187	154	149 ± 29	23.0-50.0	30.0	32.7 ± 7.9	NA	NA	NA
Lipid (%)	0.490-0.980	0.680	0.698 ± 0.14	0.610-2.92	1.18	1.40 ± 0.69	62.9-78.2	66.5	68.8 ± 4.9
Protein (mg/mL)	62.3-89.5	73.1	74.3 ± 7.7	55.9-104	66.4	71.2 ± 14	28.8-80.3	55.2	52.7 ± 17

Table 3. Concentrations (ng/g w.w.) of PFAS contaminants in maternal plasma, pup plasma and milk samples of the hooded seal mother pup pairs from the West Ice (2008). SD = standard deviation; LOD = Limit of detection; n.d. = not detected.

	Plasma mothers (n = 15)				Plasma pups (n = 15)			Milk mothers (n = 9)			
PFAS	**LOD (plasma)**	**Min - max**	**Median**	**Mean ± SD**	**Min - max**	**Median**	**Mean ± SD**	**LOD**	**Min - max**	**Median**	**Mean ± SD**
PFHxS	0.058	0.256 - 1.89	0.696	0.845 ± 0.49	0.483 - 5.01	2.60	2.90 ± 1.1	0.263	0.394 (n=1)	n.d.	n.d.
PFOS	0.023	8.51 - 24.2	12.2	13.4 ± 4.2	6.99 - 59.8	28.3	30.4 ± 13	0.427	0.540 - 2.68	1.01	1.24 ± 0.74
PFOA	0.013	0.0250 - 0.928	0.278	0.312 ± 0.23	0.0410 - 1.86	0.336	0.537 ± 0.49	0.033	0.0680 - 0.290	0.189	0.189 ± 0.068
PFNA	0.036	1.09 - 4.40	1.99	2.29 ± 1.0	0.794 - 3.86	1.33	1.61 ± 0.81	0.033	0.0830 - 0.373	0.115	0.167 ± 0.097
PFDA	0.068	1.27 - 5.50	2.30	2.75 ± 1.2	0.978 - 3.28	1.46	1.61 ± 0.65	0.030	0.0600 - 0.301	0.133	0.163 ± 0.080
PFUdA	0.045	5.02 - 17.9	8.51	9.71 ± 3.4	4.43 - 22.4	11.1	11.9 ± 4.6	0.026	0.386 - 1.34	0.611	0.741 ± 0.36
PFDoA	0.120	0.787 - 2.57	1.41	1.45 ± 0.42	0.905 - 5.45	3.37	3.38 ± 1.1	0.089	0.108 - 0.253	0.161	0.182 ± 0.056
PFTrDA	0.130	2.73 - 9.96	4.72	5.07 ± 1.6	3.33 - 22.0	12.6	13.1 ± 4.0	0.089	0.336 - 0.964	0.463	0.512 ± 0.20

* PFHxS: Perfluorohexanesulfonate, PFOS: Perfluorooctanesulfonate, PFOA: Perfluorooctanoic acid, PFNA: Perfluorononanoic acid, PFDA: Perfluorodecanoic acid, PFUdA: Perfluoroundecanoic acid, PFDoA: Perfluorododecanoic acid, PFTrDA: Perfluorotridecanoic acid.

3.2. PFAS concentrations

All eight of the analysed PFASs were detected in all of the plasma samples of both mothers and pups (Table 3). Out of the eight PFASs, seven PFASs were detected in all nine milk samples. PFHxS was only detected in one of the nine milk samples. The mean ΣPFAS concentrations reported were 35.8 ng/g w.w. in maternal plasma, 65.5 ng/g w.w. in pup plasma and 3.19 ng/g w.w. in maternal milk.

3.2.1. Multivariate analysis – concentrations

A PCA was conducted to analyse PFAS variation among samples and the relationship between the PFASs (Figure 6). PCAs and RDAs were also conducted on each sample group (i.e. maternal plasma, pup plasma and maternal milk) separately to analyse the variation of PFASs within the groups and to relate the variation in PFASs to biological variables (Figure 8). There were no differences between contaminant concentrations in sexes of the pups (Hotelling's T2, $p = 0.5$), so the male and female pups were pooled in the analysis.

The first two components extracted in the PCA on plasma and milk samples (PC1 and PC2) accounted for 93% of the total variance in PFAS concentrations (ng/g protein) among samples (Figure 6a). PFHxS was not included in the PCA because of low detection in milk samples (< LOD). There was a clustering of the PFASs into two groups: 1) PFOS, PFOA, PFNA, PFDA, PFDoA, PFTrDA; with higher concentrations in plasma than milk, and 2) PFOA with no difference in concentrations between plasma of mothers, plasma of pups and milk samples (Figure 6a and Figure 7). The plasma of mothers and pups were more clustered than the plasma and milk samples, indicating that plasma of mothers and plasma of pups was more similar to each other than the plasma and milk samples.

a) Plasma and milk concentrations

b) Plasma concentrations

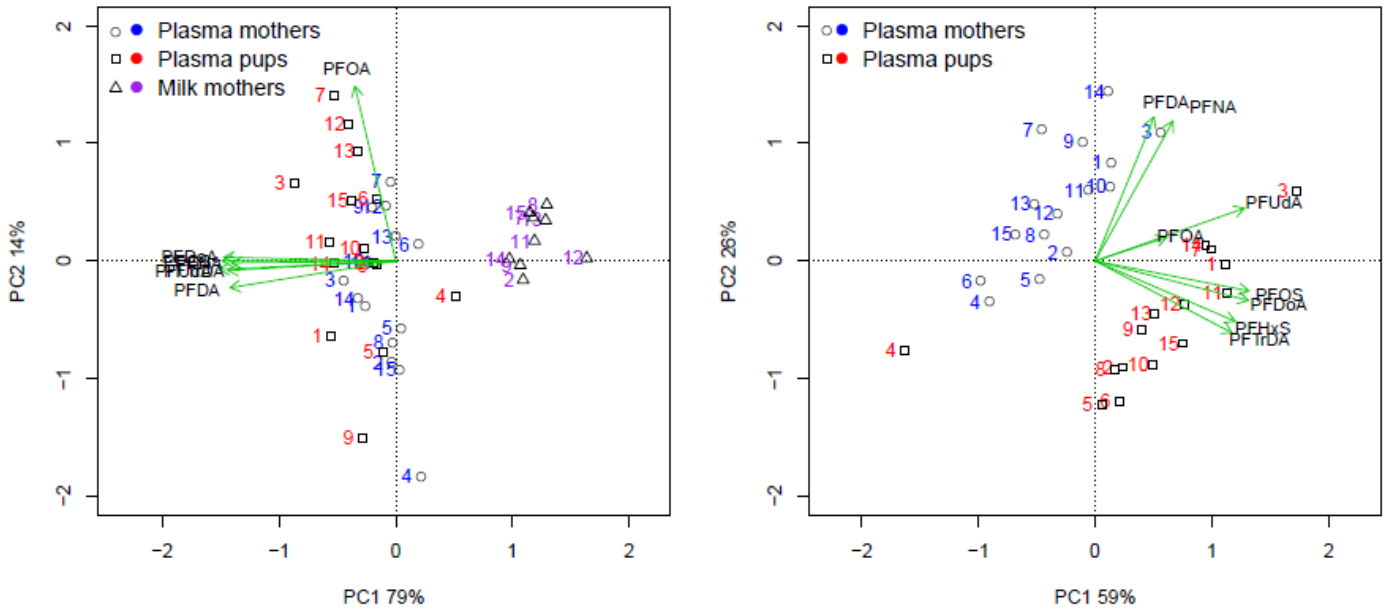


Figure 6. Biplots of a) plasma (n = 30) and milk samples (n = 9) (ng/g protein), and b) maternal plasma and pup plasma (ng/g w.w.) of hooded seals from the West Ice (2008). Sample scores (equal numbers are mother-pup pairs) and PFAS loadings are extracted on the principal components (PCs) with % of the total variance explained by each PC. Direction and length of arrows indicate respective strength and increasing variance of loading.

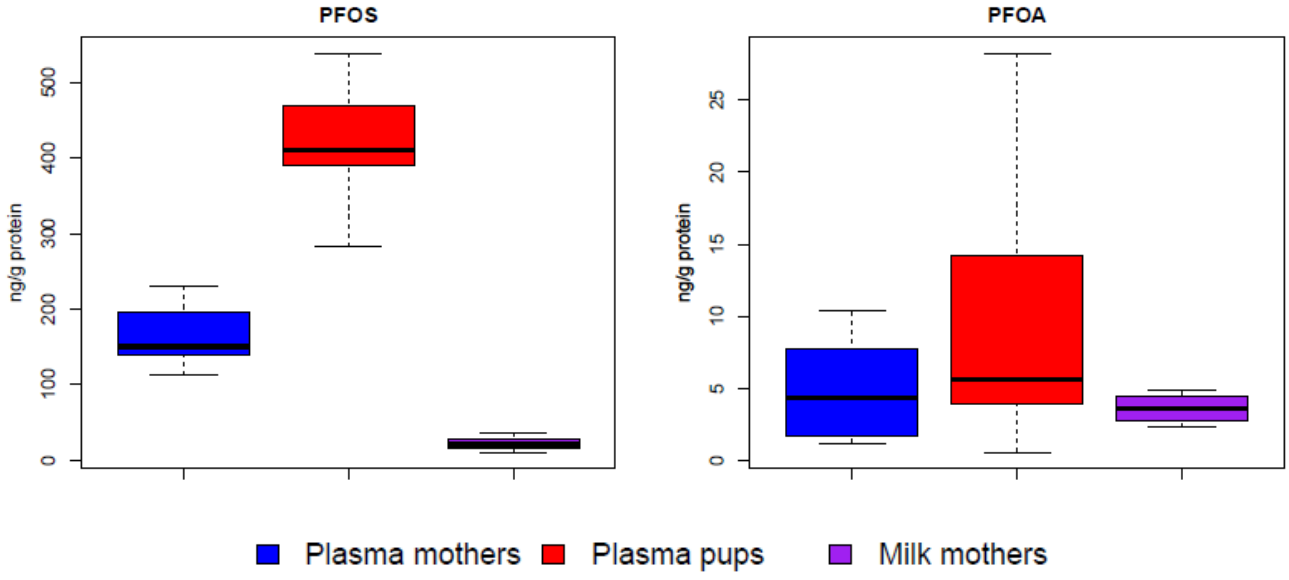


Figure 7. Boxplots (ng/g protein) of PFOS and PFOA (based on PCA biplot 6a) in plasma of mothers (n = 9), plasma of pups (n = 9) and milk samples (n = 9) of hooded seals from the West Ice (2008). The band inside the boxes represents the median. The bottom and top of the boxes represents the first and third quartiles, respectively. The whiskers extend to the most extreme data points (unless it exceeds over 1.5 times the interquartile range).

As the milk samples differed from the plasma in PFAS content, a second PCA was run for the plasma samples alone to analyse the differences between mothers and pups. The first two components extracted in the PCA on plasma samples (PC1 and PC2) accounted for 85% of the total variance in PFAS concentrations (ng/g w.w.) (Figure 6b). Levels of PFOS, PFTrDA, PFHxS and PFDoA were generally higher in pups than mothers, while levels of PFDA and PFNA were generally higher in mothers than pups. For PFOA and PFUdA there was no difference in levels between maternal and pup plasma. However, as the mother-pup samples are not independent, univariate paired t-tests were performed to test for differences between plasma of mother-pup pairs, as this was not possible to do with multivariate tests. This showed that PFUdA was significantly higher in pup plasma than maternal plasma (paired t-test, $p = 0.001$). P-values for paired t-tests are shown in Appendix B.

Because of differences in PFAS concentrations between the sample groups (RDA, permutation test, $p < 0.001$, see Appendix D, Figure 19), an RDA was run for plasma of mothers, plasma of pups and milk samples separately to relate the structure in the PFAS variance to explanatory variables (protein concentration, lipid%, lactation period, body mass, PFAS mother, PFAS milk). Only significant explanatory variables were included in the final model (permutation tests). However, for illustration, all explanatory variables were included as passive variables in the biplots, where variables significantly contributing to explain the variation in PFAS concentrations among samples were marked with yellow boxes (Figure 8).

Plasma mothers

The first two components extracted in the PCA on plasma of mothers (PC1 and PC2) accounted for 83% of the total variance in PFAS concentration (figure 8a). None of the explanatory variables significantly explained the overall PFAS variation (RDA, $p > 0.05$). However, PFOA levels increased with increasing body mass (GLM, $R^2 = 0.61$, $p = 0.003$).

Plasma pups

The first two components extracted in the PCA on plasma of pups (PC1 and PC2) accounted for 65% of the total variance in PFAS concentration (Figure 8b). The ΣPFAS concentration in the mother's plasma ("PFAS mother") was the only significant explanatory variable and explained 31% of the total variation in PFAS concentration (RDA, $p = 0.004$). PFDA and PFUdA concentrations increased with increasing ΣPFAS concentration in the mothers (GLM, PFDA: $R^2 = 0.86$, $p = 0.001$; PFUdA: $R^2 = 0.51$, $p = 0.03$). PFTrDA concentrations increased with increasing ΣPFAS concentrations in the milk ("PFAS milk") (GLM, $R^2 = 0.60$, $p = 0.01$).

Milk mothers

The first two components extracted in the PCA on milk samples (PC1 and PC2) accounted for 81% of the total variance in PFAS concentration (Figure 8c). The PFAS variation was significantly explained by ΣPFAS concentration in the mother's plasma ("PFAS mother"), the lactation period and the protein concentration (RDA, $p<0.001$) and explained 83% of the total variance. PFDA and PFUdA concentrations in milk increased with increasing ΣPFAS concentration in the plasma of mothers (GLM, PFDA: $R^2 = 0.66$, $p = 0.008$; PFUdA: $R^2 = 0.94$, $p < 0.001$). PFTrDA and PFOA concentrations increased with increasing milk protein concentrations and during the lactation period (GLM, PFTrDA: $R^2 = 0.70$, $p = 0.005$; PFOA: $R^2 = 0.58$, $p = 0.02$).

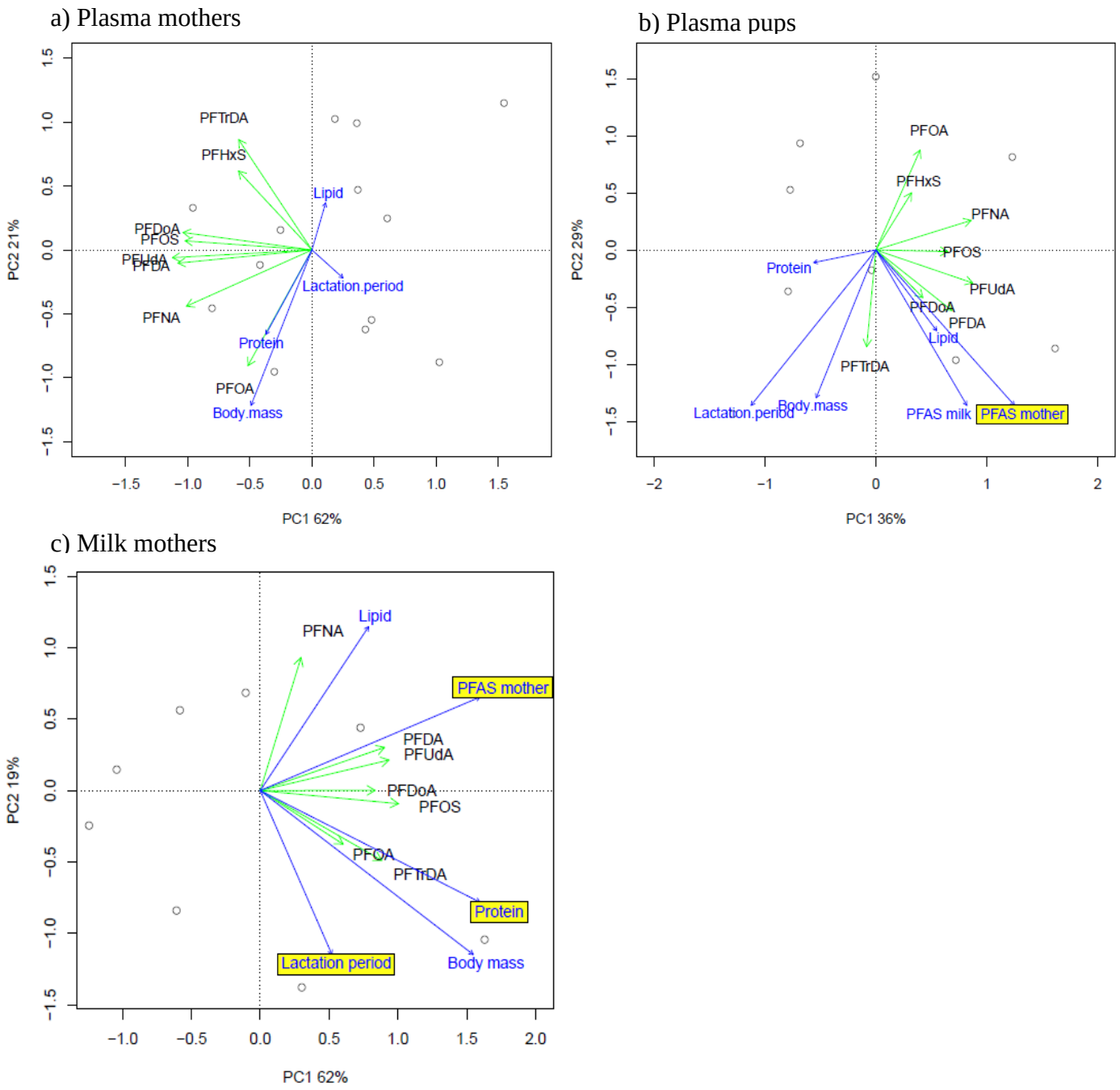


Figure 8. Biplot of PFAS concentrations (ng/g w.w.) in hooded seal a) plasma of mothers (n = 15), b) plasma of pups (n = 9), and c) milk samples (n = 9) from the West Ice (2008) with explanatory variables as passive arrows (blue arrows) and significant explanatory variables marked with yellow boxes. The % of the total variance explained by each principal component (PC1 and PC2) is given on each axis. The PCAs were based on logarithmically transformed concentrations. Direction and length of arrows indicate respective strength and increasing variance of loading.

3.3. PFAS patterns

PFOS was the predominant PFAS in all matrices and comprised 37% of Σ_8PFAS in plasma of mothers, 45% of Σ_8PFAS in plasmas of pups and 39% of Σ_7PFAS in the milk samples. PFOS, PFUdA and PFTrDA were the three most predominant PFASs in all three matrices (Figure 9). In plasma and milk samples of mothers, PFUdA had the highest relative occurrence after PFOS, while in plasma samples of pups, PFTrDA had the highest relative occurrence after PFOS (Figure 9).

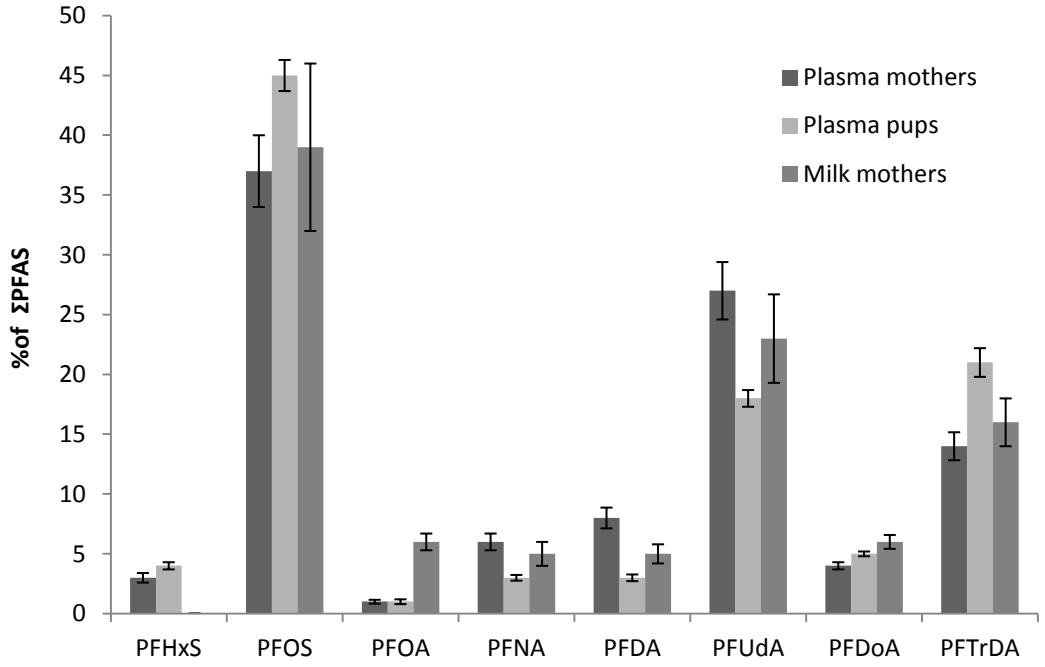


Figure 9. Pattern of PFASs in maternal plasma (n = 15), pup plasma (n = 15) and maternal milk samples (n = 9) of hooded seals from the West Ice (2008). Values are presented as mean percentage (%) of ΣPFAS for each contaminant (± SE).

3.3.1. Multivariate analysis – patterns

A PCA was conducted to analyse variation in patterns among samples (the percentage of ΣPFAS) and the relationship between the patterns of different PFASs (Figure 10). PCAs and RDAs were also conducted on each sample group (i.e. maternal plasma, pup plasma, maternal milk) separately to analyse the variation in PFAS pattern within the groups and to relate the variation in PFAS-pattern to biological variables (Figure 11).

The first two components extracted in the PCA on patterns (PC1 and PC2) accounted for 75% of the total variance (Figure 10). There was a clustering of the PFAS contaminants into three main groups; 1) PFHxS and PFOS (the sulfonates) correlated with each other and had higher relative contribution in pups than mothers and milk, 2) PFDA, PFNA and PFUdA (the intermediate chained acids) correlated with each other and had higher relative contribution in plasma of mothers than plasma of pups and milk, and 3) PFOA and PFDoA had higher relative contribution in the milk samples than plasma samples.

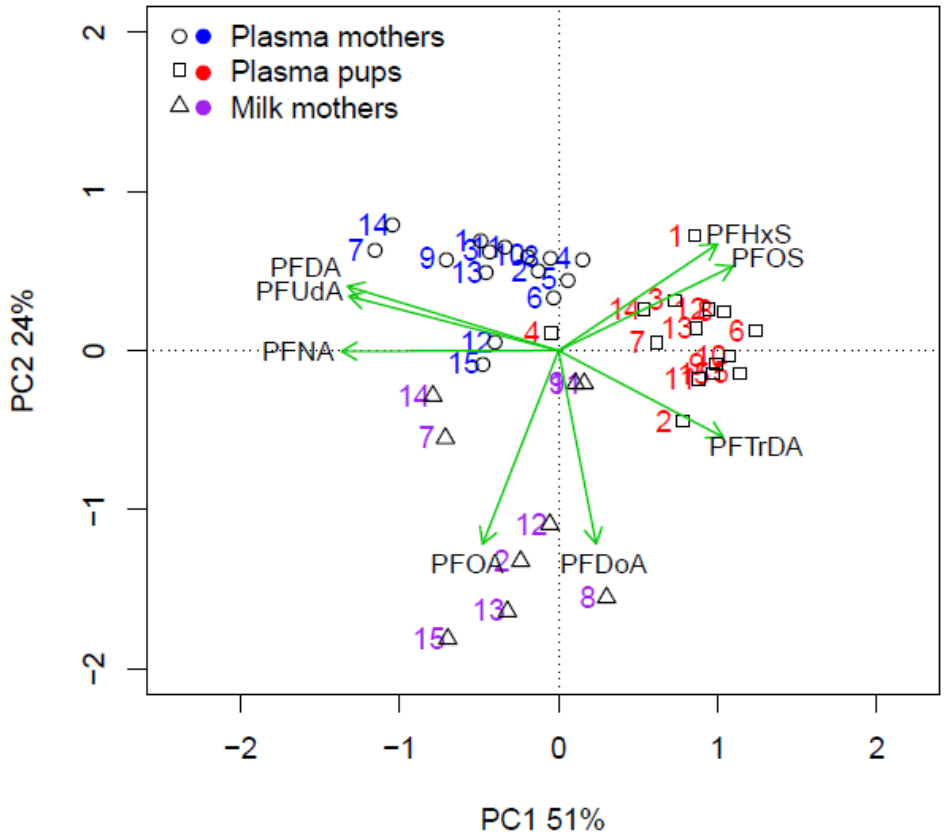


Figure 10. Biplot of maternal plasma (n = 15), pup plasma (n = 15) and milk samples (n = 9) (equal numbers are mother-pup pairs) and PFAS loadings on the extracted principal components (PCs). The % of the total variance explained by each PC is given on the axes. The principal component analysis (PCA) was based on % of total PFAS in hooded seals from the West Ice (2008). Direction and length of arrows indicate respective strength and variance of loading.

Because of differences in PFAS pattern between the sample groups (RDA, permutation test, $p < 0.001$, see Appendix D, Figure 20), RDAs were run for plasma of mothers, plasma of pups and milk samples separately to relate the structures in the variance to explanatory variables (protein concentration, lipid%, lactation period, body mass, PFAS mother, PFAS milk).

Plasma mothers

The first two components extracted in the PCA on plasma of mothers (PC1 and PC2) accounted for 71% of the total variance in PFAS pattern (Figure 11a). None of the explanatory variables significantly explained the overall variation in PFAS pattern (RDA, $p > 0.05$). The percentage of PFNA in maternal plasma increased with increasing protein concentrations (GLM, $R^2 = 0.43$, $p = 0.05$).

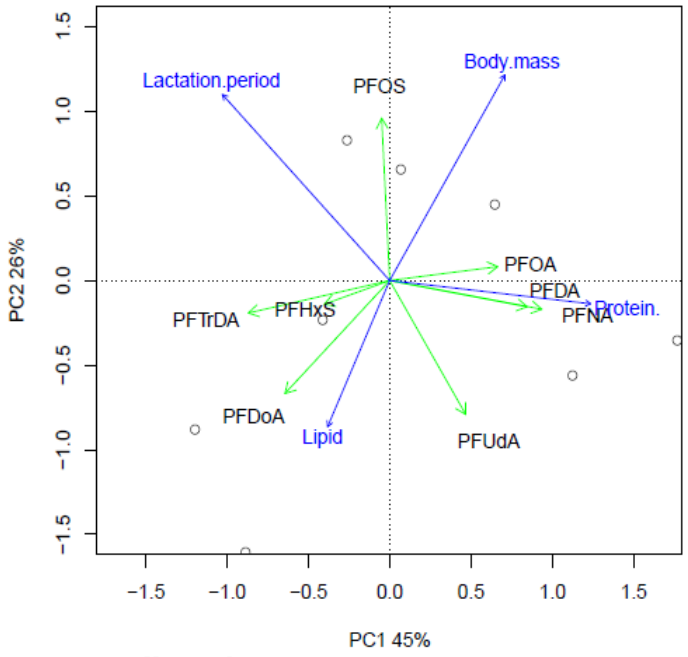
Plasma pups

The first two components extracted in the PCA on plasma of pups (PC1 and PC2) accounted for 60% of the total variance in PFAS pattern (Figure 11b). The PFAS pattern was significantly explained by the lactation period (RDA, $p = 0.05$), and explained 23% of the overall variation in PFAS pattern. The percentage of PFNA in pup plasma decreased during the lactation period (GLM, $R^2 = 0.64$, $p = 0.01$).

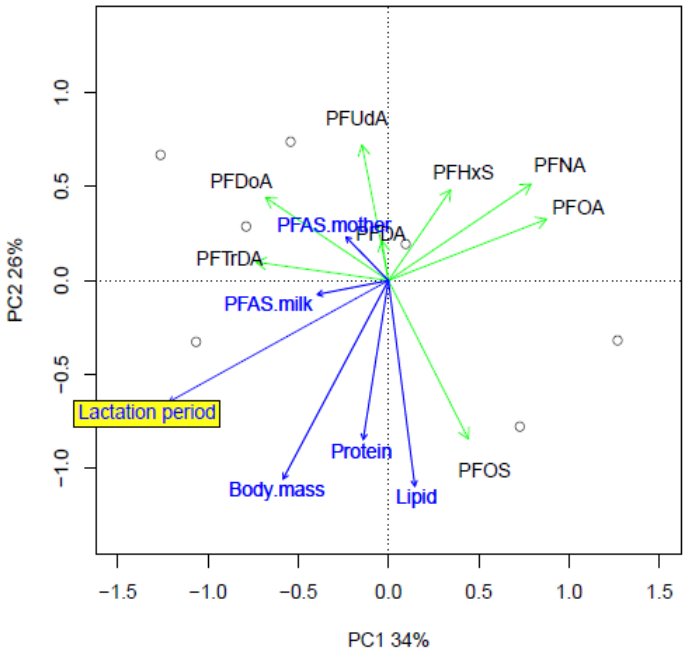
Milk mothers

The first two components extracted in the PCA on milk samples (PC1 and PC2) accounted for 67% of the total variance in PFAS pattern (Figure 11c). The lactation period, the ΣPFAS concentration in the mother's plasma ("PFAS mother") and the body mass of the mothers were the significant explanatory variables (RDA, $p < 0.001$), and explained 68% of the variation in PFAS pattern. The percentage of PFNA in the milk decreased during the lactation period, and decreased with increasing body mass of the mothers (GLM, PFNA: $R^2 = 0.63$, $p = 0.01$). The percentage of PFTrDA decreased with increasing ΣPFAS concentration in the mother's plasma (GLM, $R^2 = 0.76$, $p = 0.002$).

a) Plasma mothers



b) Plasma pups



c) Milk mothers

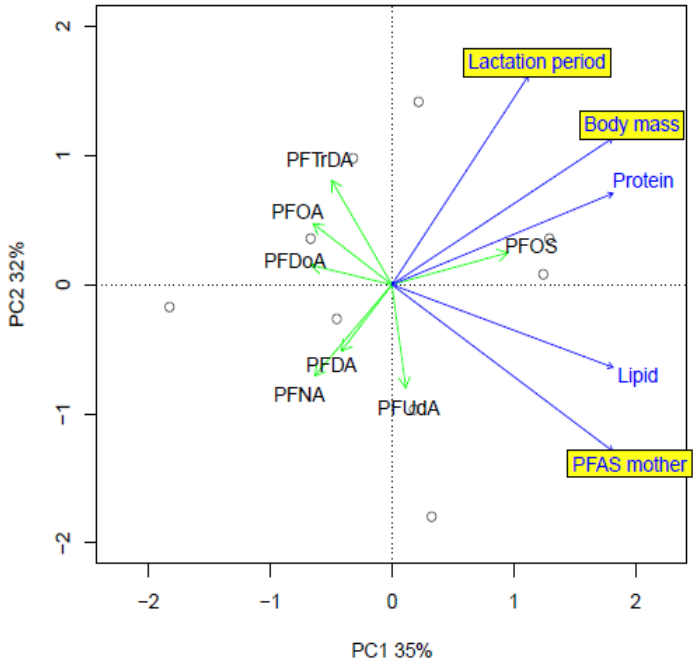


Figure 11. Biplot of PFAS pattern in hooded seal a) plasma of mothers (n = 15), b) plasma of pups (n = 9), and c) milk samples (n = 9) from the Wet Ice (2008) with explanatory variables as passive arrows (blue arrows) and significant explanatory variables marked with yellow boxes. The % of the total variance explained by each principal component (PC1 and PC2) is given on each axis. The PCAs were based on % of total PFAS. Direction and length of arrows indicate respective strength and increasing variance of loading.

3.4. Maternal transfer of PFASs

PFOS, PFNA, PFDA, PFUdA and PFDoA concentrations (w.w.) correlated positively between plasma of mothers and pups (PFOS: $r_p = 0.67$, $p = 0.005$; PFNA, PFDA, PFUdA, PFDoA $r_p = 0.82$ - 0.90, $p < 0.001$). PFOS concentrations (protein normalised) correlated positively between maternal plasma and milk (PFOS: $r_p = 0.76$, $p = 0.02$). None of the PFASs (protein normalised) correlated between pup plasma and milk.

Ratios between contaminant levels in the different matrices were calculated to examine transfer rates for individual PFASs, and to relate this to carbon chain length (Figure 12).

$Plasma_{pup}$:$plasma_{mother}$ ratios were above 1 for all contaminants, except for PFNA and PFDA (Figure 12a). The $plasma_{pup}$:$plasma_{mother}$ ratio of PFNA and PFDA was 0.70 (± 0.04) and 0.64 (± 0.07) respectively, meaning that the levels were higher in mothers than pups.

PFHxS was only detected in one milk sample and was therefore poorly/not transferred from the mother's plasma to the milk. PFNA and PFDA had the highest $plasma_{mother}$:$milk_{mother}$ ratio and was least efficiently transferred from the mother's plasma to the milk. These were followed by PFUdA and PFOS, while PFOA was the most efficiently transferred from the mother's plasma to the milk, illustrated by a ratio closer to one. PFOA levels in milk constituted 75% of levels in plasma of mothers (protein normalised). For the other PFASs, levels in milk ranged from 8% to 19% of levels in plasma of mothers (excluding PFHxS).

There was a decrease in $plasma_{pup}$:$plasma_{mother}$ ratio with increasing fluorinated chain length for the sulfonates. For the acids there was a decrease in $plasma_{pup}$:$plasma_{mother}$ ratio with increasing fluorinated chain length from C_8 to C_{10}, and an increase in $plasma_{pup}$:$plasma_{mother}$ ratio with increasing fluorinated chain length from C_{10} to C_{13} (Figure 12a).

There was an increase in $plasma_{mother}$:$milk_{mother}$ ratio with increasing fluorinated chain length for the sulfonatess. For the acids There was an increase in $plasma_{mother}$:$milk_{mother}$ ratio with increasing fluorinated chain length from C_8 to C_{10}, and a decrease in $plasma_{mother}$:$milk_{mother}$ ratio with increasing chain length from C_{10} to C_{13} (Figure 12b).

There was an increase in $plasma_{pup}$:$milk_{mother}$ ratio with increasing chain length for both the sulfonates and the acids. The highest $plasma_{pup}$:$milk_{mother}$ ratio was found for PFOS (Figure 12c).

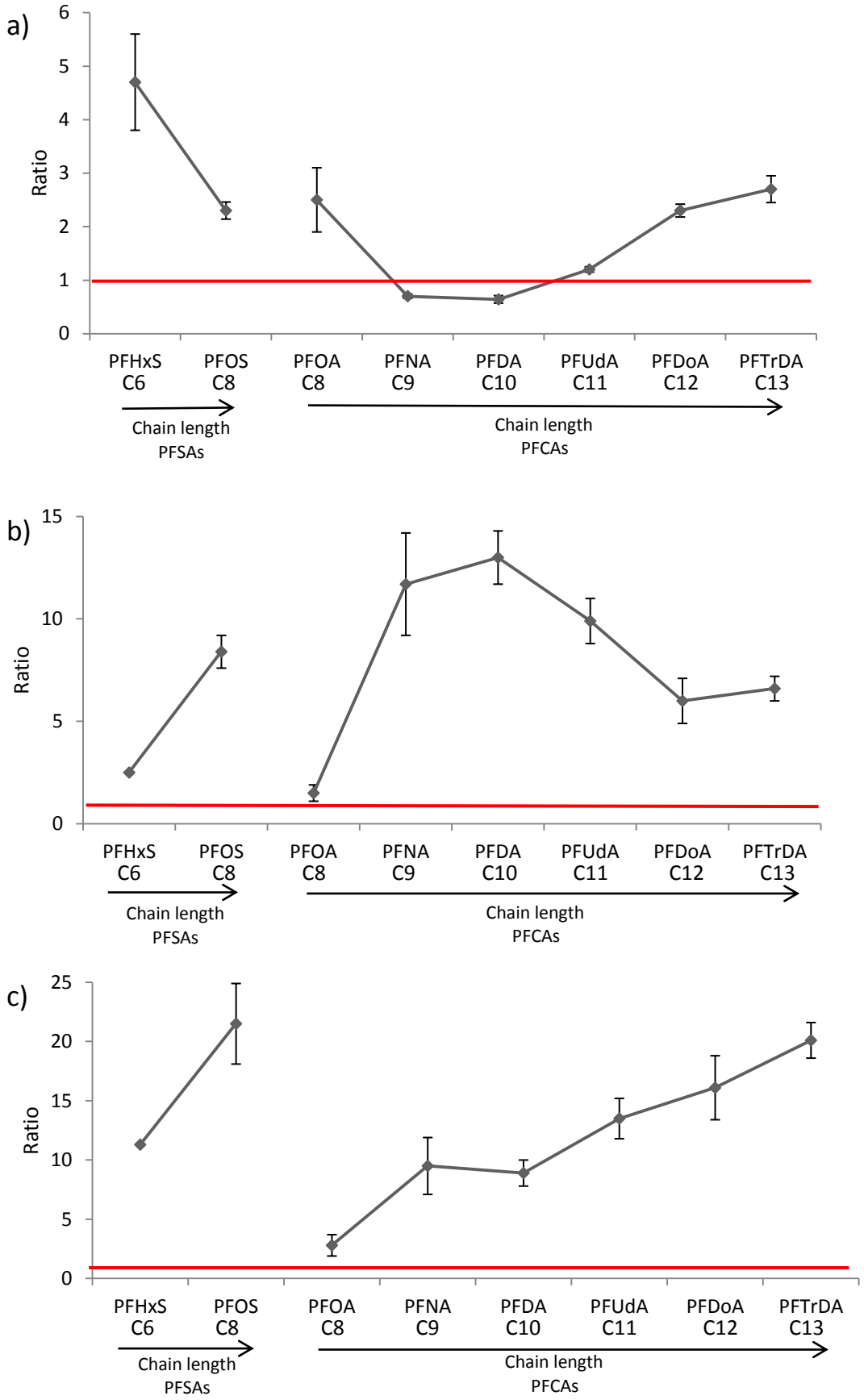


Figure 12. Ratios of a) $plasma_{pup}$:$plasma_{mother}$, b) $plasma_{mother}$:$milk_{mother}$, and c) $plasma_{pup}$:$milk_{mother}$ of hooded seals from the West Ice (2008) presented as mean ratios (± SE). Ratios were based on mean concentrations (ng/g w.w. for plasma:plasma ratios, ng/g protein for plasma:milk ratios). The C6-C13 labelling indicates the various PFAS compounds' carbon chain length. PFSAs = perfluoroalkyl sulfonates, PFCAs = perfluoroalkyl acids. The red line indicates 1:1 relationship.

3.5. Associations between PFASs and thyroid hormones

Gabrielsen *et al.* (2011) reported that all thyroid hormone levels were higher in pups than in mothers. An overview of the different thyroid hormone concentrations and thyroid hormone ratios in plasma of mothers and pups is shown in Appendix E.

3.5.1. Associations in mothers

A PCA and RDA was conducted on thyroid hormone concentrations and thyroid hormone ratios in maternal plasma of hooded seals to analyse the variation of thyroid hormones within the groups and to relate the variation in thyroid hormones and thyroid hormone ratios to PFASs and biological variables (Figure 13).

a) TH concentrations in maternal plasma

b) TH ratios in maternal plasma

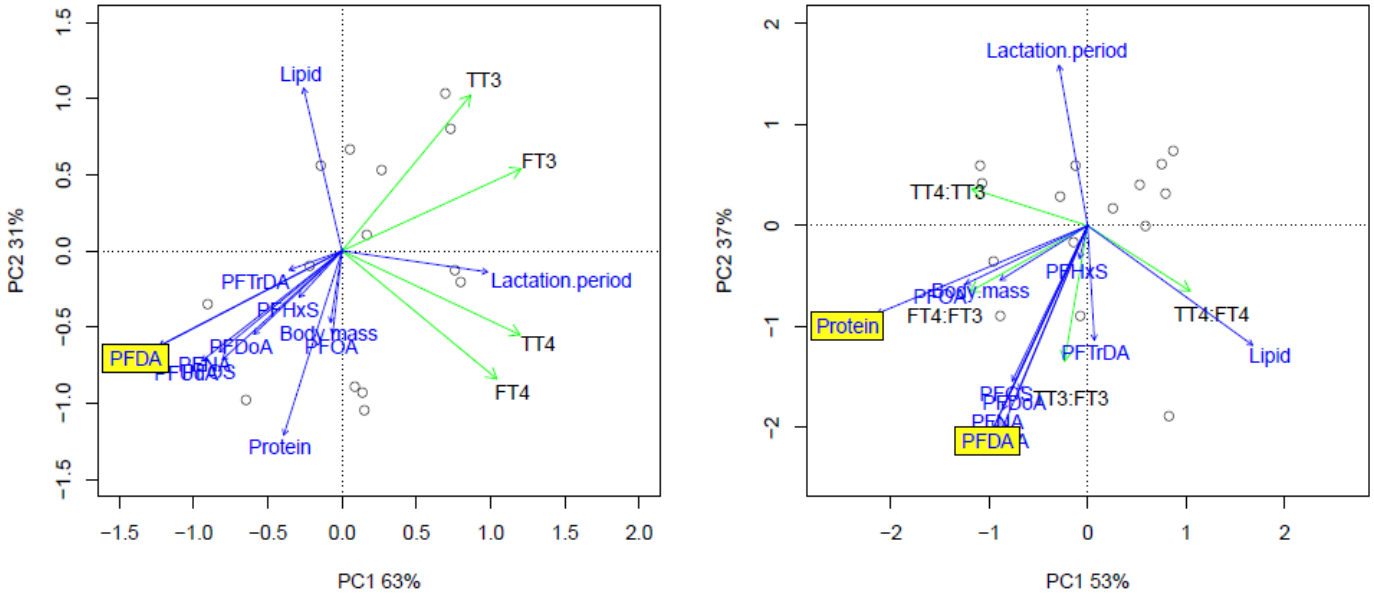


Figure 13. Biplot of a) thyroid hormone (TH) concentrations and b) thyroid hormone (TH) ratios in plasma of hooded seal mothers (n = 15) from the West Ice (2008) with PFASs and biological variables as explanatory variables (passive arrows in blue). Significant explanatory variables are marked with yellow boxes. The % of the total variance explained by each principal component (PC1 and PC2) is given on each axis. The PCAs were based on logarithmically transformed concentrations (nmol/L for TT4 and TT3, pmol/L for FT4 and FT3). Direction and length of arrows indicate respective strength and increasing variance of loading.

Effects on thyroid hormone concentrations

The first two components extracted in the PCA on thyroid hormone concentrations in plasma of mothers (PC1 and PC2) accounted for 94% of the total variance (Figure 13a). PFDA was the only significant explanatory variable (RDA, p = 0.008), and explained 31 % of the total variance. TT3 levels decreased with increasing levels of PFDA, PFUdA and PFOS (GLM, PFDA: $R^2 = 0.29$, p = 0.04; PFUdA: $R^2 = 0.26$, p = 0.05; PFOS: $R^2 = 0.27$, p = 0.05), and FT3 levels decreased with increasing levels of PFDA, PFNA, PFUdA and PFOS (GLM, PFDA: $R^2 = 0.46$, p = 0.006; PFNA: $R^2 = 0.36$, p = 0.02; PFUdA: $R^2 = 0.44$, p = 0.007; PFOS: $R^2 = 0.30$, p = 0.04).

Since both thyroid hormones and PFASs are protein associated, and since proteins were negatively correlated with T3 in the PCA, the protein level may be a possible confounder. After correcting for proteins, FT3 levels were no longer predicted by PFOS concentrations (GLM, p = 0.07), and TT3 levels were no longer predicted by any of the PFASs (GLM, p > 0.05). The remaining significant PFAS regressions after adjustment were: PFDA (GLM, $R^2 = 0.49$, p = 0.01), PFNA (GLM, $R^2 = 0.37$, p = 0.04) and PFUdA (GLM, $R^2 = 0.45$, p = 0.02). The significant PFAS regressions are shown as scatter-plots in Figure 14.

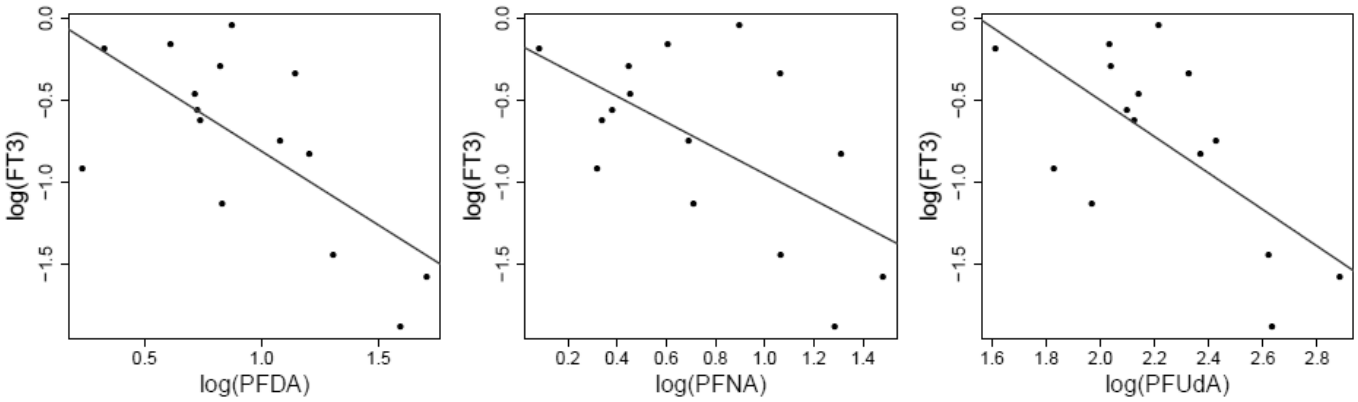


Figure 14. Scatter-plot of the linear regression analyses (GLM) for FT3 (log pmol/L) and significant explanatory variables; PFDA, PFNA and PFUdA (log ng/g w.w.) in maternal plasma of hooded seal (n = 15) from the West Ice (2008). The relationships are adjusted for protein concentration. Black lines represent regression lines.

Effects on thyroid hormone ratios

The first two components extracted in the PCA on thyroid hormone ratios in maternal plasma (PC1 and PC2) accounted for 90% of the total variance (Figure 13b). PFDA and protein concentration were the significant explanatory variables (RDA, p = 0.009), and explained 56% of the overall variance in thyroid hormone ratios. TT3:FT3 ratios increased with increasing levels of PFNA, PFDA and PFUdA (GLM, PFNA: $R^2 = 0.29$, p = 0.04; PFDA: $R^2 = 0.39$, p = 0.02; PFUdA: $R^2 = 0.36$, p = 0.02). FT4:FT3 ratios increased with increasing protein concentrations (GLM, $R^2 = 0.45$, p = 0.006,).

No correction for proteins was done for the relationships between PFNA, PFDA and PDUdA and TT3:FT3 ratio, as protein was only associated with FT4:FT3, but none of the other PFASs affecting thyroid hormone ratios. The significant PFAS regressions are shown as scatter-plots in Figure 15.

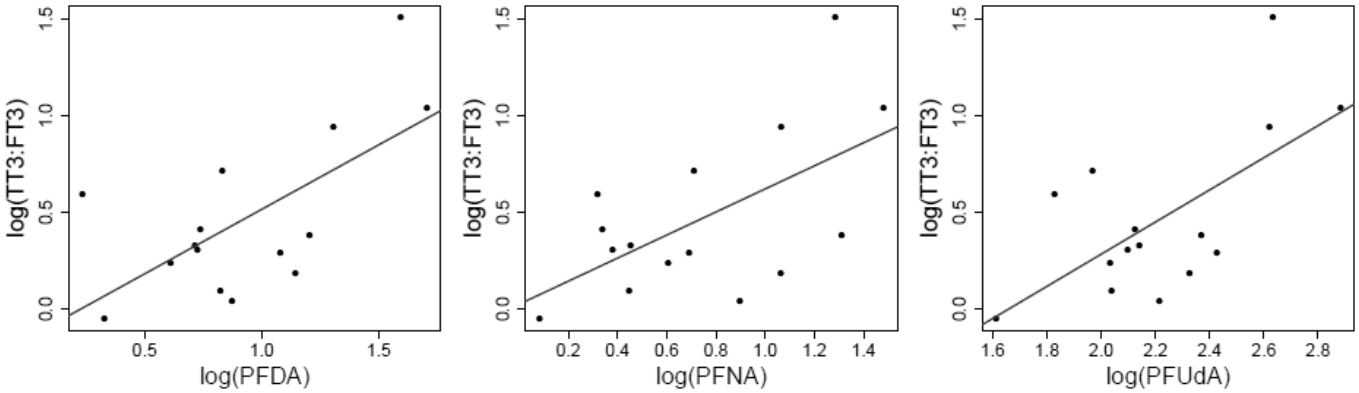


Figure 15. Scatter-plot of the linear regression analyses (GLM) for TT3:FT3 ratios (log-transformed) and significant explanatory variables; PFDA, PFNA and PFUdA (log ng/g w.w.) in maternal plasma of hooded seal (n = 15) from the West Ice (2008). Black lines represent regression lines.

3.5.2. Associations in pups

A PCA and RDA was conducted on thyroid hormone concentrations and thyroid hormone ratios in plasma of hooded seal pups to analyse the variation of thyroid hormones within the groups and to relate the variation in thyroid hormones and thyroid hormone ratios to PFASs and biological variables (Figure 16).

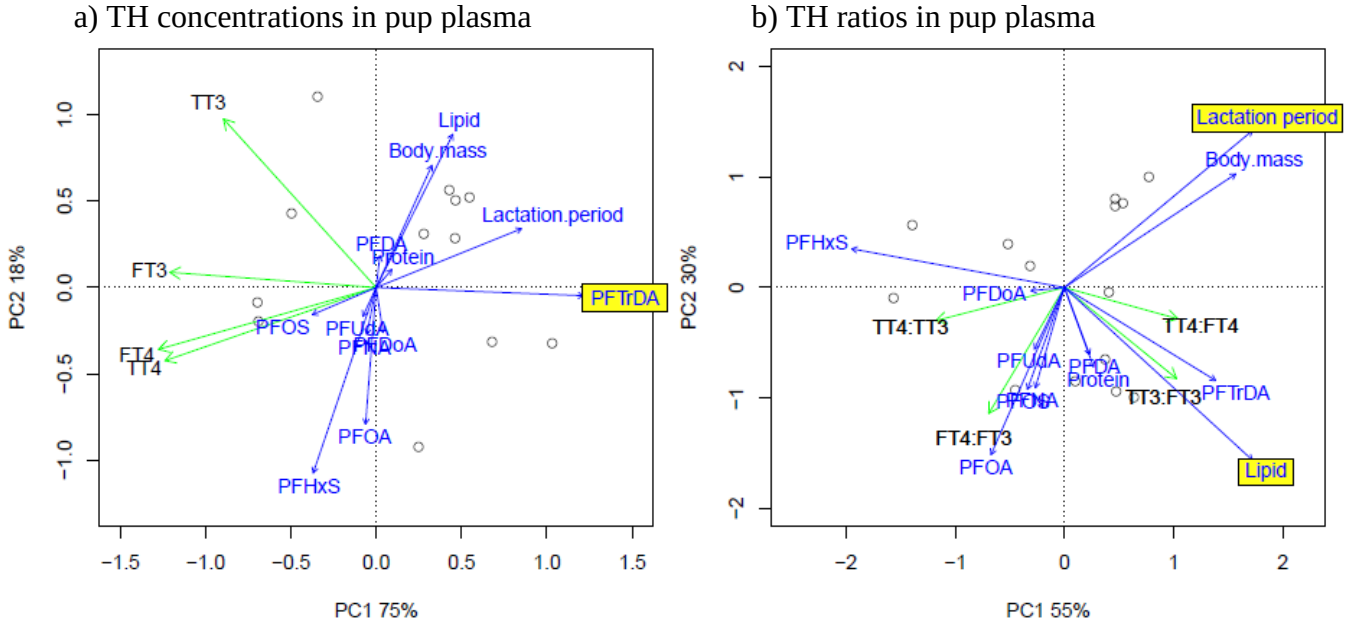


Figure 16. Biplot of a) thyroid hormone (TH) concentrations and b) thyroid hormone (TH) ratios in plasma of hooded seal pups (n = 15) from the West Ice (2008) with PFASs and biological variables as explanatory variables (passive arrows in blue). Significant explanatory variables are marked with yellow boxes. The % of the total variance explained by each principal component (PC1 and PC2) is given by each axis. The PCAs were based on logarithmically transformed concentrations (nmol/L for TT4 and TT3, pmol/L for FT4 and FT3). Direction and length of arrows indicate respective strength and increasing variance of loading.

Effects on thyroid hormone concentrations

The first two components extracted in the PCA on thyroid hormone concentrations in plasma of pups (PC1 and PC2) accounted for 93% of the total variance (Figure 16a). PFTrDA was the significant explanatory variable explaining the overall variation in thyroid hormone levels (RDA, p = 0.02) and explained 24% of the total variance. FT4 and FT3 levels decreased with increasing PFTrDA levels (GLM for FT4: $R^2 = 0.37$, p = 0.02; for FT3: $R^2 = 0.35$, p = 0.03). Levels of FT4 and TT4 decreased during the lactation period (GLM for FT4: $R^2 = 0.40$, p = 0.02; for TT4: $R^2 = 0.30$, p = 0.04).

Because of the lack of influence of proteins on the thyroid hormone variables in the PCA, no protein corrections were conducted. The significant PFAS regressions are shown as scatter-plots in Figure 17.

Effects on thyroid hormone ratios

The first two components extracted in the PCA on thyroid hormone ratios in plasma of pups (PC1 and PC2) accounted for 85% of the total variance (Figure 16b). The lactation period and lipid% were the significant explanatory variables (RDA, $p = 0.002$), and explained 53% of the total variance in thyroid hormone ratios. TT3:FT3 ratios increased with increasing levels of lipids in the plasma (GLM, $R^2 = 0.65$, $p < 0.001$). FT4:FT3 ratios decreased during the lactation period (GLM, $R^2 = 0.48$, $p = 0.006$). FT4:FT3 ratios increased with increasing levels of PFOA, although not significantly when tested with univariate analysis (GLM, $R^2 = 0.24$, $p = 0.07$). TT4:FT4 ratios increased with increasing PFTrDA concentrations (GLM, $R^2 = 0.47$, $p = 0.007$).

Because of the lack of influence of proteins on the thyroid hormone variables in the PCA, no protein corrections were conducted. The significant PFAS regression is shown as a scatter-plot in Figure 17.

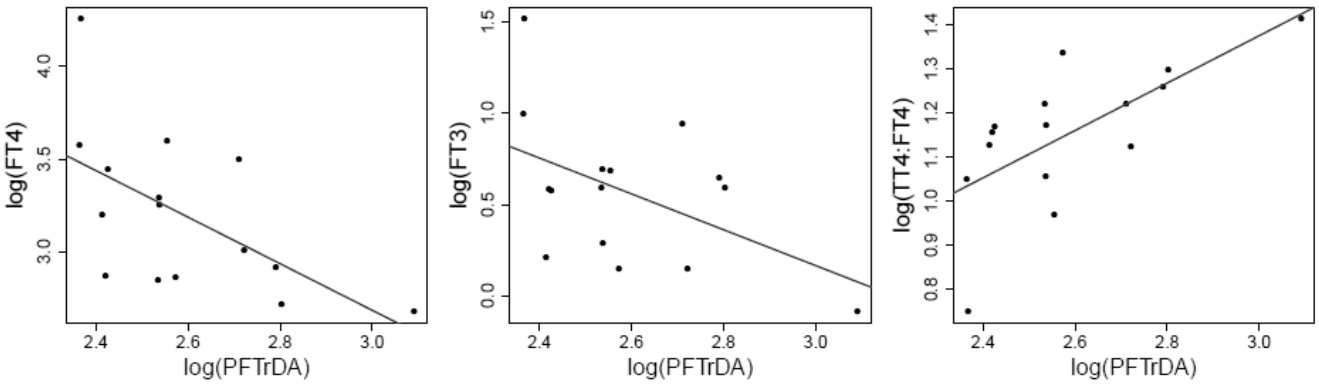


Figure 17. Scatter-plot of the linear regression analyses (GLM) for FT3 and FT4 (log pmol/L) and TT4:FT4 ratios and significant explanatory variables; PFTrDA (log ng/g w.w.) in pup plasma of hooded seal (n = 15) from the West Ice (2008). Black lines represent regression lines.

4. Discussion

The present study reports, for the first time, concentrations of PFASs in hooded seal mother-pup pairs from the West Ice. There are no previous studies on pinnipeds that investigate associations between exposure to PFASs and associated toxicological endpoints. The present study quantified levels and patterns of PFASs in plasma and milk, and aimed to determine if the PFASs are subject to maternal transfer via milk and/or placenta. The study also investigated possible associations between concentrations of PFASs and thyroid hormone concentrations in blood of hooded seal mothers and pups.

4.1. Levels and patterns of PFAS in milk and plasma

4.1.1. Levels of PFASs

Reported concentrations of PFASs in arctic pinnipeds are scarce. There are especially few studies on levels of PFASs in pups and there is, to the author's knowledge, no studies reporting levels of PFASs in pinniped milk. The maternal plasma levels of PFASs in the present study were within the range of the levels reported in blood/plasma of other pinnipeds from the Arctic (Table 4). There is only one study reporting PFAS levels in blood from pinniped pups in the Arctic: northern fur seals (*Callorhinus ursinus*) from Alaska (Kannan *et al.* 2001). PFOS levels reported in northern fur seal pups were lower than PFOS levels in hooded seal pups from the present study (Table 4). Comparisons across studies may be confounded by different sample sizes, analytical methods, number of PFASs that are included, and/or sampling from different matrices or different areas. However, PFOS, the most common PFAS, is reported in almost all studies on PFAS levels in wildlife and was therefore compared among studies. Table 4 includes studies on PFOS levels in blood/plasma from pinnipeds in the Arctic. Adult males were excluded where data on males and females were not pooled, because males often show higher PFAS levels than females (Harada *et al.* 2004, Yeung *et al.* 2006, Calafat *et al.* 2007). PFOS levels in hooded seal females were lower than levels reported in harbour seals (*Phoca vitulina*), in the lower range of levels reported in grey seals (*Halichoerus grypus)*, and higher than levels reported in ringed seals (*Phoca hisoida*), northern fur seals and bearded seals (*Erignathus barbatus*) (Table 4).

PFAS levels reported in plasma in hooded seal mothers in the present study were within the range of levels found in adult harbour seal females from Svalbard (Routti *et al.* 2014) for PFHxS, PFOA, PFNA, PFDA and PFTrDA. Levels of PFNA and PFDA in adult females

from the present study were above levels reported in ringed seals from the Canadian Arctic (Powley *et al.* 2008). The PFAS levels reported in harbour seals from Svalbard and ringed seals from the Canadian Arctic were obtained from only 4 and 5 individuals, respectively, which makes it is hard to draw a general trend based on such a small sample size.

Table 4. Reported levels (range; ng/g w.w.) of PFOS in blood/plasma from pinnipeds in the Arctic. Median values are given in parentheses, unless noted otherwise. n=sample size; n.r.= not reported.

Species	Matrix	Location	n	Sampling period	Age group	Sex	PFOS	Reference
Hooded seal	Plasma	West Ice	15	2008	Pup	F+M	7-60 (28)	Present study
Hooded seal	Plasma	West Ice	15	2008	Adult	F	9-24 (12)	Present study
Harbour seal	Plasma	Svalbard (Norway)	4	2009-2010	Juvenile	n.r.	27-46 (38)	(Routti *et al.* 2014)
Harbour seal	Plasma	Svalbard (Norway)	4	2009-2010	Adult	F	25-29 (35)	(Routti *et al.* 2014)
Ringed seal	Blood	Spitsbergen (Norway)	10 8	1996 1998	Adult	F+M	8 (± 2.5)[ab] 10 (± 2.7)[ab]	(Kannan *et al.* 2001)
Ringed seal	Plasma	Canadian Arctic	24	n.r.	n.r.	n.r.	<3-12	(Giesy and Kannan 2001)
Ringed seal	Plasma	Norwegian Arctic	18	n.r.	n.r.	n.r.	5-14 (9)[c]	(Giesy and Kannan 2001)
Ringed seal	Blood	Canadian Arctic	5	2004	n.r.	n.r.	2.5-8.6	(Powley *et al.* 2008)
Northern fur seal	Blood	Alaska (USA)	19	1995	Pup	F+M	6-12[b]	(Kannan *et al.* 2001)
Northern fur seal	Blood	Alaska (USA)	8	1995	Pup + subadult	F+M	<6[b]	(Kannan *et al.* 2001)
Northern fur seal	Blood	Alaska (USA)	10	1995	Adult	F	<6[b]	(Kannan *et al.* 2001)
Bearded seal	Blood	Canadian Arctic	1	2004	n.r.	n.r.	(1.3)	(Powley *et al.* 2008)
Grey seal	Plasma	Canadian Arctic	12	n.r.	n.r.	n.r.	11-49 (28)[c]	(Giesy and Kannan 2001)

[a]mean(±SD); [b]ng/mL; [c]mean given in parentheses

All eight PFASs analysed in the present study were detected in all of the plasma samples from both mothers and pups, while PFHxS, the PFAS with the shortest carbon chain length, was absent in eight out of nine milk samples. This is in accordance with what was reported in human milk from South-Korea, where PFHxS was detected in only two out of the 35 samples (Kim *et al.* 2011). Short chained PFASs (such as PFHxS) are infrequently detected in biological tissues in field studies (Martin *et al.* 2004b, Bossi *et al.* 2005b, Houde *et al.* 2006b, Van de Vijver *et al.* 2007), and have not been found to bioaccumulate in experimental studies

(Martin *et al.* 2003a, Martin *et al.* 2003b, Conder *et al.* 2008). The low bioaccumulation and biomagnification potential for short chained PFASs might explain the relatively low concentrations of PFHxS in the plasma samples of hooded seals herein, and the low detection in the milk samples.

PFAS levels were higher in plasma of both mothers and pups than in milk for all PFASs, except for PFOA, in the present study. The high concentrations in maternal plasma, compared to milk, are in accordance with previous studies in humans (Kärrman *et al.* 2007, Fromme *et al.* 2010), and in accordance with the expectations (hypothesis H1). The data support that PFASs bind with high affinity to blood proteins, which limits the ability to enter the milk (Jensen 1991, Jones *et al.* 2003).

Levels of PFOS, PFHxS, PFDoA, PFUdA and PFTrDA were higher in pups than mothers, while levels of PFDA and PFNA were higher in mothers than pups. This was not in accordance with the expectations (hypothesis H2). According to the expectations there would be higher levels of PFASs in maternal plasma than pup plasma, because of exposure and accumulation over a longer period of time. The relationship between levels of PFASs in hooded seal mothers and pups differ from polar bears (*Ursus maritimus*) from Svalbard (Bytingsvik *et al.* 2012) and lab experiments on rats (Hinderliter *et al.* 2005), where PFAS levels in general were higher in mothers than newborn offspring. In previous studies on hooded seals from eastern Canada (Wolkers *et al.* 2006), concentrations of lipophilic compounds, such as PCBs and DDTs, were lower in pups than mothers. However, a similar pattern with lower PFOS concentrations in adult females was reported in Baikal seals (*Pusa sibirica*) in Russia (Ishibashi *et al.* 2008a, Ishibashi *et al.* 2008b), bottlenose dolphins *(Tursiops truncates)* from the Florida coast (Kannan *et al.* 2001), Baltic grey and ringed seals (Kannan *et al.* 2002), and harbour porpoises *(Phocoena phocoena)* in the North Sea (Van de Vijver *et al.* 2005). A higher ΣPFAS concentration in pups than mothers was also reported in harbour seal liver from the northeast Atlantic (Shaw *et al.* 2009).

Higher levels of PFHxS, PFOS, PFDoA, PFUda and PFTrDA in pups than mothers implies that the elimination capacity of these PFASs in adult females may be relatively high (Houde *et al.* 2006a) while the pups may lack urinary and faecal excretion during the foetal stage (Galatius *et al.* 2011). In addition, the hooded seal mother fasts during the entire lactation period (Bowen *et al.* 1987) and is therefore not continuously accumulating PFASs during this period, as opposed to the pups. Consequently, during a short period of growth and

development, the pups may be exposed to large amounts of contaminants through the milk and placenta which they have a low capacity to eliminate, leading to accumulation of contaminants in the pups, while the mothers may excrete PFASs (Wolkers *et al.* 2006).

4.1.2. PFAS patterns in plasma and milk

As expected (hypothesis H3), PFOS was the predominant PFAS in all sample groups, and comprised approximately 40% of the total PFAS concentration. This is in accordance with the high relative occurrence of PFOS compared to other PFASs in previous wildlife studies (Bossi *et al.* 2005a, Butt *et al.* 2010, Houde *et al.* 2011), including studies on pinnipeds (Powley *et al.* 2008, Routti *et al.* 2014). Although PFOS was the most predominant of the eight PFASs (ΣPFAS) in the current study, the relative occurrence of PFOS herein was lower than for most other studies. PFOS comprised 75-80% of ΣPFAS in sea ducks *(Merginae),* ringed seals and beluga whale *(Delphinapterus leucas)* liver (Kelly *et al.* 2009). In harbour seal plasma from Svalbard PFOS comprised over 60% of the ΣPFAS (Routti *et al.* 2014). As the studies mentioned above included even a higher number of PFASs than the current study, the lower contribution of PFOS to ΣPFAS in hooded seal was not explained by the number of analytes. A part of the explanation might be that several of the above mentiond studies have reported liver concentrations, and not plasma concentrations, which may have a different accumulation pattern than plasma. The study on harbour seals from Svalbard, on the other hand, reported plasma levels and still detected a higher relative contribution of PFOS than the present study (Routti *et al.* 2014). This might indicate that there are species specific differences in toxicokinetics and accumulation for different PFASs, leading to different patterns, or reflect dietary differences or variations in the PFAS composition in the local environment where the animals reside.

The PFAS patterns in the three different matrices (maternal plasma, pup plasma, maternal milk) differed. PFUdA was the major contributor to the PFCA burden in maternal plasma and milk, while PFTrDA was the major contributor to the PFCA burden in pup plasma. This is in contrast to the observations on liver of polar bears (Smithwick *et al.* 2005) and ringed seals (Martin *et al.* 2004b) where PFNA was found to be the dominant PFCA. In harbour seal plasma from Svalbard PFUnDA (not analysed in the present study) was the major contributor to the PFCAs, followed by PFNA and PFDA. This strengthens the argument that there might be species differences in toxicokinetics for different PFASs, dietary differences or differences in the PFAS composition in the local environment.

The lactation period did not significantly explain the overall PFAS pattern in maternal plasma, while in pup plasma and maternal milk, the lactation period was significant. In pup plasma and in milk, the relative contribution of PFNA decreased throughout the lactation period. Since the relative amount of PFNA was explained by the protein concentration in maternal plasma, this could indicate that this substance binds strongly to blood proteins, which limits the incorporation into the milk, which in turn leads to lower exposure of PFNA for the pups via milk, and hence lower relative contribution of PFNA throughout the lactation period.

4.2. Maternal transfer of PFASs

All PFASs detected in maternal plasma were also detected in pup plasma. As the pups spend the entire lactation period on ice without entering the water (Lydersen *et al.* 1997), maternal transfer is the only source of PFASs to pups. These findings confirm maternal transfer of PFASs from hooded seal mothers to the pups, and are in accordance with the expectations (hypothesis H4), and findings from previous studies (Hinderliter *et al.* 2005, Bytingsvik *et al.* 2012).

The maternal plasma:cord blood contaminant concentration ratio has been suggested as a measure of transplacental transfer efficiency (Beesoon *et al.* 2011). A previous study showed that the PFOS concentration in maternal plasma and cord blood were highly correlated (R^2=0.876), indicating transplacental transfer (Inoue *et al.* 2004). In the present study, $plasma_{pup}$:$plasma_{mother}$ concentration ratios were used as a measure of maternal transfer efficiency. However, the ratio cannot be used as a measure of transplacental transfer efficiency, as the PFASs in pup plasma also may be a result of lactational transfer.

The combination of a small sample size of milk in the present study, and the fact that the pup plasma samples were sampled at only one time point (no repeated sampling throughout the lactation period), makes it challenging to determine the importance or magnitude of lactational versus placental transfer of PFAS in the present study. However, the lactation period (i.e. age of pup) was used as a possible predictor variable in the multivariate analysis to investigate general trends during the lactation period.

Maternal plasma concentration (ΣPFAS concentrations) was the only significant variable explaining the PFAS concentrations in pup plasma. This was not surprising, since the mothers have a high investment in the pups during the 11 months of pregnancy (Oftedal *et al.* 1993).

Hooded seal pups moult *in utero* and are born with a subcutaneous blubber layer. As the weaned pups do not enter water until about one month, they need sufficient energy stores to ensure normal development and survival. In order to accomplish this during only four days of nursing, prenatal deposition of energy is crucial (Oftedal *et al.* 1993). Hooded seal pups are considered to be, physically, one of the most developed of all *Phocidae* pups at birth, and the energy density in new-born hooded seal pups is the highest of any neonatal mammal (Oftedal *et al.* 1993). All of this favours prenatal transfer of PFASs to the pups. In addition, the finding that neither ΣPFAS concentration in milk nor the lactation period were significant predictors for the PFAS concentrations in the plasma of pups, suggest that the placental transfer is of significant importance. This is in agreement with the expectations (hypothesis H5).

PFAS concentrations in milk were explained by the maternal ΣPFAS plasma concentration, the lactation period and the protein content in the milk. This indicates that the magnitude of contaminant transfer via the milk depends on the contaminant body burden of the mother and nursing duration, and is in accordance with reports on other POPs, such as PCBs and PBDEs (Wolkers *et al.* 2006). What was more surprising, however, was that the lactation period did significantly explain the PFAS concentration in the milk. In domesticated animals with suckling offspring, the amount of protein mobilization from the liver and other tissues increased as a response to the increased nutritional demand as milk production increased. An increase in protein mobilization could lead to an increased release of PFASs from the liver and other tissues (Kim and Easter 2001). The longer the lactation period, the higher the energy demand and thus more proteins are mobilized from the liver. This means that higher levels of PFASs may be present in the mothers' blood, susceptible to excretion into the milk, and subsequent higher PFAS levels would be detected in the milk. Excretion of PFASs into milk may be accomplished by two ways which have been identified as transport mechanisms for chemical contaminants; binding to milk protein or to the surface of fat (Jensen 1991). This may explain why the protein concentration in the milk correlated with several of the PFASs; the more milk proteins - the more incorporation of PFASs into the milk. Albumin is known to be an important milk protein in many species. Still, the implications of chemical binding to milk albumin and subsequent transfer to the young requires further investigation (Jones *et al.* 2003).

The opposite pattern for the $plasma_{pup}$:$plasma_{mother}$ ratio and the $plasma_{mother}$:$milk_{mother}$ ratio, indicates that the PFASs that are efficiently transferred from the mother to the pup, are also more efficiently transferred from the maternal plasma to the milk, and vice versa. This

indicates that there is a similar pattern in transfer efficiency, or in other words: a similar limitation of transfer from maternal plasma to milk, and from maternal plasma to pup plasma via the placenta.

The high $plasma_{pup}$:$milk_{mother}$ ratios have several implications. Firstly, there is a bioaccumulation of the PFASs, i.e. an increase in concentration from the diet (milk) to the pup. Secondly, it indicates that the pups' body burden of PFASs is probably not solely attributed to what is in the milk, but also to transplacental transfer. This is supported by the differences in matrix specific ratios.

4.2.1. Transfer efficiency for different PFASs

The transfer rates from mother to pup differed among PFASs and for different carbon chain lengths. This is in accordance with previous studies (Ohmori *et al.* 2003, Kim *et al.* 2011), and in consensus with the expectations (hypothesis H6). In the present study, the highest transfer efficiencies ($plasma_{pup}$:$plasma_{mother}$ ratio) were observed for the shorter chained sulfonates, and for the short- and long chained acids. A similar, U-shaped, pattern of maternal transfer efficiency with increasing chain length for the acids was also reported in polar bears from Svalbard (Bytingsvik *et al.* 2012) and in humans from China (Zhang *et al.* 2013). In humans and animals, binding affinities of PFASs to proteins are known to increase with increasing carbon chain length (Jones *et al.* 2003, Qin *et al.* 2010). This could explain the trend observed for the sulfonates, but not the acids. However, when perfluoroalkyl acid binding affinities were tested by Bischel *et al.* (2011), binding affinities to blood proteins increased from C_2 to C_8, and decreased from C_9 to C_{13}. The decrease in protein binding from C_9 to C_{13} is likely due to steric hindrances associated with longer and more rigid perfluoroalkyl chains (Bischel *et al.* 2011). Thus, the U-shaped trend of transfer efficiency of the acids observed in the present study may be related to binding affinities to proteins. These results suggest the importance of protein binding for the distribution of PFASs between mother and foetus. However, only a few studies have examined the binding affinities of a wide range of PFASs to blood proteins (Jones *et al.* 2003, Qin *et al.* 2010, Bischel *et al.* 2011), therefore, further studies are needed to explore the mechanism of maternal PFAS transfer.

When comparing the two groups of PFAS, the sulfonates and the acids, it was clear that there was a higher relative occurrence of sulfonates (PFHxS and PFOS) in pup plasma compared to maternal plasma and milk. This might indicate a more efficient transfer from mother to pup

for the PFASs containing a sulfonate group, as compared to the PFASs with an acid group. In mother-pup pairs of harbour seals from the Northwest Atlantic, a similar pattern was found with a higher transfer efficiency for the sulfonates as compared to the acids (Shaw *et al.* 2009). However, previous studies on blood protein binding affinities show that sulfonates show stronger binding affinity than acids with equivalent chain lengths (Bischel *et al.* 2011), and if protein binding inhibits transfer across the placenta, one would expect an opposite pattern. On the other hand, carrier proteins, such as transthyretins (TTRs), are involved in the transfer of thyroid hormones from mothers to the foetus across the placenta (Landers *et al.* 2009). This means that the grater binding affinity of sulfonates would in fact increase the trans-placental transfer rate if they bind to this protein complex.

There are three plausible mechanisms by which PFASs with different carbon chain lengths show different half lives; (1) distribution to various organs is different between PFASs, (2) plasma protein binding is different between PFASs (Kudo *et al.* 2001), and (3) a selective excretion through elimination routes for different PFASs (Ohmori *et al.* 2003). A study on Baikal seal (*Pusa sibirica*) from Russia (Ishibashi *et al.* 2008a) reported higher concentrations of PFNA and PFDA in liver than concentrations in serum. The aqueous solubility of C_6 to C_8 perfluoroalkyl acids appears to facilitate rapid urinary excretion, while the relative hydrophobicity of the longer chained acids appears to favour biliary enterohepatic recirculation (Goecke-Flora and Reo 1996). Their results suggest that PFNA and PFDA are more efficiently retained in the liver and less stable in serum than PFOS, and hence a compound-specific persistence and retention of PFASs in liver is plausible. The retention of PFNA and PFDA in liver may explain why these PFASs seem to be less efficiently transferred from mother to pup.

4.3. Effects of PFASs on thyroid hormones

4.3.1. Associations between PFASs and thyroid hormones

The levels of total and free T3 in maternal plasma decreased with increasing PFDA, PFNA and PFUdA levels, and the levels of free T4, and free T3 in pup plasma decreased with increasing levels of PFTrDA. This is in accordance with the expectations (hypothesis H7). Negative associations between thyroid hormones and PFASs have been reported in previous experiments on rats and mice (Lau *et al.* 2003, Thibodeaux *et al.* 2003, Yu *et al.* 2009).

There are several ways and mechanisms in which PFASs may interfere with the thyroid hormone homeostasis. Weiss *et al.* (2009) suggested competitive binding of PFASs to TTR. Thyroid hormones are associated (not covalently) with the transport protein complex, TTR. This complex functions as a circulating reservoir to buffer changes in thyroid hormone levels (Van den Berg 1990, Van den Berg *et al.* 1991). The presence of PFASs in the blood would, according to Weiss' hypothesis, lead to increased concentrations of circulating free thyroid hormones (FT4 and FT3). The free fraction of thyroid hormones is then subjected to clearance, and hence a reduction in free and total thyroid hormone level (FT4, FT3, TT4 and TT3) in the blood would be expected. This is in accordance with the present study, when addressing the unadjusted results (i.e. not corrected for proteins). However, as both thyroid hormones and PFASs are proteinophilic, the levels of PFAS and thyroid hormones in the plasma may be affected by the protein levels. Protein could therefore be a confounding variable, where apparent associations between thyroid hormones and PFASs are in reality simply a result of higher protein levels. After correcting for the effects of proteins in the maternal plasma samples herein, statistical significance disappeared for total thyroid hormones. This implies that the level of PFASs had a negative impact on the level of free thyroid hormones, but no impact on the total level of thyroid hormones. Explanations for these findings may be that the present study PFAS levels were below the threshold for competitive binding to TTR, or that PFASs do not compete for binding to TTRs in the hooded seals, alternatively that TTRs are not important carriers for thyroid hormones in seals. Nevertheless, according to Zoeller *et al.* (2007), free hormone measurements theoretically provide a more reliable measure of thyroid dysfunction than measures of total hormones because the latter can be altered not only by thyroid dysfunction, but also by changes in the abundance of binding proteins, which may not represent a pathological state. On the other hand, proteins may work as a mediator or as an antecedent variable. A mediator is a variable or factor that is associated with both the dependent and independent variables, but it takes part in the causal chain between these variables. However, the difference between a confounder and a mediator cannot be distinguished on statistical grounds (Bhopal 2002). As proteins may be part of the pathway leading to a decrease in total thyroid hormones, controlling for a part of this pathway could lead to over-control, and thus mask the actual effects of PFASs on the thyroid hormone homeostasis.

There are also other mechanisms by which PFASs may affect the thyroid hormone homeostasis. A central principle of endocrinology is that hormones exert their physiological

actions through receptors. This simple fact has several implications (Kovacs and Ojeda 2012, Zoeller *et al.* 2012). It is the free fraction of thyroid hormones that is physiologically active, and which is able to enter target cells and interact with nuclear receptors (Zoeller and Crofton 2000). Thus, the observed negative relationships between free T3 and PFDA, PFNA and PFUdA in mothers and between free T4, T3 and PFTrDA in pups could lead to lower receptor activation, and hence lower thyroid hormone action. However, the hormone action is saturable, in terms of both ligand binding and effect (Kovacs and Ojeda 2012, Zoeller *et al.* 2012). This means that, although there are negative associations between PFASs and thyroid hormones, it does not necessarily lead to negative effects, as the thyroid hormone level might still be above the saturable state of the receptors. This elucidates the complexity of the thyroid hormone system.

The hooded seal pups showed higher thyroid hormone levels compared to the mothers (Gabrielsen *et al.* 2011). These high levels in newborns are in accordance with the general hyperthyroid state reported in newborn mammals (Erenberg *et al.* 1974, Cabello and Wrutniak 1990). In general, high levels of T4 are observed immediately after birth, while T3 levels are more variable (Little 1991, Haulena *et al.* 1998, Woldstad and Jenssen 1999). This is also in accordance with the results from the present study, where T4 levels were negatively correlated with the lactation period (i.e. days after birth). An implication of this may be that negative effects of PFASs on thyroid hormone levels may be difficult to detect shortly after birth.

The ratio between total and free T3 (TT3:FT3) in maternal plasma correlated positively with PFNA, PFDA and PFUdA levels, which indicates that high levels of these PFASs may lead to more protein bound T3 in relation to free T3. The ratio between free T4 and T3 (FT4:FT3) in maternal plasma was predicted by the protein concentration. This suggests that higher protein levels in plasma leads to lower conversion (deiodination) of the pro-hormone, T4 to the more active hormone, T3. This may be because thyroid hormones bound to proteins are less bioavailable to deiodinases in the peripheral tissues. The ratio between total and free T4 (TT4:FT4) in pup plasma correlated positively with PFTrDA concentrations. PFTrDA levels in pups were mainly explained by the concentrations in milk and the lactation period (positive correlation). This means that the higher levels of total T4 in relation to free T4 in pup plasma might be a result of PFTrDA exposure through milk.

The positive correlations reported herein for TT3:FT3 in mothers and TT4:FT4 in pups are in accordance with the expectations (hypothesis H7), although mostly negative associations were expected. The positive correlations may be due to PFAS induced biliary excretion of free T4 and T3. Thyroid hormone imbalance could include PFAS interference with glucuronidation or sulfonation of T4 and T3, and subsequent excretion of free thyroid hormones (Visser 1994, Brouwer *et al.* 1999). Contaminant induced increases in glucuronidation has been reported in POP exposed rats (van Raaij *et al.* 1993, Brouwer *et al.* 1998). Sulfotransferases (SULT) assist sulfation, which is important for inactivation and excretion of T4 and T3. Studies have shown that OH-PCBs interfere with the sulfation of thyroid hormones in rat liver (Brouwer *et al.* 1998, Schuur *et al.* 1999). It is possible that these mechanisms are relevant for explaining the decreased levels of free T4 and T3 in relation to total T4 and T3 associated with PFAS exposure in the hooded seal mothers and pups. However, this has not previously been shown for PFASs.

4.3.2. Implications of thyroid hormone disruption on neonates

Minor changes (or perturbations) on the thyroid hormone homeostasis may be crucial for neonates. The majority of known actions of thyroid hormones are mediated by the nuclear receptors for T3. Considering that the majority of T4 must also be converted to T3 prior to hormone action on nuclear receptors, it is clear that considerable coordination among circulating levels, hormone uptake into cells and conversion to T3 must occur (Zoeller and Crofton 2000, Zoeller *et al.* 2007). The fact that PFOS correlated positively with FT4:FT3 ratios in pups, indicates that exposure to PFOS might lead to higher levels of T4 in relation to T3, and thus possible effects on thyroid hormone actions. As thyroid hormones are very important for the development of the nervous system in foetuses and juveniles (McNabb and King 1993, Lau *et al.* 2003), the lower levels of T3 could lead to neurodevelopmental deficits, ultimately affecting behaviour and survival.

In humans, the foetal thyroid function does not start until 14-16 weeks, and significant foetal thyroid hormone production does not begin until 20 weeks after conception (Obregon *et al.* 2007). This means that during the first few months of the development, the foetus is dependent on thyroid hormone transfer from the mother. Epidemiological studies have suggested that even mild maternal thyroid hormone deficiencies during pregnancy may adversely affect the neuropsychological development of the offspring (Haddow *et al.* 1999, Pop *et al.* 1999, Li *et al.* 2010). For the present study, this implies that the negative associations observed between PFASs and thyroid hormones in the mother, may have caused

a decreased thyroid hormone delivery to the hooded seal foetus during the first months of development, which may be detrimental for the foetus.

Most of the reported effects of PFASs on thyroid hormone homeostasis are from laboratory experiments. In wildlife, however, there may be a cocktail of contaminants which may have additive, synergistic or antagonistic effects. This makes it hard to draw any cause-effect relationships when studying contaminants in wildlife. Weiss *et al.* (2009) reported possible synergistic effects of different PFAS contaminants on the thyroid hormone levels. Bytingsvik *et al.* (2013) studied effects of contaminant exposure on thyroid hormones in polar bears from Svalbard, which showed no associations between TTR-binding activity of thyroid hormones and plasma levels of PFAS (Bytingsvik *et al.* 2013). Their findings indicated that the TTR-binding activity in the plasma was mainly attributed to OH-PCBs. Previous studies on the same individual hooded seal samples as the current study reported negative associations between selected OH-PCBs and FT4:FT3 and TT3:FT3 ratios in pups, while no associations were found in mothers (Gabrielsen *et al.* 2011). Villanger *et al.* (2013) reported that TT3:FT3 ratios in hooded seal mothers and pups and TT4:FT4 ratios in hooded seal pups were associated with the levels of circulating organohalogen contaminants (OHCs), both positive and negative associations were found. For seals, the existence and importance of TTR as a thyroid hormone carrier protein in blood is not established (St. Aubin 2001). Thus, the importance of TTR-binding by PFASs as a thyroid hormone disrupting mode of action in hooded seals is uncertain. Interactions at several different levels, and a complex system with feedback mechanisms, makes the study of thyroid hormone disruption complicated.

4.3.3. Further remarks

It is challenging to identify possible effects of single groups of contaminants in wildlife. Field studies are often compromised with small sample sizes, in addition ecological variables may confound effects caused by environmental contaminants. These challenges are also present in this study. A sample size of 30 individuals (15 mother-pup pairs) may not be sufficient to assess the general effects of PFASs on thyroid hormone levels in the arctic population of hooded seals. The arctic biota is exposed to a complex mixture of anthropogenic contaminants (Letcher *et al.* 2010), and the PFASs analysed in the present study include only a selection of the PFASs used by the industries, and which may be present in nature.

Circulating hormone concentrations alone may not be the best endpoint for assessing effects on the complex thyroid hormone system. Reijnders (1994) argued that biomarkers, such as

blood parameters, must be part of an integrated approach to risk assessment, and cannot be used in isolation, since data can diverge from "normal" values for many reasons. A holistic approach investigating multiple contaminants and endpoints could elucidate the different mechanisms in which the PFASs may potentially affect the thyroid hormone system in hooded seals. It is however important to bear in mind that thyroid hormone levels in seals fluctuate with age, nutritional status and general health condition, and that the study of hormonal effects in young animals is especially challenging (Hall *et al.* 1998).

5. Conclusions

The present study reports, for the first time, levels of PFASs in plasma and milk of hooded seal mother-pup pairs. The reported levels were within the range of levels reported in similar studies. PFOS was the most predominant PFAS in all sample groups, as expected from previous wildlife studies on pinnipeds. The findings from the current study confirmed maternal transfer of PFASs from hooded seal mothers to pups via both milk and placenta, and implied that placenta was a more important transfer route than milk for most PFASs. There were different transfer ratios for PFASs with different chain lengths, where the lowest transfer efficiency was observed for the intermediate chained acids, most likely due to carbon chain length differences in protein binding affinity. Levels were generally higher in plasma than milk, supporting that binding to plasma proteins limits the incorporation into milk. There were higher levels of PFHxS, PFOS, PFDoA, PFUdA and PFTrDA in plasma of pups than mothers, due to bioaccumulation in the pups and elimination through milk and placenta in the mothers. Levels of PFNA and PFDA were higher in mothers than pups, most likely due to retention of these PFASs in the mother's liver, or less efficient maternal transfer. This is one of the first studies reporting maternal transfer of PFASs in Arctic pinnipeds. Further studies on pinniped species are therefore warranted.

The multivariate analysis revealed negative associations between total and free T3 and PFDA, PFNA and PFUdA in mothers and PFTrDA and free T4 and T3 in pups. This indicates that PFASs might disrupt the thyroid homeostasis in hooded seals and may affect thyroid hormone actions in the individuals. The positive correlations reported herein for TT3:FT3 and several PFASs in mothers and TT4:FT4 and PFTrDA in pups may be due to PFAS induced biliary excretion of free T4 and T3.

To gain a more appropriate insight into the maternal transfer of PFASs, future studies should include repeated plasma samples from the pups, throughout the lactation period. Further studies on the binding affinities of a wide range of PFASs to blood– and milk proteins are needed to explore the mechanism of maternal PFAS transfer. When studying effects of PFASs on thyroid hormone levels, a holistic approach with several different contaminants and endpoints, in addition to a larger sample size, should be used to elucidate the different mechanisms in which the PFASs potentially affect the thyroid hormone system in pinnipeds.

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Appendices

Appendix A: HPLC-MS-MS settings

The samples were run two rounds through the column, where sulfonates were detected during the first run, and acids were detected during the second run.

The injected volume was 5 µL and the flow rate was 200 µL/min. Each sample was run for 17 minutes. The ion source was a turbo spray with negative polarity. Maximum pressure for the HPLC was 5801 psi. Maximum pressure ramp was 290 psi/sec.

Instrument models:

- HPLC-MS-MS: API 3000, LC/MS/MS System
- Main column: Discovery C18 column: 15 cm x 2.1 mm x, 5 µm (Supelco, Sigma-Aldrich, Oslo, Norway)
- Pre – column: Supelguard Discovery C18 column: 2 cm x 2.1 mm x, 5 µm (Supelco, Sigma-Aldrich, Oslo, Norway)
- Autosampler model: Agilent 1100 Autosampler
- Pump model: Agilent 1100 LC Quaternary Pump

Table 5 shows the gradient for the mobile phase used to separate the PFASs in the column. Mobile phase A consisted of 0.2 mM ammonium acetate ($NH_4CH_3CO_2$) in distilled water. Mobile phase B consisted of 0.2 mM $NH_4CH_3CO_2$ in methanol (CH_3OH). The stationary phase (in the column) consisted of silica (spherical, high purity).

Table 5. The gradient for the mobile phase (A and B) during each step in the HPLC.

Step	Time (min)	A (%)	B (%)
0	8	50	50
1	1	85	15
2	5	85	15
3	10	99	1
4	17	99	1

Appendix B: Paired t-test

Table 6. P-values (two tailed) for paired t-tests comparing PFAS levels in mother-pup pairs (15 pairs) of hooded seals from the West Ice (2008).

PFAS	p-value
PFHxS	< 0.001
PFOS	< 0.001
PFOA	0.15
PFNA	< 0.001
PFDA	< 0.001
PFUdA	0.001
PFDoA	< 0.001
PFTrDA	< 0.001

Appendix C: RDA for the effect of protein concentration

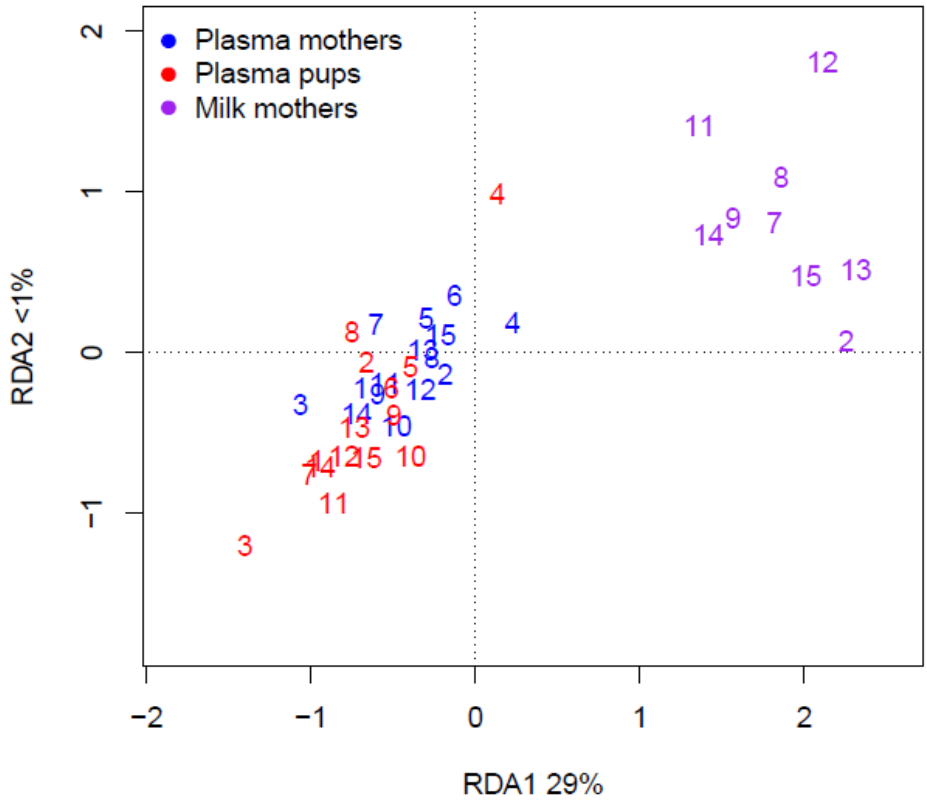

Figure 18. RDA for plasma mothers (n = 15), plasma pups (n = 15) and milk mothers (n = 9) in hooded seal with the protein level as an explanatory variable. Levels are based on ng/g w.w. Amount of variance explained by each ordination axis is given.

An RDA was run to test for the effect of protein level in separating the samples to check if the samples should be protein normalised when compared (Figure 18). The variable "protein" was significant in separating the plasma and milk samples (RDA, permutation test, $p = 0.002$), and explained 29% of the total variance. Therefore, when comparing plasma and milk directly, protein normalised (ng/g protein) concentrations were used (i.e. where w.w. concentrations were divided by protein concentrations).

Appendix D: RDA for the effect of sample group

Effects of sample group in separating individuals based on protein normalised concentrations

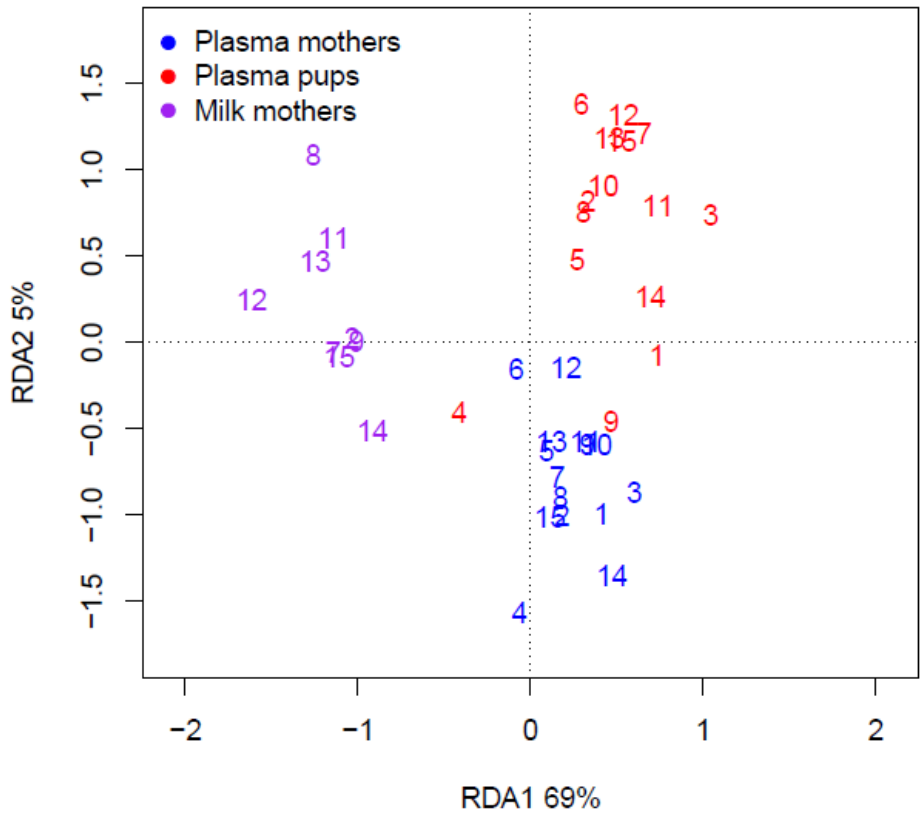

Figure 19. RDA for plasma mothers (n = 15), plasma pups (n = 15) and milk mothers (n = 9) in hooded seal with the sample group as an explanatory variable. Levels are based on ng/g protein concentrations. Amount of variance explained by each ordination axis is given.

An RDA was run to test for the effect of sample group in separating the samples to check if the samples should be run in separate PCAs and RDAs in the further analysis (Figure 19). The categorical variable "sample group" was significant (RDA, permutation test, $p < 0.001$), and explained 74% of the total variance.

Effect of sample group in separating individuals based on percentage of total PFAS

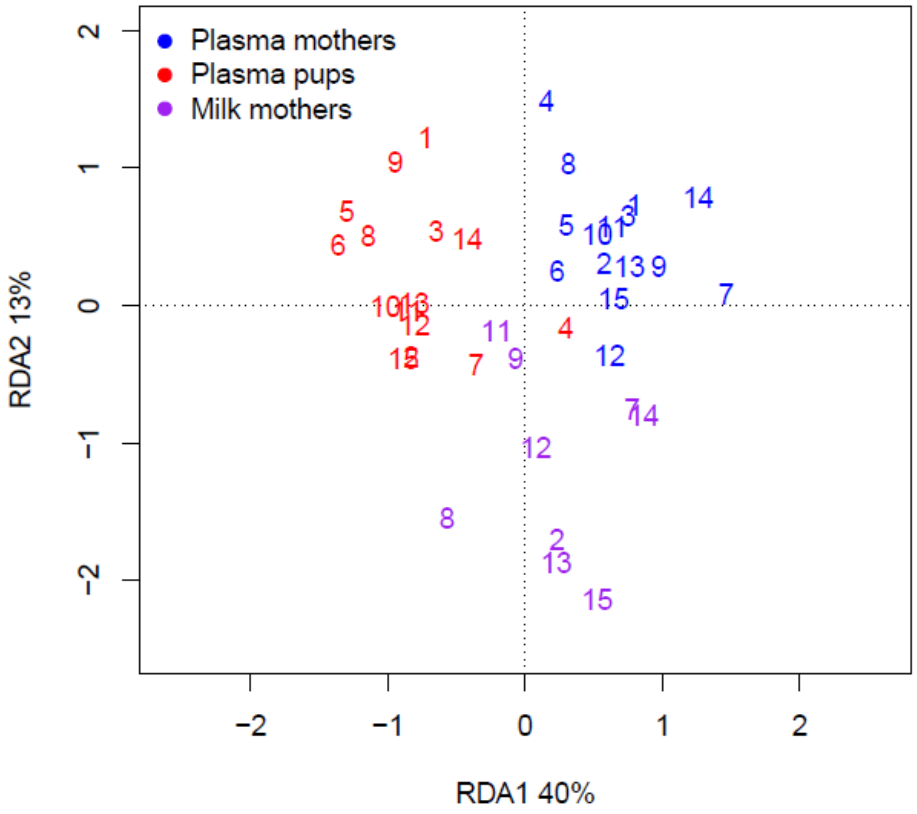

Figure 20. RDA for plasma mothers (n = 15), plasma pups (n = 15) and milk mothers (n = 9) in hooded seal with the sample group as an explanatory variable. Levels are based on percentage of total PFAS. Amount of variance explained by each ordination axis is given.

An RDA was run to test for the effect of sample group in separating the samples to check if the samples should be run in separate PCAs and RDAs in the further analysis (Figure 20). The categorical variable "sample group" was significant (RDA, permutation test, $p < 0.001$), and explained 53% of the total variance.

Appendix E: Thyroid hormone data

Table 7. Minimum - maximum, median and mean ± SD values for the different thyroid hormone concentrations and thyroid hormone ratios in plasma of mothers (n = 15) and pups (n = 15) of hooded seals from the West Ice (2008) (Gabrielsen *et al.* 2011).

	Plasma mothers			**Plasma pups**		
	Min - max	**Median**	**Mean ± SD**	**Min - max**	**Median**	**Mean ± SD**
TT4 (nmol/L)	7.81-21.4	16.1	16.1 ± 3.4	38.1-149	66.4	78.8 ± 28
FT4 (pmol/L)	1.31-5.79	3.55	3.76 ± 1.2	8.28-70.5	20.3	25.9 ± 15
TT3 (nmol/L)	0.585-1.08	0.781	0.772 ± 0.15	1.37-3.21	2.18	2.24 ± 0.50
FT3 (pmol/L)	0.152-0.957	0.538	0.539 ± 0.25	0.915-4.56	1.80	1.91 ± 0.90
FT4:FT3	3.36-17.3	8.01	8.42 ± 4.3	6.83-19.5	13.4	13.5 ± 4.0
TT4:TT3	11.4-31.3	21.7	21.3 ± 5.2	16.6-70.0	35.2	36.1 ± 13
TT4:FT4	2.81-5.98	4.67	4.53 ± 0.85	2.12-4.61	3.23	3.31 ± 0.60
TT3:FT3	0.952-4.52	1.39	1.76 ± 0.94	0.690-2.01	1.21	1.30 ± 0.41