Maternal transfer and biotransformation of persistent organic pollutants (POPs) in hooded seal *(Cystophora cristata)*

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Karl Johan Ullavik Bakken

Abstract

The Arctic has in the later years become a final destination for persistent organic pollutants (POPs) which are, due to long-range atmospheric transport, river outlets and ocean currents, brought into the pristine arctic region from southern latitudes. POPs can accumulate in biota and reach high concentrations in the upper trophic levels in the arctic food webs due to biomagnification. The hooded seal (*Cystophora cristata*) is a top-predator in the arctic marine food web and is susceptible to high POP exposures through diet, but also by placental transfer *in utero* and lactational transfer after parturition from mother to offspring. The hooded seal milk is unique by having the highest lipid percentage reported in any mammal.

The objective of this study was to examine levels and patterns of POPs and their metabolites in mother-pup pairs with respect to maternal transfer and biotransformation. Milk sampled from lactating hooded seal mothers in the West Ice breeding area in the Greenland Sea were analysed for a wide range of POPs, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides. Also, the current study have for the first time analysed OH- metabolites of PCBs and PBDEs in milk from hooded seals. Together with previously analysed levels of these contaminants in plasma of the same mother-pup pairs, these results put together were used to examine in detail the maternal transfer (placental/lactational) and uptake and biotransformation in the pups. The most dominating compounds found in hooded seal milk were PCB-153, PBDE-47 and *p,p'*-DDE, respectively, similar to findings in previous studies of seals. The results confirm a maternal transfer of the lipophilic PCBs, PBDEs and pesticides from hooded seal mothers to their pups via milk. The higher halogenated POPs and pesticides with higher lipid affinity (high log K_{OW}) appear to have lower transfer efficiency in milk and thus placental transfer may be of higher importance in explaining maternal transfer of these compounds. The lower halogenated POPs are preferably transferred to the pups during their nursing period via milk.

OH-metabolites of PCBs and PBDEs were not detected in the present hooded seal milk samples. Thus, milk transfer cannot explain the detected OH-PCBs in maternal and pup plasma for hooded seals. The study did, although detected an increase in OH-PCB ratio relative to PCB during their nursing period, suggesting a weak biotransformation in the pups. Thus, a placental transfer is suggested by the current study to be the major source of OH-metabolites found in the pups, but endogenous biotransformation in the pups may also contribute to these levels.

Abbreviations

Ar	Argon
BFRs	Brominated flame retardants
CHX	Cyclohexane
CH_4	Methane
CYP	Cytochrome P450 enzyme complex
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled, grade 1 water
ECD	Electron capture detector
GC	Gas chromatography
GLM	General linear model
H	Hydrogen
HBCDD	Hexabromocyclododecane
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
He	Helium
H_2SO_4	Sulphuric acid
l.w.	Lipid weight
LOD	Limit of detection
Log	Logarithm
MS	Mass spectroscopy
Ni	Nickel
n.d.	Not detected
nm	Nanometre
NMBU	Norwegian University of Life Sciences
NP	Norwegian Polar Institute
NTNU	Norwegian University of Science and Technology
N_2	Nitrogen
OCP	Organochlorine pesticides
OH-PCB	Hydroxylated polychlorinated biphenyl
OH-PBDE	Hydroxylated diphenyl ether

PBDE	Polybrominated diphenyl ether
PBEB	Pentabromoethylbenzene
PBT	Pentabromotoluene
PCB	Polychlorinated biphenyl
POPs	Persistent organic pollutants
PTV	Programmable temperature vaporization
rpm	Rounds per minute
SD	Standard deviation
SE	Standard error
SULT	Sulphotransferase
UDPGT	Uridine diphosphoglucuronosyl transferase
UiO	University of Oslo
UNEP	United Nations environmental programme
w.w.	Wet weight
α-HCH	α-Hexachlorohexane
β-HCH	β-Hexachlorohexane
γ-HCH	γ-Hexachlorohexane

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1 Introduction

1.1 Pollutants in the Arctic

Persistent organic pollutants (POPs) is a large group of organic contaminants known for their characteristics: they are resistant to degradation (persistent), will bioaccumulate in organisms, biomagnify in food webs and are also toxic (AMAP 2004). The umbrella term POPs consist of several different groups of compounds, such as polychlorinated biphenyls (PCBs), chlorinated pesticides (i.e. dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), hexachlorobenzenes (HCB) and brominated flame retardants (i.e. polybrominated diphenyl ethers (PBDE), pentabromotoluene (PBT) and hexabromobenzene (HBB)).

There is concern about these chemicals and in 2011 many of them were included in the Stockholm Convention- a global treaty to protect human health and environment from chemicals that remain intact in the environment for long periods of time (United Nations Environment Programme 2001). POPs are of a semi-volatile character and are therefore subjected to long-range atmospheric transport, causing these chemicals to be globally distributed. In addition to atmospheric transport, they can be transported by ocean currents, ice drift and marine fauna (AMAP 2004). Due to the wind and current system, the Arctic has become a sink for pollutants from lower latitudes, where the warm air masses from polluted southern areas in Central- and Eastern Europe, Siberia, Asia and the U.S. condense when reaching the cold arctic air. This leads to deposition of the POPs into the ice, snow and ocean (AMAP 2004). Additionally, large river outlets drain the continents and deposits into the Arctic Ocean and will contribute to the Arctic sink.

The POPs are lipophilic and combined with their persistent nature they bioaccumulate in the arctic marine fauna and biomagnify in the marine food web, causing high levels of pollutants and consequently concern for effects in top predators such as polar bears (*Ursus maritimus*), seals and seabirds (Borgå *et al.* 2004). The arctic marine food web is more complex than e.g. terrestrial food webs, and long lived mammals at a high trophic level make them vulnerable to POPs exposure. In mammals, POPs are transferred across generations form the mother to the foetus *in utero* and to the offspring through the maternal milk. As arctic marine mammals have very high lipid content in milk, ranging from 40% to 60% in seals (Lydersen *et al.*

1997), maternal transfer during the lactation period may be an important elimination route for the mothers and accumulation period for the offspring.

1.2 Chemical structure

Several of the pollutants found in the Arctic are exclusively anthropogenic and PCB and PBDE are two of the most important. The two contaminants consist of two benzene rings fused together with a singular bond and 2 or more chlorines substituted on the ring structures for the PCBs (Fig. 1). The structure is mostly similar for PBDE, whereas an oxygen atom holds the two benzene rings together with two singular bonds. The numbering follows the same rules as for PCB. 209 theoretical versions of the PCB- and PBDE molecules exist. These are known as congeners and their structural names follow strictly rules defined by the International Union of Pure and Applied Chemistry (IUPAC) (Mills Iii *et al.* 2007). The chlorine substitution is placed on different positions at the PCB molecules, defined by the positions *meta*, *ortho* and *para*.

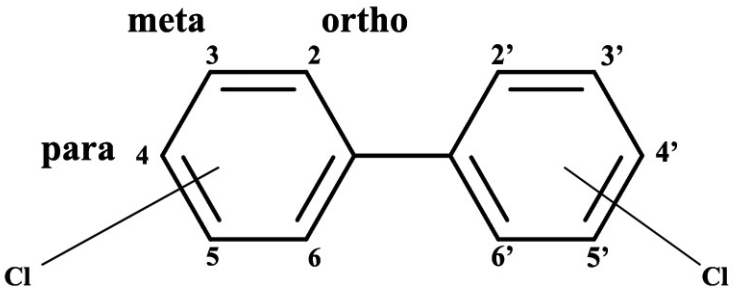

Figure 1 Chemical structure of PCB showing different positions of chlorine atoms and the numbers where chlorine substitution might occur. Private illustration.

A variety of OH-PCBs are produced after biotransformation *in vivo*. Their structure is mostly similar to the parent PCBs, but they have an extra hydroxylated group (OH) attached, usually in *para* or *meta* position (Letcher *et al.* 2000). Naming the OH-PCBs follows the same rules as for the PCBs, however, the OH- group have numbering priority in the IUPAC nomenclature system meaning that the parental compound and its metabolite can have different names (Maervoet *et al.* 2004).

1.3 Metabolism

When exposed to contaminants like i.e. xenobiotics, organisms implement an arsenal of reactions in order to eliminate the contaminants from the body. In mammals, the most important elimination route of contaminants and their metabolites is through the kidneys into the urine. Another important excretion route is via placenta to the foetus and into breast milk (AMAP 2004), whereas excretion of POPs into milk is more important than placental transfer for many seal species (Gallenberg & Vodicnik 1987; Nakashima *et al.* 1997), due to high fat percentage in the milk. In the metabolism process, the body attempts to make the lipophilic compounds more polar in order to expel them from the body. Metabolism can be divided into two main reactions depending on their action.

The first phase, phase I, starts with the induction of cytochrome P450 enzymes (CYP) where CYP1A, -2B and -3A are inducible by contaminant exposure and most likely present in seals (Goksøyr *et al.* 1992; Goksøyr 1995; Ross *et al.* 1996; Wolkers *et al.* 1998a; Wolkers *et al.* 2000; Wolkers *et al.* 2009). By adding a polar functional group, the foreign molecule can be excreted directly or be further processed in the second phase (phase II).
In phase II, a conjugation occurs where the foreign compound binds to an polar, endogenous group like uridine diphosphoglucuronosyl transferase (UDPGT) or sulfotransferase (SULT) (Letcher *et al.* 2000). In some cases, the enzymatic activity can lead to bioactivation of harmless compounds, altering them to become harmful with the ability to bind directly to i.e. proteins and bioaccumulate in the organism (AMAP 2004). This is the case for i.e. CYP-facilitated biotransformation of PCB and PBDEs to OH-PCB and OH-PBDEs, respectively (van Ommen *et al.* 1985; Letcher *et al.* 2000). Phenolic metabolites have the ability to bind to proteins in blood or other tissues, in this way being retained instead of excreted (Malmberg *et al.* (2004).

1.4 Maternal transfer

During the mammalian gestation period, maternal, dietary exposure of toxicants can be distributed to their offspring in two major ways. Prenatal exposure can take place via placental transfer *in utero* to the offspring or there might be a postnatal exposure to the pup, when the offspring is nursed by their mother. Compared to lactation, the *in utero* prenatal contaminant exposure is considered to be of minor importance (Addison & Brodie 1987;

Nakashima *et al.* 1997). Regardless of exposure route, the offspring gain al their burden from their mothers during the entire gestation period until they are weaned.

Lactational transfer is an important route of excretion for many mammals as lipid-soluble contaminants (Addison & Brodie 1987; Gallenberg & Vodicnik 1987; Nakashima *et al.* 1997) like i.e. PCB and pesticides that are bound to the fatty milk. Especially for many marine mammals, the mothers are fastening during their nursing period. This involves a mobilisation from the blubber were passive contaminants are made active and becomes available to the offspring, through the milk. The transfer of POPs from the mothers blubber to their pup via lactations is the most energetically costly component of adult mammalian reproduction and occupies a significant part of the energy budget (Millar 1977). However, the transfer efficiency (blubber to blood to milk) is dependent on the molecular configuration and physicochemical properties of the chemical (Letcher *et al.* 2010).

Although lactational transfer is important, the placental transfer is not to be neglected. Metabolites like the hydroxylated (i.e. OH-PCBs) are poorly transferred by lipids and are instead bound to proteins, making placental transfer to the foetus of certain contaminants possible (Park *et al.* 2008; Gabrielsen *et al.* 2011).

1.5 Study species: Hooded seal *(Cystophora cristata)*

The hooded seal (*Cystophora cristata*) belong to the pinniped family *Phocidae* and ranges over a large sector of the North Atlantic; along the Eastern coast of North America north of Maine, to the Western part of Europe and up the Norwegian coast towards the Arctic Ocean (Kovacs 2009). Juveniles have been recorded to migrate as far south as to Portugal and the Caribbean in the Atlantic Ocean (Mignucci-Giannoni & Odell 2001).

Usually they are solitary, but annually the aggregate together for giving birth and breeding purposes at the drifting pack ice in three distinct locations: the Gulf of St. Lawrence, including the "Front" east of Newfoundland, Davis Strait and at the West Ice in the Greenland Sea. The hooded seals are known as capital breeders where the investment to the offspring is high and the reproduction demands stored energy reserves (Andersen *et al.* 2013) as the mothers do not eat during the parturition or while nursing their pups.
After birth, the pups grow approximately 7 kg per day, more than doubling their birth weight from approximately 23 kg to 60 kg (Kovacs & Lavigne 1992; Kovacs 2009). The pups are

weaned after only 3-5 days, representing the shortest lactation period for any mammals (Bowen *et al.* 1985). Their rapid growth is possible due to intake of a lipid-rich milk, where fat constitutes 60-70% (Oftedal *et al.* 1988; Lydersen *et al.* 1997) and the efficient mass transfer from mother to pup exceeds 60% (Kovacs & Lavigne 1992; Lydersen *et al.* 1997).

The hooded seal is a top-predator in the arctic marine food web, occupying a high trophic level. Thereby they are vulnerable for biomagnification from their diet, which vary, but important species are Greenland halibut (*Reinhardtius hippoglossoides*), polar cod (*Boreogadus saida*), squid (*Gonatus fabricii*) and capelin (*Mallotus villosus*) (Haug *et al.* 2007; Kovacs 2009). They are good divers and usually dive to a depth from 100-600m, although dives down to more than 1000 meters below the surface have been reported, deeper than any other North-Atlantic phocid (Folkow & Blix 1999). With deeper depth, they are also exposed to increasing levels of organochlorines (Bidleman *et al.* 1989; Hargrave *et al.* 1992).

Though the hooded seals have few predators, the polar bear (*Ursus maritimus*) and killer whale (*Orcinus orca*) are natural ones, whereas the humans are likely the most important source of mortality (Kovacs 2009). The total stock size of hooded seals are estimated as high as 660.000 animals on a global scale (Kovacs 2009), whereas the West Ice breeding stock is estimated to be at least 250.000 animals (Rasmussen 1960; Folkow *et al.* 1996).
Due to population decline, the hooded seal became Red Listed by the International Union for Conservation of Nature (IUCN) in 2008, categorized as vulnerable (IUCN 2008). From 2007, all commercial harvesting of the hooded seal in the West Ice were stopped (Haug 2009).

The lipid rich milk and the efficient mass transfer from mother to pup makes the hooded seal a good model species in the current study.

1.6 Aims and hypotheses

1.6.1 Motivation

The diet and physiology of hooded seal makes them susceptible to bioaccumulation of contaminants, leading to biomagnification of contaminants. Earlier studies have reported lactational transfer of PCBs, PBDEs and chlorinated pesticides in hooded seals, but OH-metabolites analyses in milk have previously not been reported and it is not known if and

how these metabolites are transferred between mother and pup in hooded seals and other seal species.

1.6.2 Aim

1. Quantify levels and patterns in milk (analysed in the current study) of hooded seal mothers and compare them with previous reported plasma levels of precursors (Villanger *et al.* 2013) and their metabolites (Gabrielsen *et al.* 2011) in the same mother-pup pairs.

2. Determine if the halogenated contaminants and their metabolites are transferred from mother to pup via maternal transfer (placenta and/or milk) to their offspring, or if biotransformation in the pup is of greatest importance.

1.6.3 Hypotheses

H1: **A)** PCBs, PBDEs and pesticides are lipid soluble and I expect them to be majorly transferred from mother to pup postnatally by lactation due to the generally high lipid content of hooded seal milk (~60%).

B) Due to the protein bound association of the OH-metabolites of PCB and PBDE, I expect that milk will not constitute the main source of exposure, but that placental transfer *in utero* from mothers to pups will be more important for pup levels.

H2: For the same reason described in H1 B), I expect to find low levels in hooded seal milk of OH-metabolites of PCB and PBDEs.

H3: Due to the general low or lacking metabolic capacity in neonates, I expect the endogenous biotransformation in postnatal pups to be of minor importance compared to placental- and lactational transfer of OH-metabolites, respectively.

H4: As the higher halogenated contaminants are lipophilic, I would expect there to be a difference in transfer ratios between different contaminants, depending on their degree of halogenisation.

H5: Previous studies have reported PCB-153 and PBDE-47 to be the congeners measured in highest concentrations. I therefor expect them to be the predominant PCB and PBDE in the analysed milk samples.

2 Material and methods

2.1 Sample methods

Milk and plasma samples from hooded seal mother and their recently born pups (1-4 days old) were sampled by the Norwegian Polar Institute (NP) in the "West Ice", north east of the island Jan Mayen (approximately 73.3°N, 14.5°W) in the Greenland Sea from the research vessel "RV Lance" during March 2008 (Fig. 2). Only the mothers were sedated during sampling.

The sex of the pups was noted, and their body mass (BM) of pups and mothers were measured to the nearest half kg. Pup age was estimate to nearest day based on developmental stage (Kovacs & Lavigne 1992). The pups were sampled before weaning, thus the newborn pups had fed only on milk since birth. The mothers fast during the lactation period (Kovacs 2009). All animal handling were performed after the principles and guidelines of the National Animal Research Authority (NARA) (Forsøksdyrutvalget 2005).

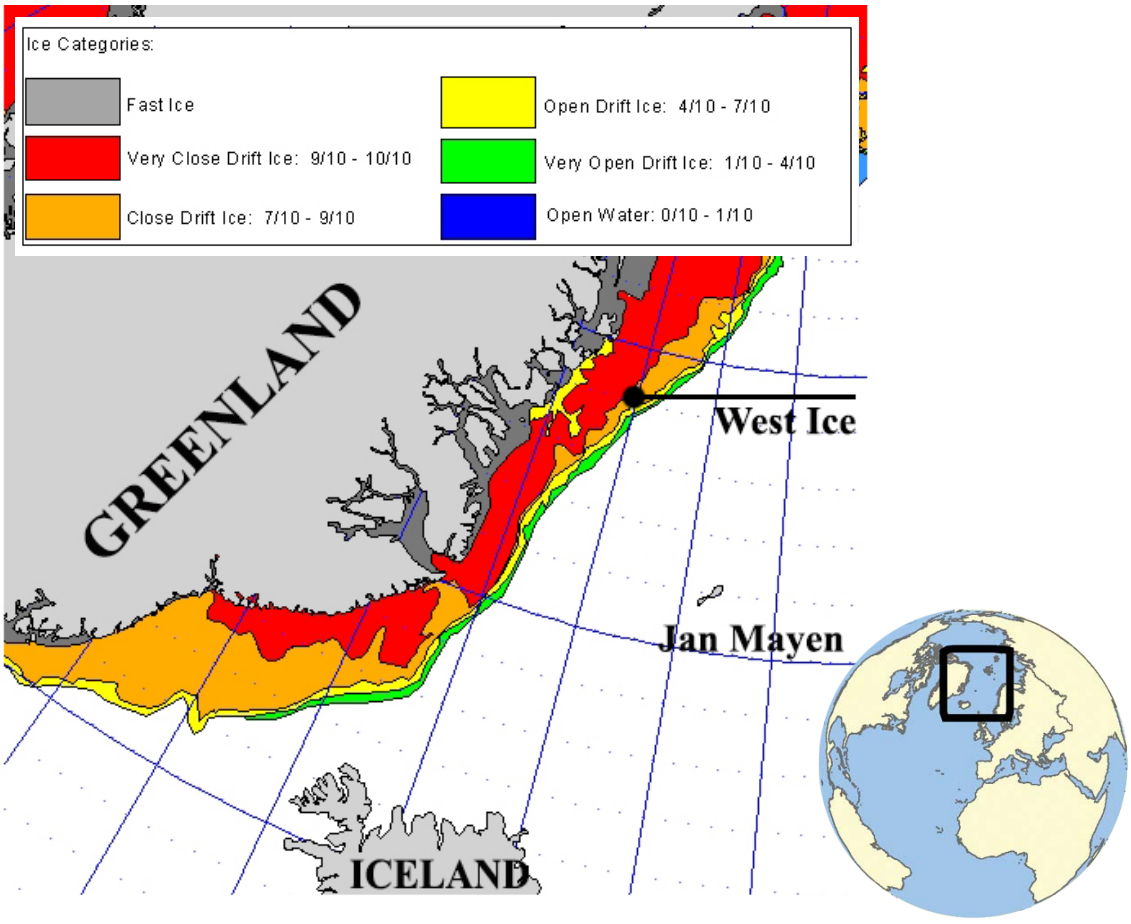

Figure 2. The sampling location in the Greenland Sea, the West Ice, located north east of the island Jan Mayen. The colours represent the condition of the ice edge at 14 March 2008 with the sampling area in the very close/close drift ice. Modified ice map from Norwegian Meteorological Institute (2015).

By injecting a syringe between the vertebrae, blood was collected from the extradural vein into a heparinised- and ethylenediaminetetraacetic acid (EDTA) coated Venoject® tubes (10mL, Terumo Corporation, Leuven, Belgium). The blood samples were centrifuged and plasma and serum were frozen at -20 ºC and later transferred to the Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway for storage at -70 ºC. Blood was sampled from 15 mothers with their respective 15 pups. Milk samples were collected (approximately 10mL) from nine of the 15 mothers while sedated, 10 minutes after an intramuscular injection of 20IU oxytocin and frozen at -20 ºC. The procedures for capturing and sampling are described in more detail in Gabrielsen *et al.* (2011). Prior to analyses in the present study the samples were transferred to the Norwegian University of Life Sciences (NMBU), campus Adamstuen, Oslo, Norway and stored at -20ºC.

2.2 Contaminants analyses

The contaminant analyses in hooded seal milk were all carried out at the Laboratory of Environmental Toxicology (MT-lab) at NMBU. The analyses was performed using a method originally developed for use in human milk first described by Brevik (1978) and modified by Polder *et al.* (2008). The method was later modified for analysing OH-metabolites (Gabrielsen *et al.* 2011). The multi-method (M-MT.2.2) includes extraction of lipids and lipophilic substances with acetone/cyclohexane; clean-up using concentrated sulphuric acid (H_2SO_4) and analyses using gas chromatograph (GC) with a mass spectrometer (MS) or electron capture detector (ECD) mounted. In addition, extraction of OH-PCBs and OH-PBDEs with base (M-MT.2.5) and derivatisation of the same OH-metabolites (M-MT.2.6) were achieved. In addition, a method was conducted for preparation of perfluorinated compounds for PFAS contaminant analyses in hooded seal milk and plasma. This was done in cooperation with Randi Grønnestad for her master's project. The results from the PFAS analyses were not used in the current study. All glassware was washed with a mixture of acetone and cyclohexane (1:1) prior to use. The hooded seal milk samples were stored in an incubator at 40ºC for one hour prior to analyses. 1mL (approximately 0.5 g) of hooded seal milk was weighed in 80mL centrifuge tubes, with 3 decimals. The Internal standards (I.S.) PCB-29, PCB-112, PCB-207; PBDE-77, -119, -181 and - $^{13}C_{12}$-209 and 4'-OH-$^{13}C_{12}$-PCB-159 and 4-OH-$^{13}C_{12}$-PCB-187 were added.

Table 1. The PCB congeners analysed in milk of hooded seal (*Cystophora cristata*) from the "West Ice" with their respective IUPAC name present.

PCB congeners	IUPAC name
PCB-28	2,4,4'-trichlorobiphenyl
PCB-52	2,2',5,5'-tetrachlorobiphenyl
PCB-74	2,4,4',5-tetrachlorobiphenyl
PCB-99	2,2',4,4',5-pentachlorobiphenyl
PCB-101	2,2',4,5,5'-pentachlorobiphenyl
PCB-105	2,3,3',4,4'-pentachlorobiphenyl
PCB-110	2,3,3',4',6-pentachlorobiphenyl
PCB-114	2,3,4,4',5-pentachlorobiphenyl
PCB-118	2,3',4,4',5-pentachlorobiphenyl
PCB-137	2,2',3,4,4',5-hexachlorobiphenyl
PCB-138	2,2',3,4,4',5'-hexachlorobiphenyl
PCB-141	2,2',3,4,5,5'-hexachlorobiphenyl
PCB-149	2,2',3,4',5',6-hexachlorobiphenyl
PCB-153	2,2',4,4',5,5'-hexachlorobiphenyl
PCB-156	2,3,3',4,4',5-hexachlorobiphenyl
PCB-157	2,3,3',4,4',5'-hexachlorobiphenyl
PCB-170	2,2',3,3',4,4',5-heptachlorobiphenyl
PCB-180	2,2',3,4,4',5,5'-heptachlorobiphenyl
PCB-183	2,2',3,4,4',5',6-heptachlorobiphenyl
PCB-187	2,2',3,4',5,5',6-heptachlorobiphenyl
PCB-189	2,3,3',4,4',5,5'-heptachlorobiphenyl
PCB-194	2,2',3,3',4,4',5,5'-heptachlorobiphenyl
PCB-196	2,2',3,3',4,4',5,6'-octachlorobiphenyl
PCB-206	2,2',3,3',4,4',5,5',6-nonachlorobiphenyl
PCB-209	Decachlorobiphenyl

Table 2. The PBDE congeners analysed in milk of hooded seal (*Cystophora cristata*) from the "West Ice" with their respective IUPAC name present.

PBDE congeners	IUPAC name
PBDE-28	2,4,4'-tribromodiphenyl ether
PBDE-47	2,2',4,4'-tetrabromodiphenyl ether
PBDE-99	2,2',4,4',5-pentabromodiphenyl ether
PBDE-100	2,2',4,4',6-pentabromodiphenyl ether
PBDE-153	2,2',4,4',5,5'-hexabromodiphenyl ether
PBDE-154	2,2',4,4',5,6'-hexabromodiphenyl ether
PBDE-183	2,2',3,4,4',5',6-heptabromodiphenyl ether
PBDE-206	2,2',3,3',4,4',5,5',6-nonabromodiphenyl ether
PBDE-207	2,2′,3,3′,4,4′,5,6,6′-nonabromodiphenyl ether
PBDE-208	2,2',3,3',4,5,5',6,6'-nonabromodiphenyl ether
PBDE-209	Decabromodiphenyl ether

Table 3. The analysed organochlorine pesticides, brominated flame retardants (BFRs) and pentachlorophenol (PCP) in milk of hooded seal (*Cystophora cristata*) from the West Ice.

Name	IUPAC names
p,p'- DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
p,p'- DDE	1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]benzene
Oxychlordane	1,5,6,8,9,10,11,11-octachloro-4-oxatetracyclo[6.2.1.$0^{2,7}$.$0^{3,5}$]undec-9-ene
trans-Nonachlor	(1R,2S,3R,4R,5S,6R,7S)-1,3,4,5,7,8,9,10,10-nonachlorotricyclo[5.2.1.$0^{2,6}$]dec-8-ene
HCB	Hexachlorobenzene
α-HCH	(1R,2R,3R,4R,5S,6S)-1,2,3,4,5,6-hexachlorocyclohexane
β-HCH	(1R,2R,3R,4R,5R,6R)-1,2,3,4,5,6-hexachlorocyclohexane
γ-HCH	(1R,2R,3S,4R,5R,6S)-1,2,3,4,5,6-hexachlorocyclohexane
Mirex	1,2,3,4,5,5,6,7,8,9,10,10-dodecachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$]decane
HBCDD	1,2,5,6,9,10-hexabromocyclododecane
PBT	2,3,4,5,6-pentabromotoluene
PBEB	1,2,3,4,5-pentakis(2-bromoethyl)benzene
DPTE	2,3-dibromopropyl 2,4,6-tribromophenyl ether
HBB	Hexabromobenzene
BTBPE	1,2-bis(2,4,6-tribromophenoxy)ethane
PCP	Pentachlorophenol

Table 4. The analysed hydroxylated PBDEs (OH-PBDEs) from milk of hooded seal (*Cystophora cristata*) shown with IUPAC names.

OH-PBDE name	IUPAC name
4 -OH-PBDE42	4-OH-2,2',3,4'-tetrabromodiphenyl ether
3 -OH-PBDE47	3-OH-2,2',4,4'-tetrabromodiphenyl ether
6 -OH-PBDE47	6-OH-2,2',4,4'-tetrabromodiphenyl ether
4'-OH-PBDE49	4'-OH-2,2',4,5'-tetrabromodiphenyl ether
2'-OH-PBDE68	2'-OH-2,3',4,5'-tetrabromodiphenyl ether

Table 5. The analysed hydroxylated PCBs (OH-PCBs) from milk of hooded seal (*Cystophora cristata*) shown with IUPAC names.

OH-PCB name	IUPAC name
4`-OH-PCB106	4'-OH-2,3,3`,4,5-pentachlorobiphenyl
4 -OH-PCB107	4-OH-2,3,3`,4`,5-pentachlorobiphenyl
4`-OH-PCB108	4'-OH-2`,3,3`,4`,5-pentachlorobiphenyl
3 -OH-PCB118	3-OH-2,3`,4,4`,5-pentachlorobiphenyl
3`-OH-PCB138	3'-OH-2,2`,3`,4,4`,5-hexachlorobiphenyl
4`-OH-PCB130	4'-OH-2,2`,3,3`,4`,5-hexachlorobiphenyl
4 -OH-PCB146	4-OH-2,2`,3,4`,5,5`-hexachlorobiphenyl
4`-OH-PCB159	4'-OH-2`,3,3`,4`,5,5`-hexachlorobiphenyl
4`-OH-PCB172	4'-OH-2,2`,3,3`,4`,5,5`-heptachlorobiphenyl
3`-OH-PCB180	3'-OH-2,2`,3`,4,4`,5,5`-heptachlorobiphenyl
4 -OH-PCB187	4-OH-2,2`,3,4`,5,5`,6-heptachlorobiphenyl

2.2.1 Lipid extraction

The samples were added: 2mL 6% sodium chloride (NaCl) (Merck, Darmstadt, Germany), 10mL 1M sulphuric acid (H_2SO_4) (Merck), 15mL acetone (VWR Chemicals, Radnor, PA, USA) and 20mL cyclohexane (CHX) (VWR Chemicals). All samples were sonicated for 2 minutes using Ultrasonic Processor (Cole-Parmer Instrument Company Ltd., Chicago, IL, USA). Between each sample the sonicator was rinsed for 10 seconds using a mixture of CHX and acetone (1:1). The samples were then centrifuged at 3000 rpm (=2095g; Allegra® X-12R, Beckman Coulter, Brea, CA, USA) for 10 minutes. The supernatant were taken off using a 10mL pipette and transferred to 50mL Zymark glassware and evaporated to ~1mL using a Zymark evaporation system (TurboVap® II Concentration Workstation, Zymark Corporation, Hopkinton, MA, USA) at 40°C with a gentle flow of nitrogen gas (0.6 bar N_2) (purity: 99.6%, AGA AS, Oslo, Norway).

The extraction was repeated once with 5mL acetone and 10mL CHX and sonicated for 1 minute, then centrifuged (3000 rpm, 10 min.). The top organic phases were added to the first extract and evaporated. The concentrated extracts in the Zymark glassware were quantitatively transferred to 5mL volumetric flasks and the volume was adjusted.

2.2.2 Lipid determination

The lipid determination was done gravimetrically. An aliquot of 1mL of the lipid extract was pipetted from the volumetric flasks to pre weighed glass vials. The rest of the lipid extract was transferred to reagent glasses. The glass vials with aliquots were put at sand bath (40°C) with a gentle flow of N_2 gas (AGA AS) over the night in a fume hood for evaporation until dry. The next day the glass vials were weighed after being tempered in room temperature for 5 minutes, and evaporation was repeated until stable weight.

The lipid percentage was calculated using the formula:

$$\frac{\left(Weight\ of\ vial\ with\ fat\ (g) - weight\ empty\ vial\ (g)\right) * mL\ total\ extract}{Weight\ initial\ sample\ (g) * 1mL\ aliquot} * 100$$

2.2.3 Clean-up

The 4mL remaining fat extracts were split into two 10mL test tubes pr. sample number for clean-up. For removal of lipids and proteins, the extracts were added 6mL 97.5% H_2SO_4 (Merck). The samples were quickly mixed using a Whirlimixer (IKA MS2 mini shaker, Staufen, Germany). After 60 minutes left in the dark, the test tubes were centrifuged (3000 rpm, 10 min.) and the supernatant was transferred to new reagent glasses and the clean-up was then repeated. About 1 mL CHX was added to the acid layer for optimisation of the extraction. Centrifugation and removal of supernatant was repeated.

2.2.4 Extraction of OH-PCBs and OH-PBDEs

To avoid interference from other similar lipophilic substances affecting the analyses, the metabolites were extracted with base to a separate phase.
The 4 mL acid cleaned extract was added 5mL 1M potassium hydroxide (KOH) (Merck) in 50% ethanol (Kemetyl Norge AS, Vestby, Norway). After a quick mixture at a Whirlimixer the samples were centrifuged (3000 rpm, 5 min.) and the lower phase containing the OH-PCB and OH-PBDE were pipetted out and transferred to 50mL reagent glasses. The extractions were then repeated. The top organic phases were further used in PCB- and PBDE analyses. To the upper organic phase, 2mL distilled (grade 1) water (dH_2O) was added and centrifuged (3000 rpm, 5 min.). The supernatant was then transferred to pre washed conical reagent glasses, calibrated with 400µL 2% decane in CHX. To the water phases containing the hydroxylated PCBs and PBDEs, H_2SO_4 was added until pH = 1. The OH-PCBs and PBDEs were then extracted to an organic phase by adding 5mL CHX to the water phase. After the OH-extracts had separated into two different phases the supernatants were transferred to Zymark glassware. The extraction was repeated once. The samples were evaporated to a volume of ~1mL. After evaporation the extracts were quantitatively transferred with CHX (2 x ~2mL) to 10mL glass reagent glass, and finally put on sand bath (40°C) with a gently flow of N_2 gas (AGA AS) to a volume of ~1mL.

2.2.5 Derivatisation of OH-PCBs and OH-PBDEs

To avoid asymmetrical peaks, "peak tailing", at the GC, the polar OH- groups of PBDE and PCB were replaced with acetyl groups (CH_3CO-) in a process called derivatisation. 1mL sample was added a 50µl mixture of acetic acid anhydride and pyridine (1:1), mixed and

placed in an incubator at 60°C for 30 minutes. After temperate to room temperature (5 min.), the samples were extracted with 2mL dH_2O, mixed and centrifuged (3000 rpm, 5 min.).

The supernatant was transferred to conical reagent glasses (10mL) calibrated with 400µl 2% decane in CHX. The test tubes with the leftovers were added a 1mL pipette CHX to include the leftovers. After mixing and centrifuged (3000 rpm, 5 min.) the organic phase was added to the reagent glasses. The samples were then evaporated to a final volume of 400µl on sand bath (40°C) with a gentle flow of N_2 (AGA AS) before they were transferred to vials with inlets ready for GC.

2.2.6 Quantification of OH-PCBs and OH-PBDEs

The OH-PCB and OH-PBDE were analysed by a high resolution GC (Agilent 6890 Series, Agilent Technologies, Santa Clara, CA, USA). 2µL sample was injected on a DB-5MS 60 meter capillary column (0.25 mm inner diameter (i.d.), 0.25µm film thickness, J&W, Agilent Technologies). Helium (He) (purity: 99.999%, AGA AS) was used as carrier gas (1.8mL/min. at a constant flow). The temperature program was as follows: 90°C (1 min. hold); 40°C/min. increase to 240°C (1 min. hold); 1.5°C/min. increase to 270°C (5 min. hold); 30°C/min. increase to 310°C (10 min. hold). Total run time was 42.08 min.

The GC system was connected to a quadrupole MS (Agilent 5975C series, Agilent Technologies). Selective ion monitoring (SIM) mode was used to detect the I.S. and the OH-PCB/PBDE. For both ion source and quadrupole, the temperatures were 150°C. Methane (CH_4) (purity: 99.995%, AGA AS) was used as a reagent gas. Target ions used were: 4-OH-PCB107: *m/z* 384; 4-OH-PCB146 and 4'-OH-PCB159: *m/z* 418; 4'-OH-PCB130 and 3'-OH-PCB138: *m/z* 346; 4'-OH-PCB172, 3'-OH-PCB180 and 4-OH-PCB187: *m/z* 452; 6-OH-PBDE47: *m/z* 79; internal standards (I.S.) 4'-OH-PCB159: *m/z* 430 and 4-OH-PCB187: *m/z* 464.

2.2.7 PCB analysis and quantification including OCPs

After the base extraction of the OH-PCB, the top organic phases left in the 50mL test tubes were used for determination of PCBs. To the organic phase, 1mL of dH_2O (grade 1) was added before centrifuged (3000 rpm, 5 min.) and the top phase was transferred to conical glass test tubes, calibrated with 400µl "keeper".

After evaporation to the 400µl final volume on sand bath (40ºC) with a gentle flow of N_2, they were transferred to headspace vials with inlets.

Quantification of PCBs and OCPs were done using a high resolution GC (Agilent 6890 Series, Agilent Technologies). Hydrogen (H) (purity: 99.999%, AGA AS) was used as carrier gas (1.2mL/min., constant flow) on a pre-column further connected to a dual column system with columns of different polarity and selectively: Column 1: J & W SPB-5, 60m, 0.25mm i.d., 0.25µm nominal film thickness (Agilent Technologies). Column 2: J&W SPB-1701, 60m, 0.25mm i.d., 0.25µm nominal film thickness (Agilent Technologies). The temperature program was as follows: start 90ºC; 25ºC/min. increase to 180ºC; 25ºC/min. increase to 220ºC; 10ºC/min. increase to 275ºC (10 min. hold). Total run time was 20.70 min. To the GC system, two nickel^{63} (Ni^{63}) µECDs (Agilent Technologies) were connected. The make-up gas was a mixture of 95% argon (Ar) and 5% CH_4 (AGA AS) at a flow rate of 60mL/min.

Due to technical disturbances for the higher chlorinated PCBs at the GC-ECD, the following were detected on the GC-MS: PCB-156, PCB-157, PCB-170, PCB-189, PCB-194, PCB-196, PCB-206, and PCB-209. 2µl sample solution was injected on a J&W DB-5MS column: 60m, 0.25mm i.d., 0.25µm film thickness (Agilent Technologies). Helium (He) (purity: 99.999%, AGA AS) was used as carrier gas (1.3mL/min., constant flow). The program was as follows: 90ºC (2 min. hold); 25ºC/min. increase to 180ºC (2 min. hold); 1.5ºC/min. increase to 220ºC (2 min. hold); 3ºC/min. increase to 275ºC (12 min. hold); 25ºC/min. increase to 300ºC (4 min. hold). Total run time was 71.60 min.

2.2.8 Quantification of BFRs

For separation and detection of BFRs, 2µl final sample solution was injected on a J&W DB-5MS capillary column: 60m, 0.25mm i.d., 0.25µm film thickness (Agilent Technologies). Carrier gas was helium (He) (purity: 99.999%, AGA AS) with a constant flow of 1.6mL/min. The temperature program was as follows: 90ºC (1 min. hold); 25ºC/min. increase to 180ºC (1 min. hold); 2.5ºC/min. increase to 220ºC; 20ºC/min. increase to 320ºC (4 min. hold). Total run time was 30.60 minutes.

For PBDE-206 and PBDE-209 only, 5µl of final extracts were injected on a GC-MS (Agilent 6890 Series/5973Network, Agilent Technologies) with a programmable temperature vaporization (PTV) injector mounted.

Separation and identification were performed on a J&W DB-5MS column: 10m, 0.25mm i.d., 0.25µm film thickness (Agilent Technologies). The program was as follows: 80°C (2 min. hold); 30°C/min. increase to 315°C (4 min. hold). Total run time was 13.83 min.

2.3 GC calculations

Calculations of GC data were performed using GC ChemStation (Version B.04.03, Agilent Technologies) and MSD ChemStation (Version E.02.01, Agilent Technologies).

2.4 Protein analysis

A modified method for quantification of proteins (Lowry *et al.* 1951) were performed in order to standardize results from the biological analyses. By measuring absorbance due to a blue colour development resulting from the reaction of proteins with an alkaline copper tartrate solution and Folin reagent, total protein concentration could be detected. The colour reaction is primarily caused by the amino acids tyrosine and tryptophan and to a lesser extent, cystine, cysteine and histidine (Lowry *et al.* 1951). The analysis was conducted at the Department of Biosciences, University of Oslo, Norway.

For the protein standard, a dilution series were made (1mg/mL, 0.5mg/mL, 0.25mg/mL and 0.125mg/mL) using a bovine gamma albumin protein standard (BSA) (Protein standard 200mg/mL, Sigma-Aldrich) diluted with 0.1M Trizma Buffer (pH = 8 at 4°C, 6.35g Trizma HCl and 1.18g Trizma Base, Sigma-Aldrich). A dilution series were made for the hooded seal milk (20x, 100x and 200x) (n = 9) and plasma (40x, 80x and 100x) (n = 30) diluted in Trizma Buffer (Sigma-Aldrich). For blank, only Trizma Buffer (Sigma-Aldrich) was used. Protein standards, blanks and samples were plated out in triplicates for each concentration dilution with 10µL in each well on a 96-well Microtiter plate (Nunclon™, VWR).

The following step consisted of adding 25µL alkaline copper tartrate solution, reagent A (Bio-Rad laboratories, Hercules, CA, USA) and 200µL Folin reagent, reagent B (Bio-Rad laboratories). Reagent B was added using a multi-channel pipette. Between the adding of the two reagents, the plate was gently shaken. The work was performed in dull light, as reagent B is light sensitive. After incubation in room temperature for 15-30 minutes, wrapped in aluminium foil, the absorbance was read at 750 nm using a Synergy MX plate reader (BioTek, Winooski, VT, USA) with Gen10 software.

A standard curve was constructed in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) by use of the outcome from the plate reader, where the diluted protein standards made it possible to calculate the protein concentrations of unknown samples by using the linear standard curve (y = a + bx). The protein concentrations output were given in µg/mL.

2.5 Conversion of units and presentation of concentrations

In order to normalise parental compounds and their hydroxylated protein associated metabolites for lipid and protein, respectively, the output from the lipid determination (given in % lipid) (section 2.2.2) were converted to the same unit as the protein concentrations, g/mL (µg/mL x 10^{-6}). This was achieved by rewriting the mass concentration formula:

$$\rho = \frac{m}{V} \rightarrow \frac{m * \rho}{V} = concentration$$

where ρ in the first equation is the mass concentration defined as the mass of a constituent (m) divided by the volume of the compound (V).

An assumption of similar density between in plasma and milk were made to be able to normalise the parental- and metabolite compounds. Both matrices were set a density of 1 g/cm^3, equal to water. Therefore, 1g (m) would equal 1mL (V), and can be removed from the equation, leaving the concentration (g/mL) left.

All contaminant concentrations presented in the current study are given as nanograms per gram lipid normalised concentrations (ng/g lipid weight), except for the protein associated OH-PCBs, which is given as protein normalised concentrations (ng/g protein weight).

2.6 Analytical quality assurance

The Laboratory of Environmental Toxicology at NMBU, the former Norwegian School of Veterinary Sciences (NVH) is accredited by Norwegian Accreditation since 1996 for the determination of organochlorines (OCs), brominated flame retardants (BFRs) and lipid content in biological matrices according to the requirements of the NS-EN/IEC 17025 (test

137). The determination of the OH-metabolites is not an accredited method, but is validated after the same criteria as the accredited methods.

A control sample of 0.5g milk was taken out for each sample analysed. In addition, 0.25g seal blubber and 2.5g seal blood were used for internal reference. For recovery, 2g cow milk and 3g egg were used.

Limit of detection (LOD) was defined as three times the average background noise in the chromatograms of the sample extracts. The instrumental detection limits ranged from 0.140 to 2.765 ng/g w.w. (pesticides and PCB), 0.040 to 23.8 ng/g w.w. (BFRs) and 0.170 to 0.880 ng/g w.w. for OH-metabolites.

2.7 Data processing

Contaminants detected (i.e. above LOD) in < 60% samples in each matrix, are reported in Table 8, but excluded from further statistical analyses (= 60% cut-off).
This includes: PCP, γ-HCH, PCB-28, -114, -157, -196; PBDE-28, -183, -206, -207, -208, -209, PBT, PBEB, DPTE, HBB, BTBPE, 3-OH-PCB-118, 4'-OH-PCB108, 4'-OH-PCB130, 4'-OH-PCB159, 3'-OH-PCB180, 6-OH-PBDE47, 2'-OH-PBDE68, 4'-OH-PBDE49, 3-OH-PBDE47 and 4-OH-PBDE42.
Except for these compounds, Table 7 show the individual 41 organohalogen contaminants that were detected in more than 60% of the samples and that were included in further statistics.
For chemicals with more than 60% of samples above LOD, missing values were replaced by randomly generated numbers between 0 and LOD for the contaminants used in further analyses (Table 6). The random numbers were generated using Microsoft Excel 2010, Norwegian edition (Microsoft Corporation) with the given function:

= RANDOM()*(b-a)+a, where a = 0 and b = detection limit (LOD).

Table 6. The chemicals with more than 60% of the samples above LOD that were given a random value between 0 and LOD. The LOD values is present together with matrix (PM = plasma mother, PP = plasma pup) and the number of the observations (*n*) that were given a random value.

Contaminant	**LOD (ng/g)**	**Matrix**	***n* random values**
α-HCH	0.008	PM	1
β-HCH	0.021	PM	2
p,p'- DDT	0.664	Milk	1
PCB-137	0.018	PM	3
		PP	3
PCB-183	0.022	PM	1
PCB-156	0.022	PP	3
		PM	5
PCB-170	0.018	PM	1
PCB-189	0.015	PP	4
PCB-194	0.021	PM	1
	0.245	Milk	1
PCB-206	0.162	Milk	2
PCB-209	0.030	PP	3
		PM	5
PBDE-47	0.015	PM	1
PBDE-99	0.019	PP	3
PBDE-100	0.006	PP	4
PBDE-153	0.013	PP	1
		PM	2
4-OH-PCB146	0.004	PP	1
3'-OH-PCB138	0.005	PP	3
		PM	2
4'-OH-PCB172	0.006	PP	1

Table 7. 41 contaminants with more than 60% of the samples per matrix above LOD which were thereby included in the further statistics. Sample size, $n = 15$ (maternal and pup plasma) and $n = 9$ (milk).

	Plasma mother	**Plasma pup**	**Milk**
HCB	✓	✓	✓
α-HCH	✓	✓	✓
β-HCH	✓	✓	✓
p,p'- DDE	✓	✓	✓
p,p'- DDT	✓	✓	✓
Oxychlordane	✓	✓	✓
trans-Nonachlor	✓	✓	✓
Mirex	✓	✓	✓
PCB-52	✓	✓	✓
PCB-74			✓
PCB-99	✓	✓	✓
PCB-101	✓	✓	✓
PCB-105			✓
PCB-110	✓	✓	
PCB-118	✓	✓	✓
PCB-128			✓
PCB-137	✓	✓	✓
PCB-138	✓	✓	✓
PCB-141	✓	✓	✓
PCB-149	✓	✓	✓
PCB-153	✓	✓	✓
PCB-156	✓	✓	✓
PCB-170	✓	✓	✓
PCB-180	✓	✓	✓
PCB-183	✓	✓	✓
PCB-187	✓	✓	✓
PCB-189		✓	
PCB-194	✓	✓	✓
PCB-206	✓	✓	✓
PCB-209	✓	✓	
PBDE-47	✓	✓	✓
PBDE-99		✓	✓
PBDE-100		✓	✓
PBDE-153	✓	✓	✓
PBDE-154	✓	✓	✓
HBCDD			✓
4-OH-CB107	✓	✓	
4-OH-CB146	✓	✓	
3'-OH-CB138	✓	✓	
4'-OH-CB172	✓	✓	
4-OH-CB187	✓	✓	

2.8 Statistical analyses

Statistical analyses were conducted using R (version 3.20, the R project for statistical computing, Auckland, New Zealand). Microsoft Excel 2010 (Microsoft Corporation) was used to create barplots. To test for normality, the Shapiro-Wilk test was used. Many of the data were normally distributed, and those who weren't were log-transformed to achieve normally distribution.

Homogeneity of variance was tested with the Levene's test (package "Lawstat", R).
To test for differences in contaminant levels among groups, the parametric, Two Sample t-test was used.
When the assumptions for the t-test were not met, regarding normality and homoscedasticity, the data were log-transformed. When normal distribution was achieved but not homoscedasticity, the Welch's t-test was used. Kruskal-Wallis test was used when it was not possible to achieve normal distributed data or homoscedasticity.

The differences in plasma concentrations of OH-PCBs between mother-pup pairs were tested using a parametric paired t-test, and when the assumptions for normality were not met even after log-transformation, the non-parametric Wilcoxon signed rank test were performed.
To analyse correlation, the non-parametric Spearman rank correlation (denoted RS) was used to measure the degree of association between two variables.
The Spearman test does not make any assumptions about the distribution and is more robust towards outliers than the Pearson correlation test. When analysing the OH-metabolite/precursor ratio versus days, the linear regression model (GLM) was used to calculate association as the days were treated as factor variables.

When having more than two groups (matrixes) to compare to one or two factor variables, a One or Two-Way ANOVA (Analysis of Variance) was used, respectively. The One-way ANOVA was followed by Tukey's honestly significant difference post hoc test (TukeyHSD) to test for significant differences. TukeyHSD is suitable for multiple comparison of means as it adjusts the p-value, reducing the risk of getting a type I error.

The level of significance was set to $p < 0.05$. All p-values are two-tailed.

3 Results

3.1 Quality assurance

The analytical quality was monitored using a number of tests according to the accreditation. Every analytical series included three blank samples (solvents only) to control contamination from solvents, equipment and air; one blind sample (cow's milk) and two cow's milk samples spiked with different POPs for recovery test. In addition, the internal reference sample of seal blubber was included for monitoring repeatability and reproducibility of the analysis.
The relative recovery rates are 94% to 217% (PCB), 75% to 208% (BFRs), 18% to 129% (pesticides) and for OH-metabolites 54% to 140%, whereas for OH-PBDE it was 92% to 150%.

The recovery of PCB-156 and PCB-157 was too high. They were both detected on GC-MS, and no particular reasons for the deviations were found. When correcting for recovery (by technicians at the MT –lab, NMBU), the values of both congeners in the control sample were too low compared to their actual value. Therefore, caution must be taken considering the two congeners. Also the recovery for *p,p'*- DDT (pesticides) was too low due to decomposition in the GC injector. The value of *p,p'*- DDT in the control sample (seal blubber) was also too low.

3.2 Biological variables

The mothers weighted 149.1 ± 28.9 kg (mean ± SD) whereas the pups weighed 32.73 ± 8.0 kg (mean ± SD). The age of the pups were positively correlated to their body mass (BM) (R_S = 0.85, $p < 0.001$) and the group consisted of 6 males and 9 females. There were no significant differences in body mass between the sexes of the pups (t-test: $p = 0.23$). The mean age of the pups was 2.7 ± 0.8 days.

The mean percent plasma lipids in pups were significantly higher (1.40 ± 0.69%) than the corresponding maternal sample (0.7 ± 0.1%) (t-test: $p < 0.001$), possibly reflecting the pup's constant feed for milk. Mean lipid percentage (68.7 ± 4.92%) was significantly higher in milk than maternal plasma (Welch's t-test: $p < 0.001$). There were no significant difference (t-test: $p = 0.320$) in protein levels between plasma of mothers and pups (74.3 ± 7.7mg/mL and 71.2

± 13.5mg/mL, respectively). The level of proteins in the milk (52.7 ± 17mg/mL) were significantly lower (Welch's t-test: $p < 0.005$) than protein levels in maternal and pup plasma.

3.3 Environmental contaminants

A total of 68 contaminants were analysed for in milk, where 37 contaminants were detected (above LOD; see Table 8) and 5 of these were removed from further statistics as they were below the limit of detection in < 60% of the samples.
In 3 of the 37 contaminants detected in milk that were above limit of detection (a minimum of 6 out of 9 individuals) were assigned a random value for the missing values (Table 6).
OH- metabolites of PCB and PBDE were not detected in any of the milk samples. Contrary to OH-PCBs, the OH-PBDE have not previously been detected in maternal or pup plasma either (Villanger *et al.* 2013). The plasma values were first analysed and described by Villanger *et al.* (2013) (precursors) and Gabrielsen *et al.* (2011) (OH-PCBs only) and are presented in Table 8.

There were 15 contaminants that were detected in both plasma and milk: HCB, *p,p'*-DDE, Oxychlordane, *trans*-Nonachlor, Mirex, PCB-52, -101, -99, -149, -118, -153, -141, -138, -187 and PBDE-154. Altogether 32 contaminants, including the five OH-PCBs, were found above cut off levels in plasma of both mothers and pup, whereas five contaminants were found only in pup plasma, and not in their associated mother's plasma or milk; PBDE-99 and -100, PCB-209, -189 and -156. PCP and PCB-196 were detected in milk from one mother each (not the same mother). All PCBs analysed and detected in maternal plasma were also detected in pup plasma, with the exception of PCB-189, which was detected in pup plasma only.
An overview of the contaminants that were detected in the matrices is given in Table 8.

∑PCB level were significant higher relative to ∑PBDE[1] (Tukey: $p < 0.001$) and ∑Pesticides[2] (Tukey: $p < 0.001$). The pattern between ∑PCB and ∑PBDE are similar, with highest levels in pup plasma, followed by maternal plasma and milk, respectively. For ∑OCP the pattern is different with highest levels in pup plasma, followed by milk and maternal plasma.

[1] ∑PBDE include: PBDE-47, PBDE-153 and PBDE-154.
[2] ∑Pesticides include: HCB, β-HCH, *p,p'*-DDE, *p,p'*-DDT, Oxychlordane, *trans*-Nonachlor and Mirex.

Table 8. Concentrations (ng/g ww) of the contaminants detected in milk and plasma of hooded seal (*Cystophora cristata*) mother-pup pairs. Contaminants with lack of detection in all matrices, such as e.g. OH-PBDEs, are not given. Mean values and standard deviations (SD) are shown, together with minimum and maximum values. Contaminants over LOD in ≥ 60% of the individuals are highlighted in bold.

	Analysed in the current study					**Reported in Villanger *et al.* (2013)**									
	Milk (*n* = 9)					**Maternal plasma (*n* = 15)**					**Pup plasma (*n* = 15)**				
	n > LOD	Mean	SD	Min.	Max.	n > LOD	Mean	SD	Min.	Max.	n > LOD	Mean	SD	Min.	Max.
PCP	1[a]	0.267	-	0.267	0.267	0[a]	-	-	-	-	0[a]	-	-	-	-
HCB	**9[b]**	**8.62**	1.90	5.89	11.2	**15[b]**	**0.089**	0.028	0.052	0.162	**15[b]**	**0.195**	0.140	0.073	0.627
α-HCH	0[a]	-	-	-	-	**14[b]**	**0.017**	0.007	0.000*	0.027	**15[b]**	**0.053**	0.047	0.017	0.193
β-HCH	**9[b]**	**4.25**	1.06	3.14	6.38	**13[b]**	**0.033**	0.016	0.013	0.073	**15[b]**	**0.080**	0.073	0.029	0.265
p,p'-DDE	**9[b]**	**250**	82.7	82.5	371	**15[b]**	**1.59**	0.521	0.398	2.44	**15[b]**	**6.28**	4.38	1.64	15.3
p,p'-DDT	**8[b]**	**1.22**	0.793	0.271	3.09	**15[b]**	**0.224**	0.080	0.068	0.349	**15[b]**	**0.835**	0.642	0.190	2.13
Oxychlordane	**9[b]**	**28.8**	8.61	16.8	48.1	**15[b]**	**0.216**	0.067	0.089	0.401	**15[b]**	**0.707**	0.473	0.281	1.99
trans-Nonachlor	**9[b]**	**51.7**	27.5	1.29	91.2	**15[b]**	**0.389**	0.172	0.128	0.793	**15[b]**	**1.46**	1.16	0.462	3.92
Mirex	**9[b]**	**5.83**	3.97	2.03	15.4	**15[b]**	**0.096**	0.049	0.020	0.221	**15[b]**	**0.274**	0.206	0.061	0.795
∑OCP	**9[b]**	**350**	114	141	513	**15[b]**	**2.65**	0.811	0.835	4.13	**15[b]**	**9.90**	6.95	2.90	23.9
PCB-28	0[a]	-	-	-	-	0[a]	-	-	-	-	1[a]	0.024	-	0.024	0.024
PCB-52	**9[b]**	**14.1**	3.74	7.48	18.9	**15[b]**	**0.198**	0.035	0.146	0.274	**15[b]**	**0.428**	0.286	0.165	1.22
PCB-74	**9[b]**	**3.71**	0.944	1.99	4.93	0[a]	-	-	-	-	8[a]	0.115	0.085	0.010	0.261
PCB-101	**9[b]**	**21.8**	7.70	9.38	33.1	**15[b]**	**0.315**	0.080	0.150	0.477	**15[b]**	**0.998**	0.754	0.295	2.61
PCB-99	**9[b]**	**33.1**	9.70	15.0	46.7	**15[b]**	**0.253**	0.072	0.082	0.381	**15[b]**	**0.804**	0.535	0.232	2.02
PCB-110	0[a]	-	-	-	-	**15[b]**	**0.115**	0.029	0.063	0.179	**15[b]**	**0.355**	0.320	0.078	1.15
PCB-149	**9[b]**	**13.1**	3.82	7.25	18.4	**15[b]**	**0.168**	0.045	0.068	0.241	**15[b]**	**0.587**	0.490	0.171	1.71
PCB-118	**9[b]**	**10.1**	3.61	4.23	14.8	**15[b]**	**0.101**	0.029	0.034	0.156	**15[b]**	**0.327**	0.266	0.080	0.83
PCB-153	**9[b]**	**104**	48.0	35.7	201	**15[b]**	**1.08**	0.373	0.269	1.70	**15[b]**	**3.46**	2.18	0.807	7.93
PCB-105	**9[b]**	**4.65**	1.14	2.40	5.69	0[a]	-	-	-	-	0[a]	-	-	-	-
PCB-141	**9[b]**	**1.39**	0.584	0.533	2.28	**15[b]**	**0.026**	0.011	0.009	0.054	**15[b]**	**0.078**	0.078	0.015	0.263
PCB-137	**9[b]**	**2.47**	1.14	1.07	4.62	**12[b]**	**0.023**	0.012	0.003	0.043	**13[b]**	**0.065**	0.052	0.003	0.177
PCB-138	**9[b]**	**62.8**	26.3	22.2	112	**15[b]**	**0.556**	0.212	0.172	0.965	**15[b]**	**1.73**	1.13	0.476	4.10
PCB-187	**9[b]**	**11.6**	6.46	3.54	25.2	**15[b]**	**0.181**	0.064	0.049	0.291	**15[b]**	**0.585**	0.387	0.124	1.39
PCB-183	**9[b]**	**4.99**	4.18	0.165	13.8	**14[b]**	**0.095**	0.045	0.004	0.183	**15[b]**	**0.306**	0.201	0.057	0.766
PCB-128	**9[b]**	**4.18**	1.80	2.11	7.67	0[a]	-	-	-	-	0[a]	-	-	-	-
PCB-156	**9[b]**	**2.85**	1.31	1.01	4.94	**10[b]**	**0.023**	0.015	0.000*	0.048	**13[b]**	**0.079**	0.058	0.011	0.185
PCB-157	4[a]	1.467	0.174	1.27	1.65	0[a]	-	-	-	-	0[a]	-	-	-	-
PCB-180	**9[b]**	**22.1**	15.9	5.18	58.4	**15[b]**	**0.425**	0.209	0.076	0.910	**15[b]**	**1.38**	0.906	0.249	3.51
PCB-170	**9[b]**	**11.8**	7.89	2.94	29.7	**14[b]**	**0.137**	0.064	0.025	0.256	**15[b]**	**0.417**	0.280	0.076	1.11
PCB-196	1[a]	6.11	-	6.11	6.11	0[a]	-	-	-	-	0[a]	-	-	-	-
PCB-189	2[a]	0.20	0.001	0.194	0.195	6[a]	0.026	0.005	0.017	0.033	**12[b]**	**0.025**	0.017	0.004	0.054
PCB-194	**8[b]**	**1.33**	1.30	0.181	4.46	**14[b]**	**0.054**	0.033	0.011	0.136	**15[b]**	**0.198**	0.148	0.029	0.570
PCB-206	**7[b]**	**0.39**	0.225	0.082	0.775	**15[b]**	**0.032**	0.012	0.020	0.063	**15[b]**	**0.090**	0.059	0.020	0.198
PCB-209	4[a]	0.269	0.092	0.195	0.388	**10[b]**	**0.034**	0.019	0.004	0.081	**12[b]**	**0.083**	0.061	0.005	0.198
∑PCBs[c]	**9[b]**	**331**	140	123	605	**15[b]**	**3.32**	1.18	1.27	5.84	**15[b]**	**12.0**	7.83	3.11	26.4

	Analysed in the current study (cont.)					Reported in Villanger *et al.* (2013) (cont.)									
	Milk (*n* = 9)					**Maternal plasma (*n* = 15)**					**Pup plasma (*n* = 15)**				
	n > LOD	Mean	SD	Min.	Max.	n > LOD	Mean	SD	Min.	Max.	n > LOD	Mean	SD	Min.	Max.
HBCDD	**9**[b]	6.92	3.14	2.63	12.1	0[a]	-	-	-	-	4[a]	0.216	0.085	0.155	0.340
PBEB	0[a]	-	-	-	-	1[a]	0.013	-	0.013	0.013	0[a]	-	-	-	-
PBDE-47	**9**[b]	**3.69**	1.63	1.12	6.19	**14**[b]	**0.041**	0.014	0.006	0.067	**15**[b]	0.149	0.131	0.024	0.440
PBDE-99	**9**[b]	**0.824**	0.417	0.248	1.70	4[a]	0.118	0.193	0.019	0.407	**10**[b]	0.040	0.040	0.002	0.138
PBDE-100	**9**[b]	**0.353**	0.175	0.101	0.649	6[a]	0.050	0.091	0.008	0.235	**11**[b]	0.022	0.026	0.001	0.095
PBDE-153	**9**[b]	**0.253**	0.170	0.055	0.630	**13**[b]	**0.024**	0.024	0.004	0.106	**14**[b]	0.054	0.047	0.011	0.155
PBDE-154	**9**[b]	**0.445**	0.272	0.101	1.05	**15**[b]	**0.027**	0.021	0.007	0.098	**15**[b]	0.088	0.078	0.014	0.287
PBDE-183	0[a]	-	-	-	-	4[a]	0.377	0.486	0.086	1.10	5[a]	0.194	0.091	0.094	0.300
PBDE-206	0[a]	-	-	-	-	1[a]	0.521	-	0.521	0.521	0[a]	-	-	-	-
PBDE-207	0[a]	-	-	-	-	5[a]	0.046	0.039	0.023	0.115	5[a]	0.086	0.068	0.028	0.198
PBDE-208	0[a]	-	-	-	-	3[a]	0.017	0.004	0.014	0.021	3[a]	0.059	0.084	0.009	0.156
PBDE-209	0[a]	-	-	-	-	3[a]	0.219	0.104	0.138	0.336	3[a]	0.976	1.33	0.201	2.51
∑PBDE[d]	**9**[b]	**5.57**	2.60	1.63	10.21	**15**[b]	**0.101**	0.057	0.028	0.257	**15**[b]	0.353	0.307	0.067	1.07
						Reported in Gabrielsen *et al.* (2011)									
						Maternal plasma (*n* = 15)					**Pup plasma (*n* = 15)**				
						n > LOD	Mean	SD	Min.	Max.	n > LOD	Mean	SD	Min.	Max.
4-OH-CB107	0[a]	-	-	-	-	**15**[b]	**1.16**	0.459	0.265	1.79	**15**[b]	0.577	0.213	0.115	0.848
3-OH-CB118	0[a]	-	-	-	-	0[a]	-	-	-	-	1[a]	0.011	-	0.011	0.011
4-OH-CB146	0[a]	-	-	-	-	**15**[b]	**0.040**	0.018	0.015	0.069	**15**[b]	0.019	0.010	0.000*	0.034
3'-OH-CB138	0[a]	-	-	-	-	**12**[b]	**0.013**	0.007	0.001	0.027	**10**[b]	0.013	0.008	0.001	0.027
4'-OH-CB172	0[a]	-	-	-	-	**15**[b]	**0.023**	0.014	0.007	0.057	**13**[b]	0.014	0.009	0.003	0.033
4-OH-CB187	0[a]	-	-	-	-	**15**[b]	**0.138**	0.082	0.043	0.289	**14**[b]	0.060	0.045	0.015	0.140
∑OH-PCB[e]	0[a]	-	-	-	-	**15**[b]	**1.38**	0.529	0.338	2.04	**15**[b]	0.684	0.266	0.141	1.06

a) OHC detected in < 60% of the individuals in each group are classified as not detected (n.d.) and current values are based on the numbers of individuals with levels > LOD.

b) OHC detected in ≥ 60% of the individuals in each group and missing values were given a random value between zero and LOD and included in mean and SD, and further statistics.

c) ∑PCB includes PCB-52, -101, -99, -110, -149, -118, -153, -141, -137, -138, -187, -183, -156, -180, -170, -194, -206 and -209 in mother plasma, while the additional PCB-189 was detected in pup plasma. In milk, the following were included: PCB-52, -74, -101, -99, -149, -118, -153, -105, -141, -137, -138, -187, -183, -128, -156, -180, -170, -194 and -206.

d) ∑PBDE includes PBDE-47, -99, -100, -153 and -154 in plasma pup and milk, while only PBDE-47, -153 and -154 were detected in maternal plasma.

e) ∑OH-PCB includes 4-OH-CB107, 4-OH-CB146, 3'-OH-CB138, 4'-OH-CB172 and 4-OH-CB187 in mother and pup plasma (3-OH-CB118, 4'-OH-CB108, 4'-OH-CB130, 4'-OH-CB159, 3'-OH-CB180 were n.d.). No OH-metabolites were detected in milk.

* Significant numbers not visible due to space.

3.3.1 PCBs

The lipid normalised concentrations (ng/g lw) of ∑PCB[3] were 558 (± 201 SD), 832 (± 315 SD) and 487 (± 219 SD) for maternal- and pup plasma, and milk, respectively (Fig. 3). The milk samples were available from 9 of 15 mothers, however there were no significant differences between the two sample sizes with respect to plasma contaminant values (t-test: all $p = 0.316 – 0.815$), and the sub set of 9 mother-pup pairs was considered representative for the larger sample pool. The mean ∑PCB level was 49% higher in plasma of pups compared to the maternal plasma (Tukey: $p < 0.02$), while the milk levels were 71% and 14% times lower compared to pups and mother plasma (Tukey: $p < 0.008$), respectively. There were no significant difference in ∑PCB between maternal plasma and milk (Tukey: $p = 0.78$), respectively.

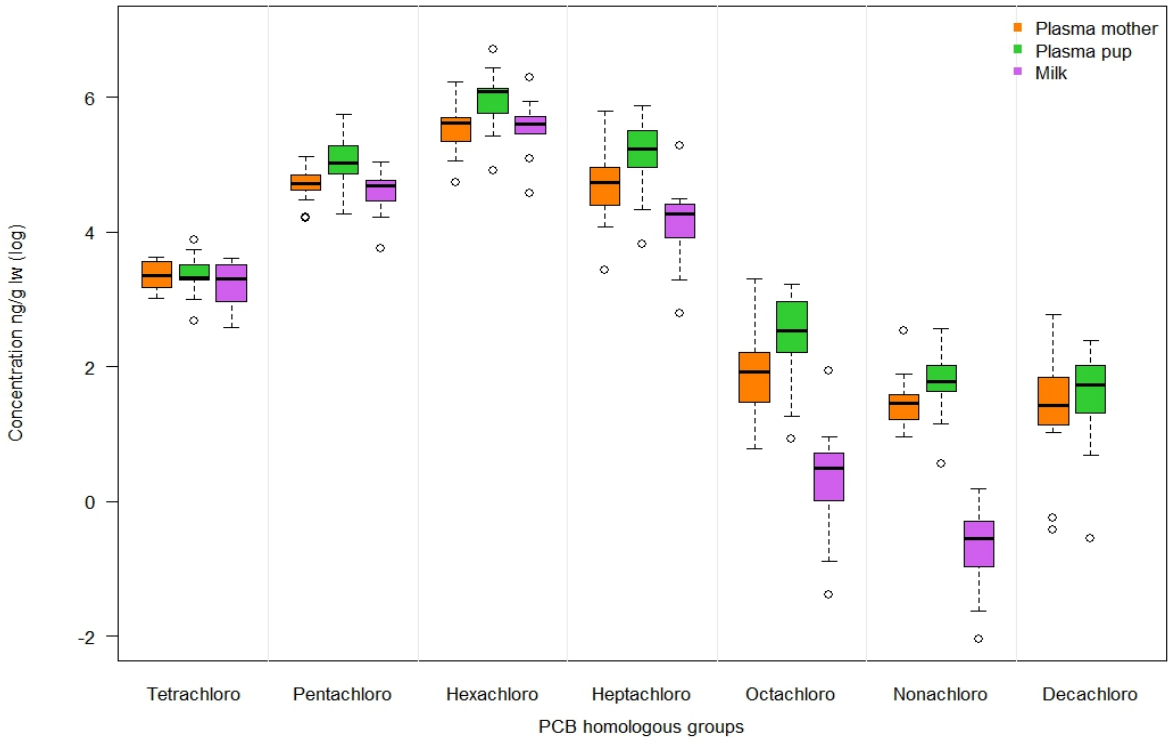

Figure 3. Log-transformed concentrations (ng/g lw) of the PCBs grouped in homologue groups based on the chlorination. In the decachloro group, milk was not detected. Sample size: $n = 15$ (plasma), $n = 9$ (milk).

[3] ∑PCB all three matrices include: PCB-52, PCB-101, PCB-99, PCB-149, PCB-118, PCB-153, PCB-141, PCB-137, PCB-138, PCB-187, PCB-183, PCB-156, PCB-180, PCB-170, PCB-194 and PCB-206. In addition PCB-74, PCB-105 and PCB-128 (milk). Additional PCB-110 and PCB-209 (maternal plasma) + PCB-189 (pup plasma).

The dominant PCB congeners were PCB-153, PCB-138 and PCB-180, with highest concentrations of PCB-153. Arranging the ∑PCBs into homologues group based on their chlorination demonstrated a decrease in milk concentrations with increasing chlorination (Fig. 4).

Relative to ∑PCB, highest level was observed in the hexachloro group and milk showed highest levels of the matrices in the group. The relative contribution of both the pentachloride and heptachloride group to ∑PCB were similar with a lower level observed in the latter group.

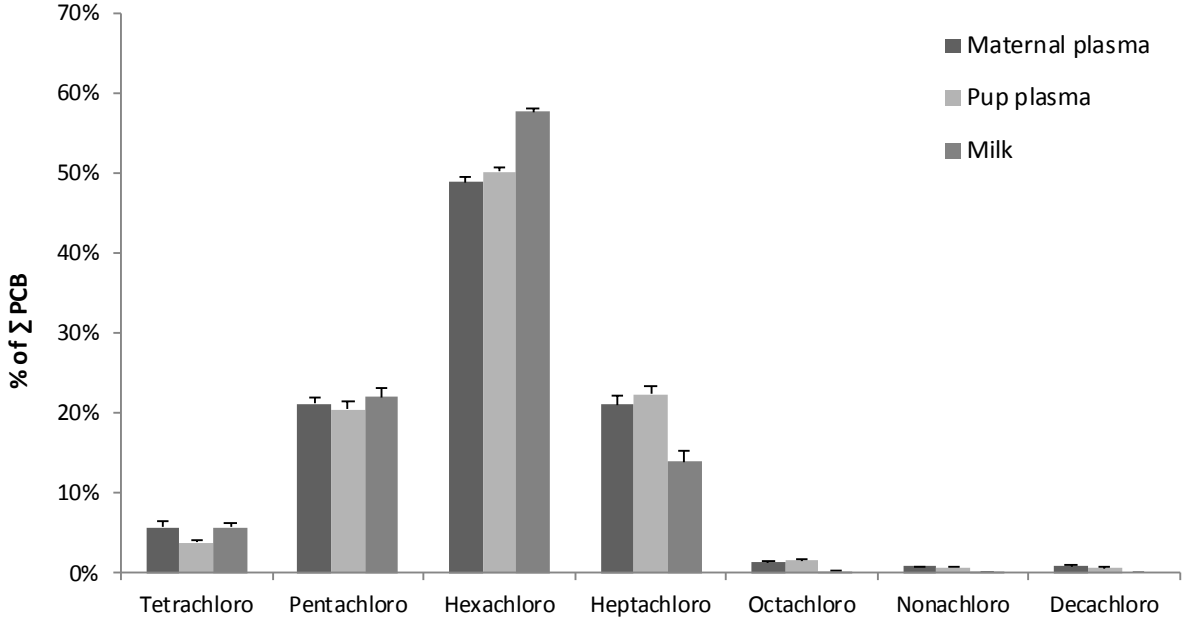

Figure 4. Proportions of the homolog PCB groups of hooded seal (*Cystophora cristata*) presented as values relative to the ∑PCB (column + error bars = mean + SE). Sample size *n* = 15 (plasma), *n* = 9 (milk). In the decachloro group, the milk levels was under LOD in less than 60% of the individuals and not shown here.

3.3.2 PBDEs

Five congeners were detected above LOD in more than 60% of the samples for each of the matrices, whereas for PBDE-99 and PBDE-100 the maternal plasma less than 60% of the samples with concentrations below LOD (these were noted at not detected in maternal plasma).

Highest milk concentrations were seen for PBDE-47, followed by PBDE-99 and PBDE-154 (Fig. 5). There were no differences between plasma- and milk concentrations for the tetra- and pentabrominated groups, whereas a significant difference was seen in the hexachlorinated group (Fig. 6).

No clear patterns were detected when comparing each congener to ∑PBDE (Fig. 6), whereas for the hexabromo group it seemed to be a similar pattern between PBDE-153 and -154. The relative contribution of the milk was high in the tetrabromo group and was decreasing with increased bromination.

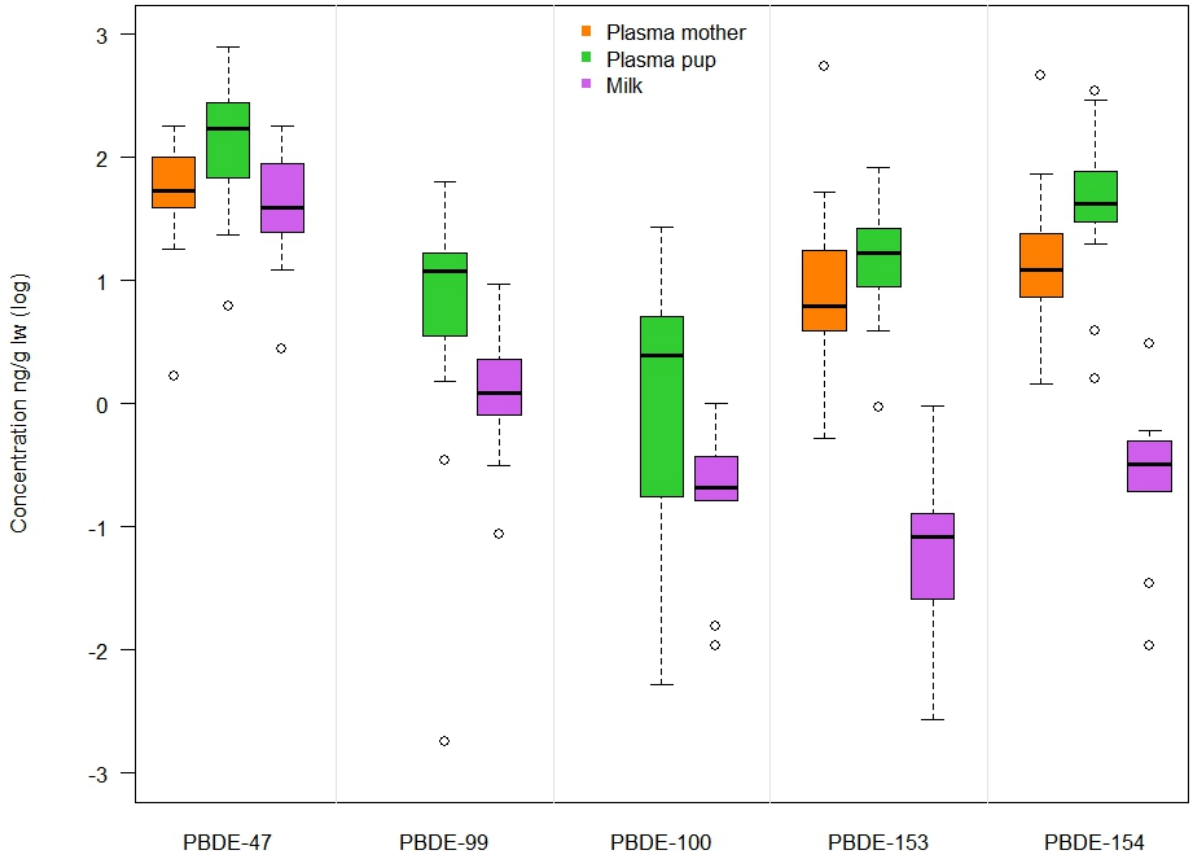

Figure 5. Log-transformed concentrations (ng/g lw) of the PBDEs detected in plasma and milk of hooded seal. PBDE-99 and -100 were not detected in maternal plasma.

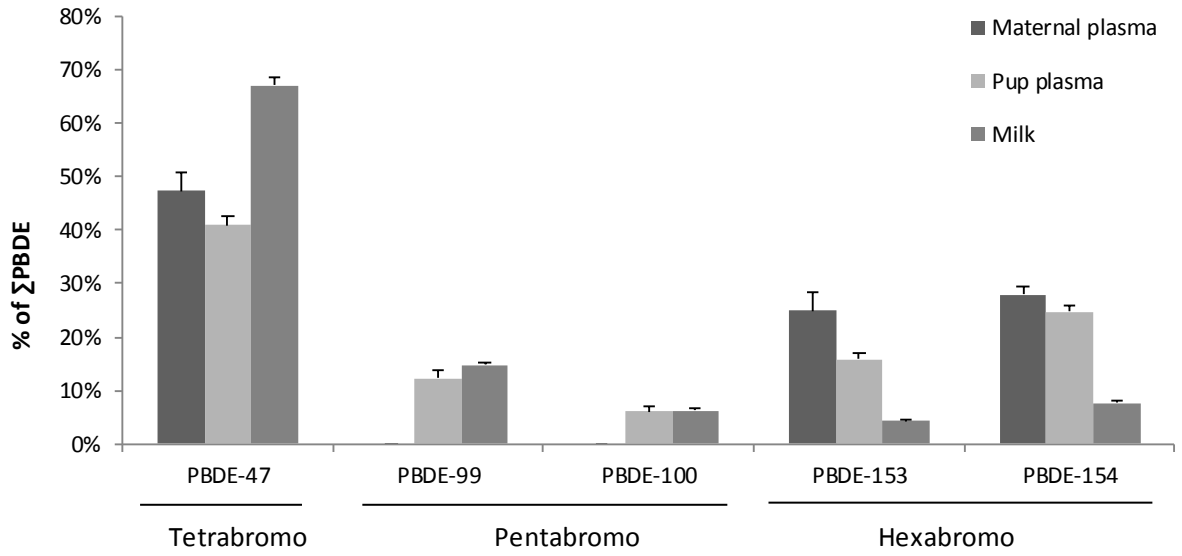

Figure 6. Maternal- and pup plasma (n = 15) and milk (n = 9) PBDE congeners from hooded seal are shown as a percentage of $_5\sum$PBDE. PBDE-99 and -100 were under detection in maternal plasma. Positive standard errors (SE) of means are present. The 5 congeners are grouped into three groups based on their bromo substitution.

3.3.3 Pesticides

Total pesticides (∑Pesticides) concentrations measured in milk were between 196 – 795 ng/g whereas maternal and pup plasma levels were 170 – 609 ng/g, 255 – 1086 ng/g, respectively. Among the 10 pesticides analysed, α-HCH and γ-HCH were not detected in milk, whereas α-HCH was detected in plasma of mother and pups. PCP was detected in milk of only one individual mother, while not detected in plasma of mother or pups. *p,p'*-DDE was the most abundant pesticide, followed by *trans*-Nonachlor.

The ∑Pesticides levels were significantly higher in pup plasma compared to maternal plasma (Tukey: $p < 0.001$), whereas for milk-plasma, there were no significant difference in mothers ($p = 0.159$) and pup ($p = 0.120$), respectively. The maternal plasma (Fig. 7) levels of *p,p'*-DDE, *p,p'*- DDT, α-HCH, oxychlordane and *trans*-Nonachlor were lower than in pup plasma (all $p < 0.04$). For the mothers, the *p,p'*- DDE levels were higher in milk than in plasma ($p < 0.02$) whereas the *p,p'*- DDT was lower in milk than plasma ($p < 0.001$). Oxychlordane and *trans*-Nonachlor levels did not vary between maternal plasma and milk ($p = 0.08$ and 0.30, for oxychlordane and *trans*-Nonachlor, respectively).

Mirex concentrations were higher in pup plasma compared to milk (Tukey: $p < 0.009$), whereas there were no difference between maternal plasma versus pup plasma or milk, respectively (Tukey: $p = 0.21$ - 0.24).

The octanol-water partition coefficient (log K_{OW}), a measure of lipid solubility where higher chlorination are the most lipophilic (Mackay & Fraser 2000), ranged from 3.78 to 6.96 for α-HCH and *p,p'*- DDE, respectively (Fig. 7). The chlordanes (oxychlordane and *trans*-Nonachlor) were both higher than the rest of the pesticides with regard to their expected position relative to the other pesticides based on their K_{OW}.

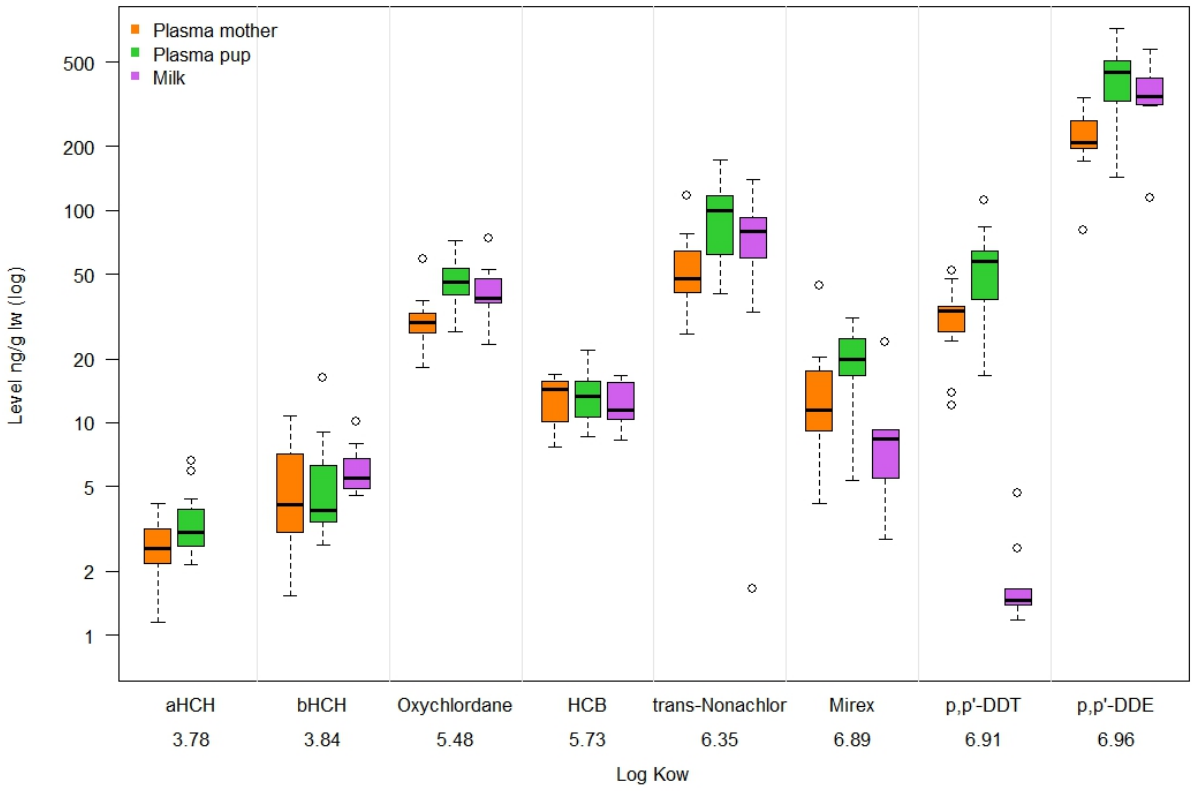

Figure 7. Pesticide concentrations (ng/g lw) in plasma and milk of hooded seal given in increasing order of octanol-water partition coefficient (log K_{OW}) here presented as factor variables. Sample size: n = 15 (plasma) and n = 9 (milk). The log K_{OW} values were reported in De Bruijn *et al.* (1989), whereas the chlordanes and Mirex were reported in U.S. National Library of Medicine (2015). Plasma levels from Villanger *et al.* (2013).

The pesticide pattern demonstrated highest contribution of p,p- DDE to ∑Pesticides, with highest contribution in milk (Fig. 8) and the relative contribution increases with log KOW, although dominated by DDE and the chlordanes.

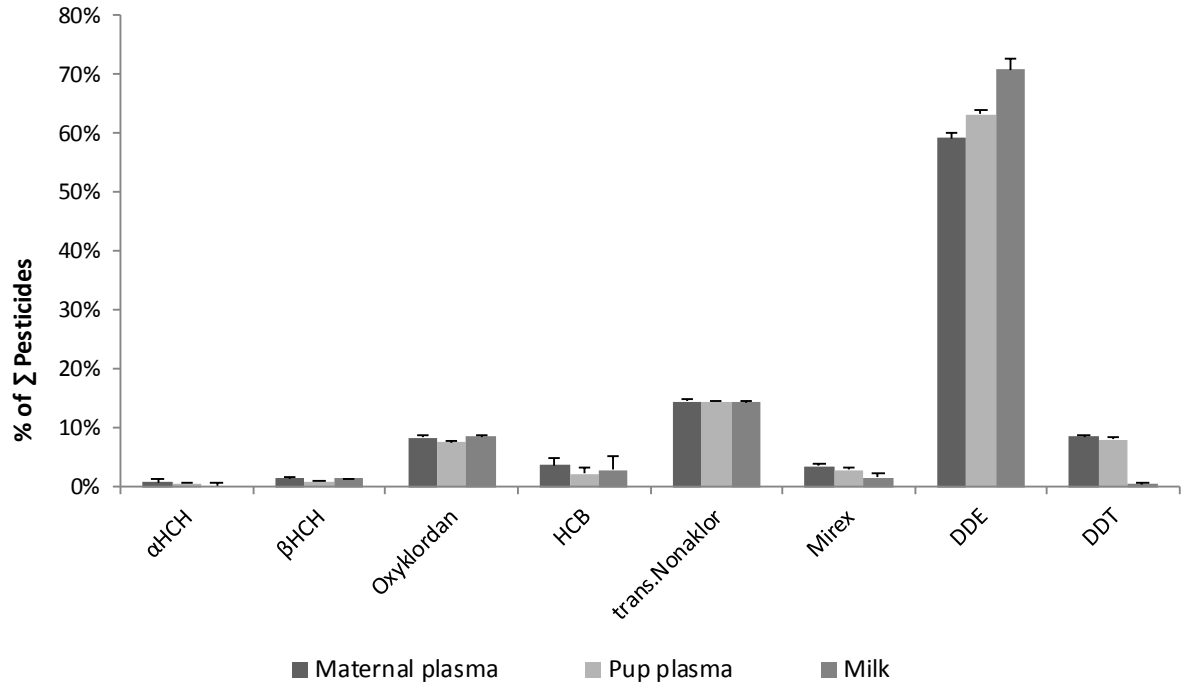

Figure 8. Proportions of the individual pesticides in hooded seal presented as values relative to ∑Pesticides (mean lipid weight + SE) given in increasing order (K_{OW}). Sample size: *n* = 15 (plasma), *n* = 9 (milk).

3.3.4 OH-PCBs

No OH-PCBs were detected in any of the milk samples of hooded seal (present study), whereas 4-OH-PCB107, 3'-OH-PCB138, 4-OH-PCB146, 4'-OH-PCB172, 4-OH-PCB187 were found in plasma (w.w.) of mothers and pups (Gabrielsen *et al.* 2011). When protein normalised, the OH-PCBs (Fig. 9) were higher in mothers for 4-OH-PCB107, 4'-OH-PCB172, 4-OH-PCB187 (t- test: all $p < 0.001$) and 4-OH-PCB146 (Wilcoxon signed rank test: $p < 0.001$). Protein normalised ∑OH-PCB levels were highest in maternal plasma (Welch's t-test: $p < 0.001$).

The OH-metabolites are metabolic products derived from one or multiple precursor PCB congeners presented in Table 9. As OH-PCBs were detected in plasma of both mother and pups in hooded seals (Gabrielsen *et al.* 2011) a correlation was performed between the respective protein normalised OH-PCBs and their possible lipid normalised precursors (Fig. 10).

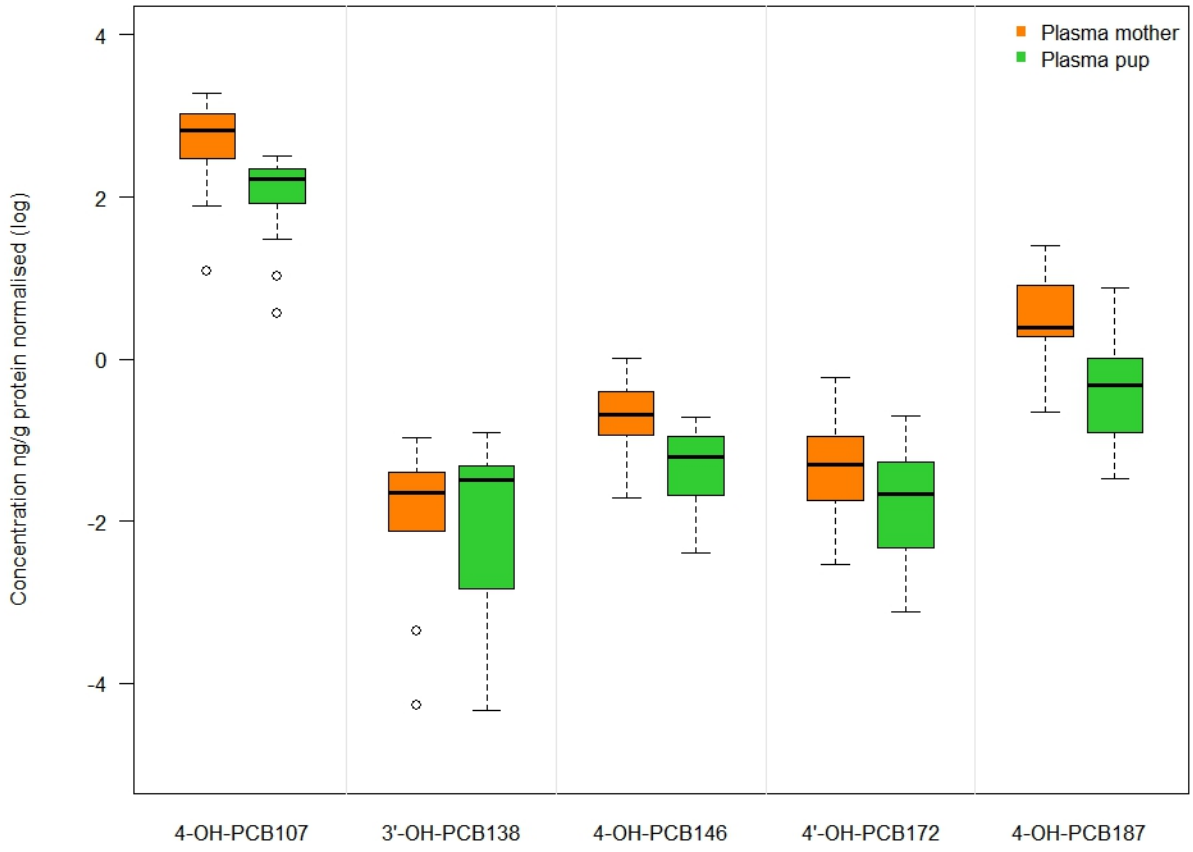

Figure 9. Log-transformed concentrations (ng/g protein normalised) of the 5 OH-PCB detected in plasma of 15 mother- pup pairs. Plasma data (w.w.) from Gabrielsen *et al*. (2011).

Significant negative correlations were found in maternal plasma between three of the metabolites and their suggested precursors: 4-OH-PCB146, 4-OH-PCB187 and 4'-OH-PCB172 (R_S: 0.78 – 0.81, $p < 0.003$). For the latter metabolite, a correlation was also found in pup plasma (R_S: 0.72, $p < 0.003$) (Fig. 8).

The respective lipid or protein normalised data resulted in stronger correlations between precursors and their metabolites (i.e. higher R_S), compared to non-normalised correlations on wet weight concentrations.

Table 9. OH-PCBs detected in hooded seal plasma (Gabrielsen *et al*. 2011) and their identified or suggested PCB precursor congeners (Bergman *et al*. 1994; Sjödin *et al*. 1998; Letcher *et al*. 2000; Sandau *et al*. 2000; Sjödin *et al*. 2000).

OH-PCBs	Precursor PCBs
4 -OH-PCB107	PCB-105*, PCB-107**, PCB-118
4 -OH-PCB146	PCB-138, PCB-146**, PCB-153
3'-OH-PCB138	PCB-138
4'-OH-PCB172	PCB-170
4 -OH-PCB187	PCB-183, PCB-187

* Only detected in milk.
** Not detected in this study.

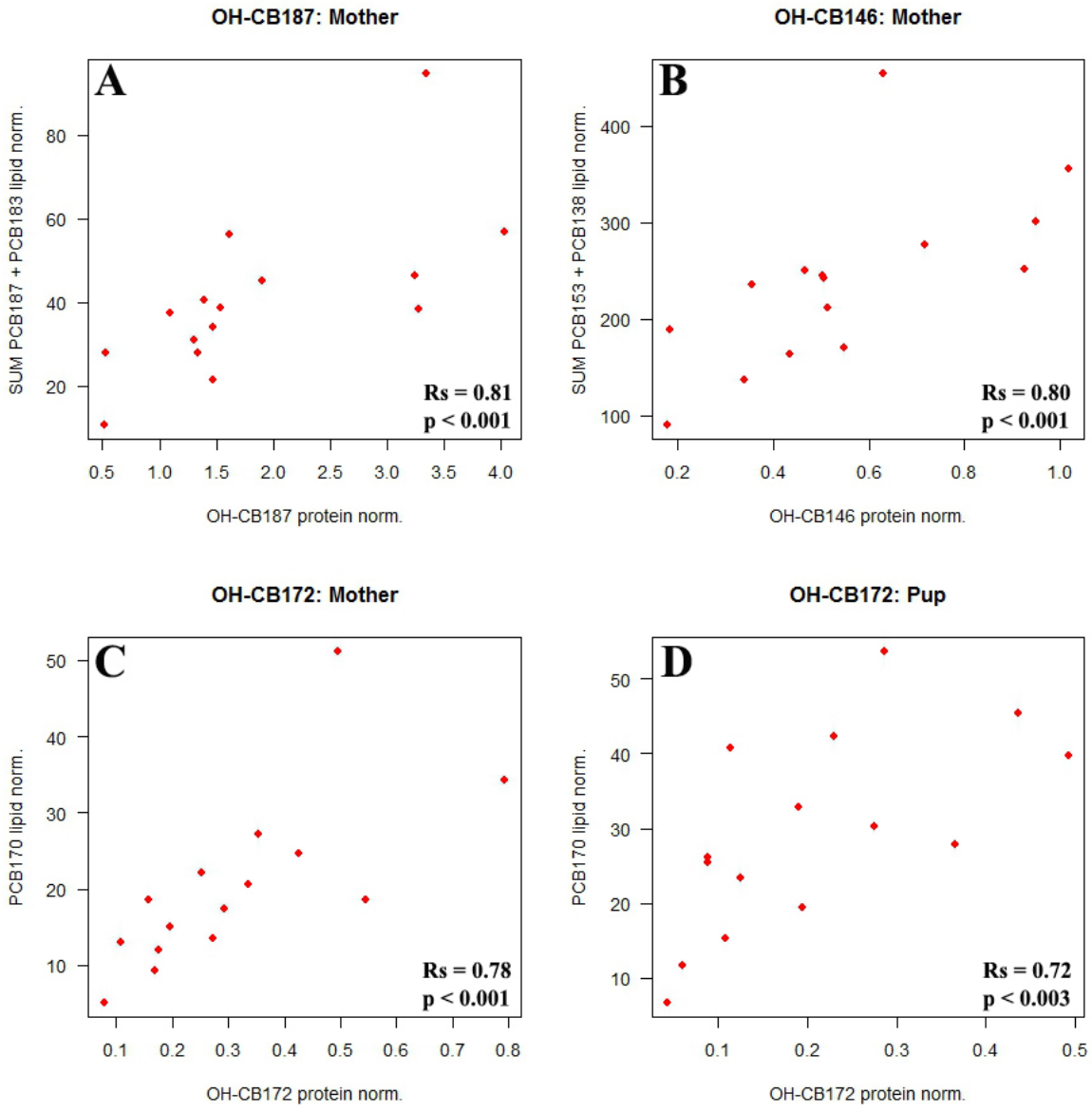

Figure 10. Significant OH-PCBs and their respective precursors found in plasma of hooded seal. **A)** Sum of PCB-183 and PCB-187 plotted against OH-PCB146, **B)** Sum of PCB-153 and PCB-138 plotted against OH-PCB146, **C-D)** PCB-170 plotted against OH-PCB172 in mothers (C) and pups (D). All precursors were lipid normalized whereas the OH-PCBs were protein normalized. Correlation (R_S) and significance levels are seen in the bottom, right corner. Plasma concentration data from Gabrielsen *et al*. (2011).

3.4 Maternal transfer to pup

All contaminants found in the mothers were also found in the pups. The maternal transfer was investigated by comparing the different matrices to see how the respective levels and patterns were distributed between milk and plasma, in pups and mothers. The PCBs and PBDEs were both grouped in homolog groups depending on their degree of halogenation.

By calculating the $plasma_{mother}$:milk ratio, the transfer from the maternal plasma into the milk was investigated for the various contaminants, whereas the $plasma_{pup}$:milk ratio describes how effective the transfer is from milk to the pup. The plasma ratios in pup:mother was used as a measure of maternal transfer efficiency. It difficult to discriminate the placental from the milk transfer in the present study as many of the POPs measured in pup plasma could have originated from both transport processes. However, evaluating compounds with these ratios we can highlight compounds with high pup:mother plasma ratios (i.e. high transfer efficiency), and for compounds where milk appear to be less significant the placental transfer might be the more important transfer route.

3.4.1 PCBs

Higher $plasma_{mother}$:milk ratios were seen with increasing chlorine substitution ranging from 5 to 9 chlorines (Fig. 11A). The ratio doubled from 7- to 8 chlorines and from 8 to 9 chlorines. With increasing chlorination there is also an increase in K_{OW} value. While only the 4 chlorinated PCB group were < 1, the others were > 1 and highest ratios were seen for the 8- and 9 chlorinated PCB groups. Highest variance for $plasma_{mother}$:milk ratios was seen in the 9 chlorinated group.

The $plasma_{pup}$:milk ratio (Fig. 11B) was quite similar to the $plasma_{mother}$:milk ratios, demonstrating a slightly higher ratio, whereas the pattern was similar. The ratio of both the 7 and 8 chlorinated homolog group in $plasma_{pup}$:milk were higher than in the $plasma_{mothers}$:milk.

The $plasma_{pup}$:$plasma_{mother}$ ratios (Fig. 11C) were all higher than 1 and the highest ratio was seen for the lowest chlorinated PCB homolog group, whereas the lowest was seen for the highest chlorinated PCB homolog group. Except this, no apparent patterns were found related to chlorination degree.

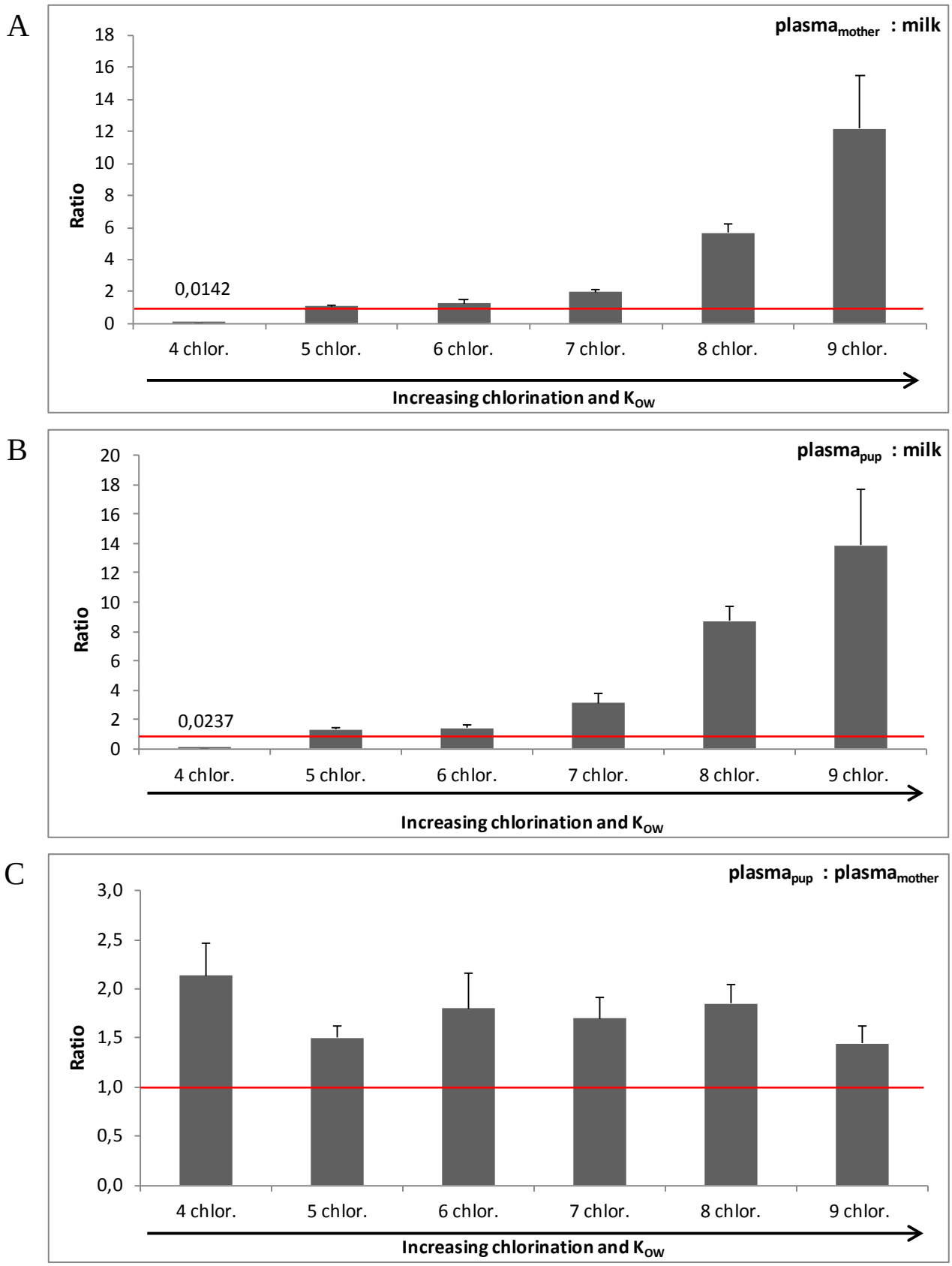

Figure 11. PCB ratios of A) plasma$_{mother}$:milk, B) plasma$_{pup}$:milk, C) plasma$_{pup}$:plasma$_{mother}$ of hooded seal presented as mean ratios with positive standard errors. Ratios are based on ng/g lipid normalized concentrations. The arrows indicate the increasing chlorine substitution of in PCBs. Each chlorine group consists of: 4 chlor. (PCB-52); 5 chlor. (PCB-101, -99, -118); 6 chlor. (PCB-149, -153, -141, -137, -138, -156); 7 chlor. (PCB-187, -183, -180, -170); 8 chlor. (PCB-194) and 9 chlor. (PCB-206). Samples size: *n* = 15 (plasma:plasma), *n* = 9 (plasma:milk).

3.4.2 PBDEs

As PBDE-99 and PBDE-100 were both under the limit for detection for more than 40% of the samples of maternal plasma, they were excluded from the statistics, but are here included for comparison reasons.

In the $plasma_{mother}$:milk, all ratios were over 1, implying higher concentrations in maternal plasma compared to milk (Fig. 12A). Lowest ratio was seen in the tetrabromo group, PBDE-47 and the highest was seen in PBDE-153. Both PBDE-99 and PBDE-100 were higher than PBDE-153, but a very high standard error were seen in both.

All congeners were above 1 in $plasma_{pup}$:milk (Fig. 12B). The highest ratio was seen in the hexabrominated group; PBDE-153 followed by PBDE-154, whereas the lowest ratio was observed in the lowest brominated group, PBDE-47. The pentabrominated group, consisting of PBDE-99 and -100 were both demonstrating similar ratios. The ratios in $plasma_{mother}$:milk were approximately 5 times lower than in $plasma_{pup}$:milk.

When comparing the plasma levels between pups and mothers ($plasma_{pup}$:$plasma_{mother}$), all congeners demonstrated high- and quite similar ratios: 1.8- 2 times higher ratios in pups than mothers (Fig. 12C). Lowest ratio was observed in the lowest brominated group, PBDE-47, and highest ratios were seen in the highest brominated group represented by PBDE-154.

The two excluded congeners PBDE-99 and -100 were both demonstrating ratios under 1 with the highest standard errors of the three PBDE homologue groups.

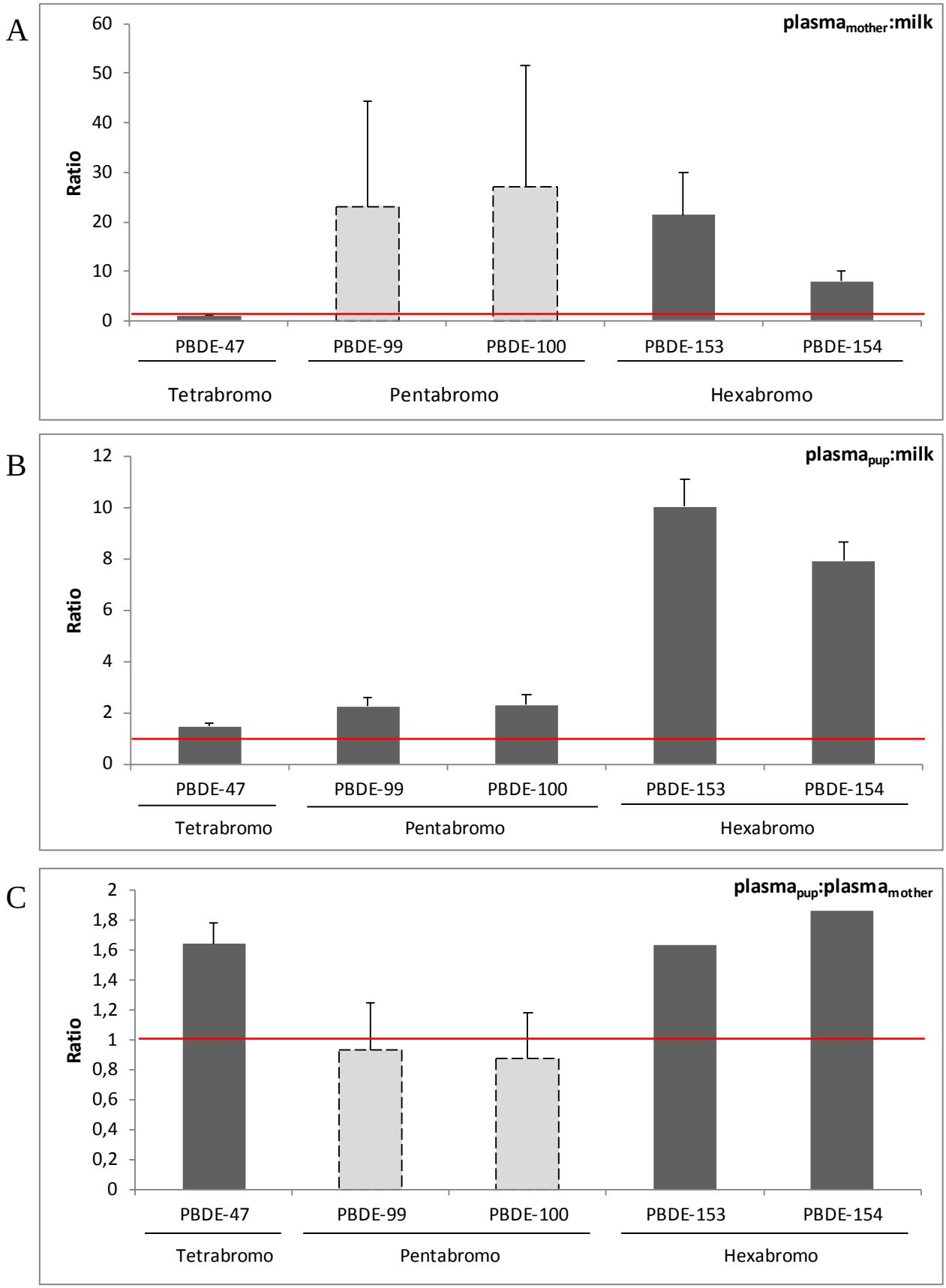

Figure 12. PBDE ratios of A) plasma$_{mother}$:milk, B) plasma$_{pup}$:milk, C) pup:mother plasma of hooded seal presented as mean ratios with standard errors. Ratios are based on ng/g lipid normalized concentrations. Plasma:plasma ratios are based on 15 observations, while plasma:milk are based on 9. The red line shows the 1:1 relationship between mother and pups. The two light grey bars (PBDE-99 and -100) in A) and C) were both taken out of statistics due to few observations, but are here included for comparison reasons.

3.4.3 Pesticides

In $plasma_{mother}$:milk, ratios > 1 were seen in three of the pesticides (Fig. 13A); *trans*-Nonachlor, Mirex and *p,p'*- DDT, with highest ratio in the latter pesticide; 6 times higher than for *trans*-Nonachlor. The ratio of HCB was 0.99, quite close to an equal relation between the two matrices.

The $plasma_{pup}$:milk ratios (Fig. 13B) were similar to the mothers, whereas the general ratios were slightly higher in the pups. Oxychlordane and *p,p'*- DDE ratios were > 1. Highest ratio was seen in *p,p'*- DDT, 1.4 times higher than in $plasma_{mother}$:milk. The pesticide α-HCH was not possible to include in the ratios for $plasma_{mother}$:milk and $plasma_{pup}$:milk, as it was not detected in milk.

For $plasma_{pup}$:$plasma_{mother}$ (Fig. 13C) all pesticide ratios were > 1. Lowest ratio was seen in HCB, whereas highest ratio was seen in DDE. Except the highly elevated chlordane ratios, there seemed to be a decrease in the ratios from α-HCH (K_{OW} = 3.78) up to HCB (K_{OW} = 5.73), followed by an increase up to *p,p'*- DDE (K_{OW} = 6.96).

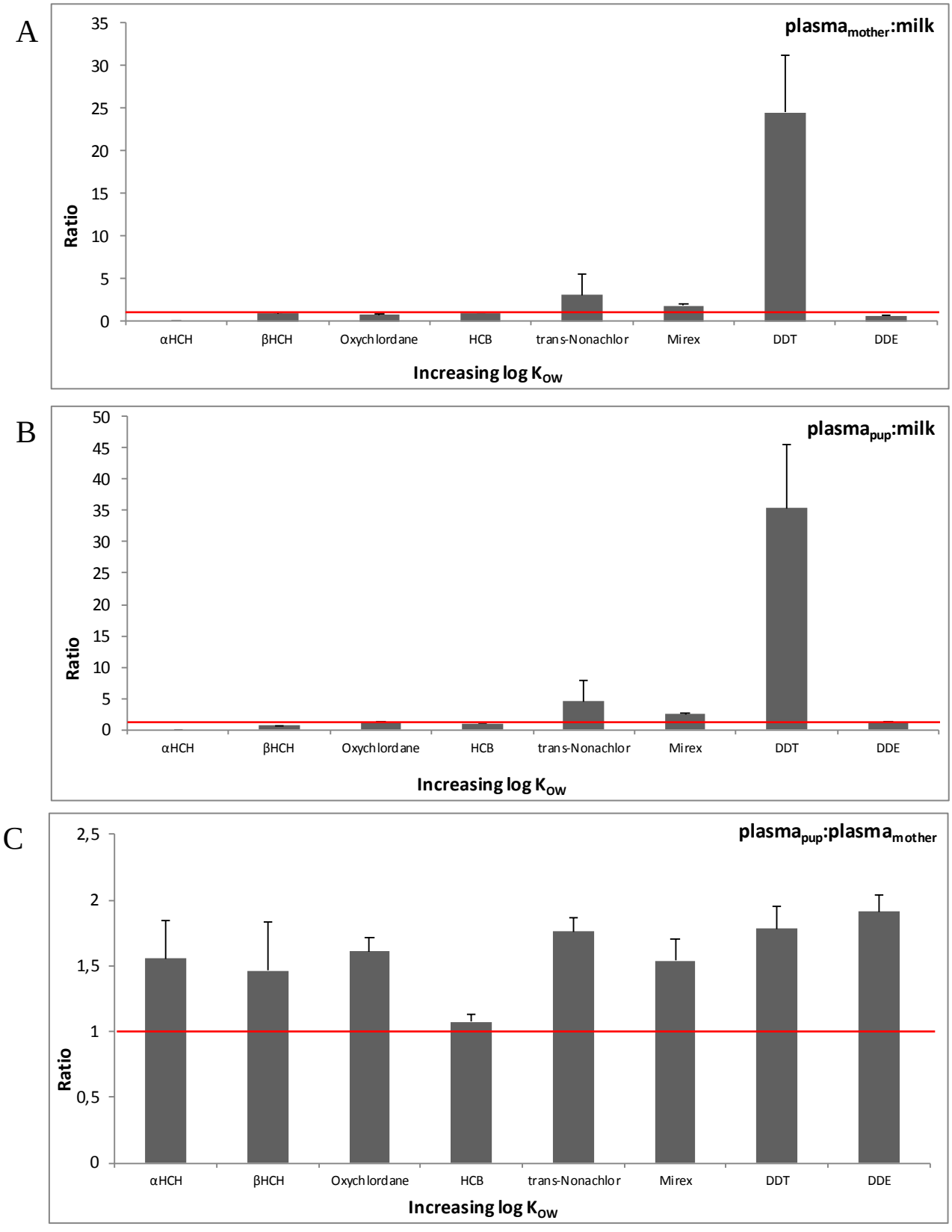

Figure 13. Ratios of A) pup:mother, B) plasma$_{pup}$:milk, C) plasma$_{mother}$:milk of hooded seal presented as mean ratios with standard errors. The pesticides are given in order of increasing K_{OW} (not shown). Ratios are based on ng/g lipid normalized concentrations. Sample size: plasma:plasma (n = 15), plasma:milk (n = 9). The red line shows the 1:1 relationship between mother and pups.

3.4.4 OH-PCBs

In mothers, higher levels of OH-PCBs were found compared to pups (Fig. 15). For all OH-PCBs, except 4-OH-PCB107 (R_S: 0.40, $p = 0.13$), correlation was found between mothers and pups (R_S: 0.61 – 0.88, $p < 0.02$). 3'-OH-PCB138 was the only metabolite where the levels were similar between mothers and pups (Fig. 15).

The OH-PCB/PCB plasma ratios in pups correlated with lactation duration in, i.e. the days the pup had been nursed (Fig. 16), except for OH-PCB172/PCB-170. This demonstrates an increase in OH-metabolites relative to their PCB precursors. No correlations were found for the mothers. Highest correlation in pups was seen for 4-OH-PCB107/PCB-118 (Linear regression: $r^2 = 0.43$, $p < 0.008$).

3.5 Biotransformation in pup

Endogenous biotransformation is regarded the major source of OH-metabolites in PCBs in marine mammals (Routti *et al.* 2008).

The PCBs can be divided into five metabolic groups in marine mammals (Fig. 14), based on their chlorine patterns (Boon *et al.* 1997) and have further support in previous studies (Wolkers *et al.* 1998b; Li *et al.* 2003). The two dominant OH-PCBs in plasma were 4-OH-PCB107 and 4-OH-PCB187 (Gabrielsen *et al.* 2011). PCB-105, PCB-107 and PCB-118 are suggested precursors for 4-OH-PCB107, whereas PCB-183 and PCB-187 are suggested for 4-OH-PCB187 (Letcher *et al.* 2000; Sjödin *et al.* 2000; Sandau *et al.* 2002).

PCB-105 and -118 in group III, show low relative contributions to PCB-153, whereas their metabolite (4-OH-PCB107) was the dominant metabolite.
Relative contribution of PCB-183 and -187 in group I were higher than for PCB-105 and -118, whereas their metabolite (4-OH-PCB187) were the second dominant metabolite.

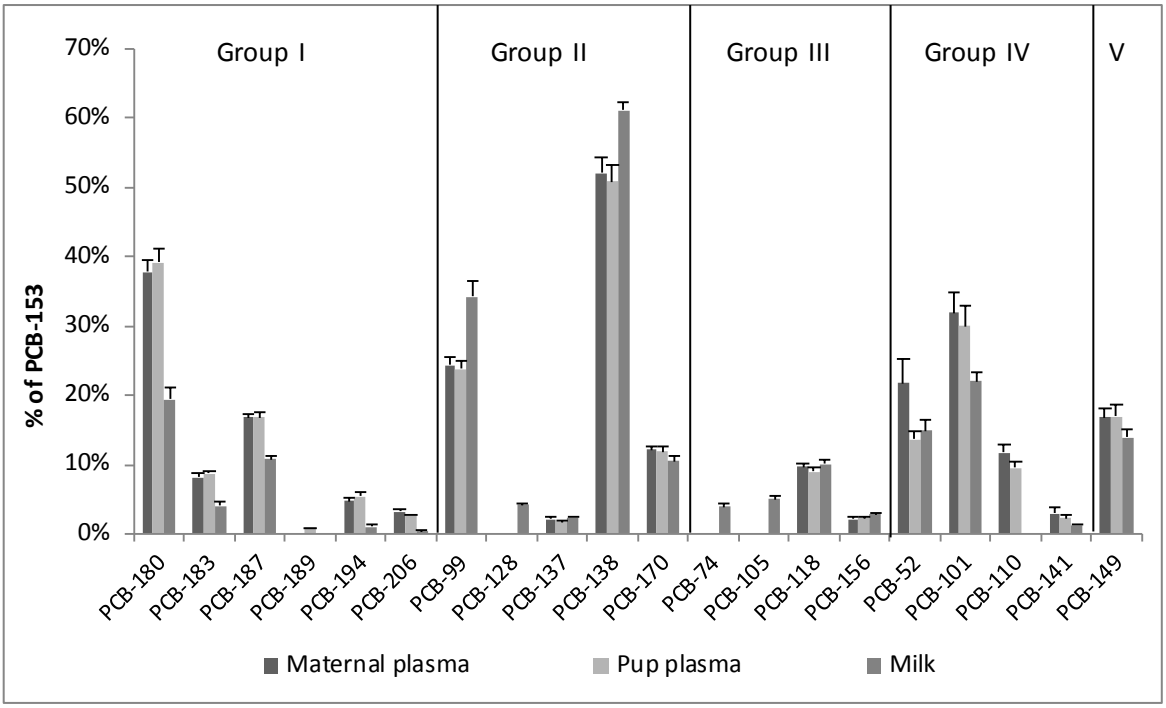

Figure 14. PCB pattern in three matrices in hooded seal presented as values relative to PCB-153 (mean + SE). Sample size: n = 15 (plasma) and n = 9 (milk).

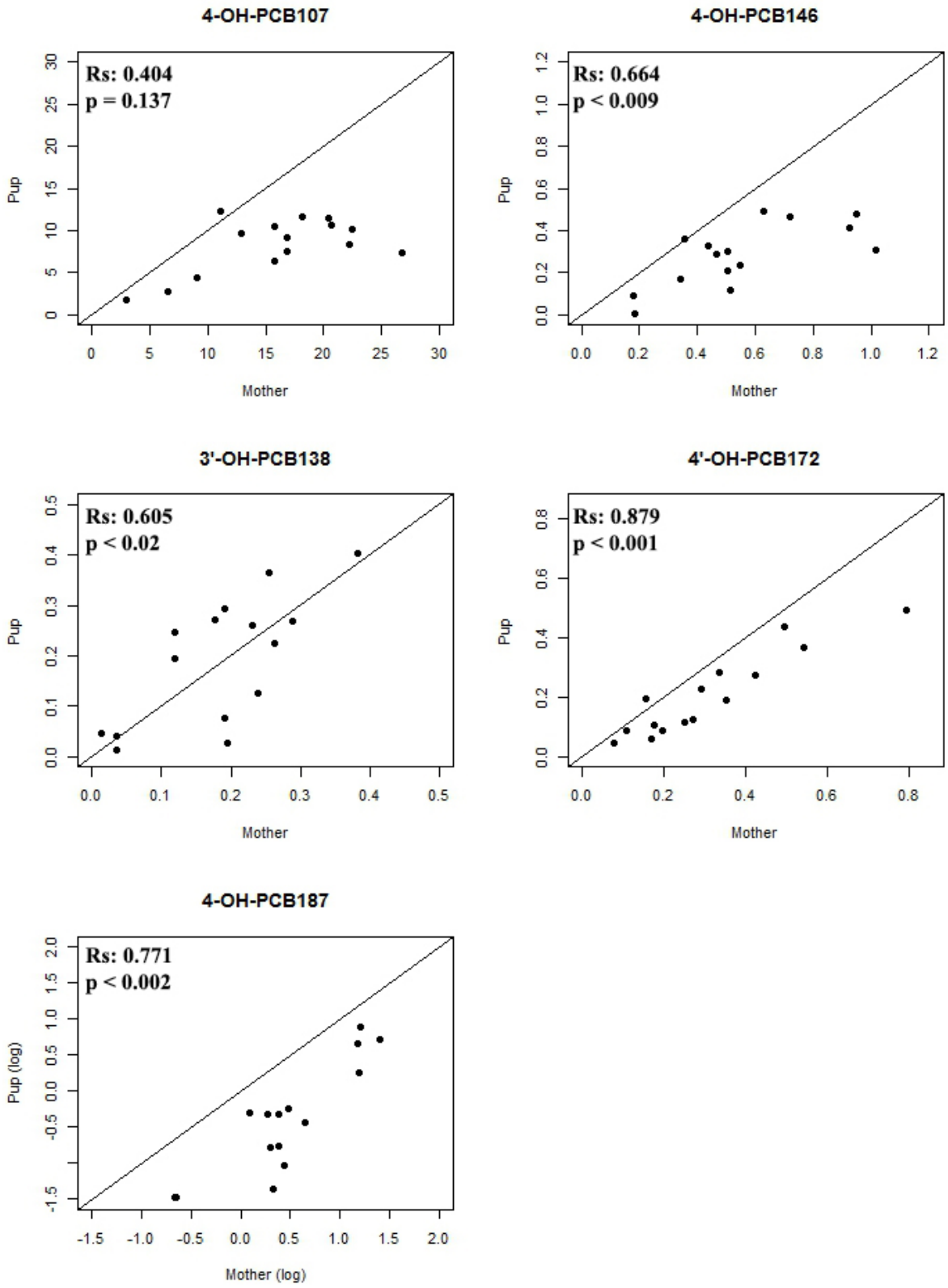

Figure 15. The 5 protein normalised OH-PCBs detected plotted between maternal and pup plasma. Both axes have equal values. OH-PCBs values for OH-PCB187 were log-transformed. Sample size: n = 15. Correlation (R_S) and significance level are shown. The diagonal line (0,1) show the 1:1 relationship between maternal and pup plasma. Plasma concentration data from Gabrielsen *et al.* (2011)

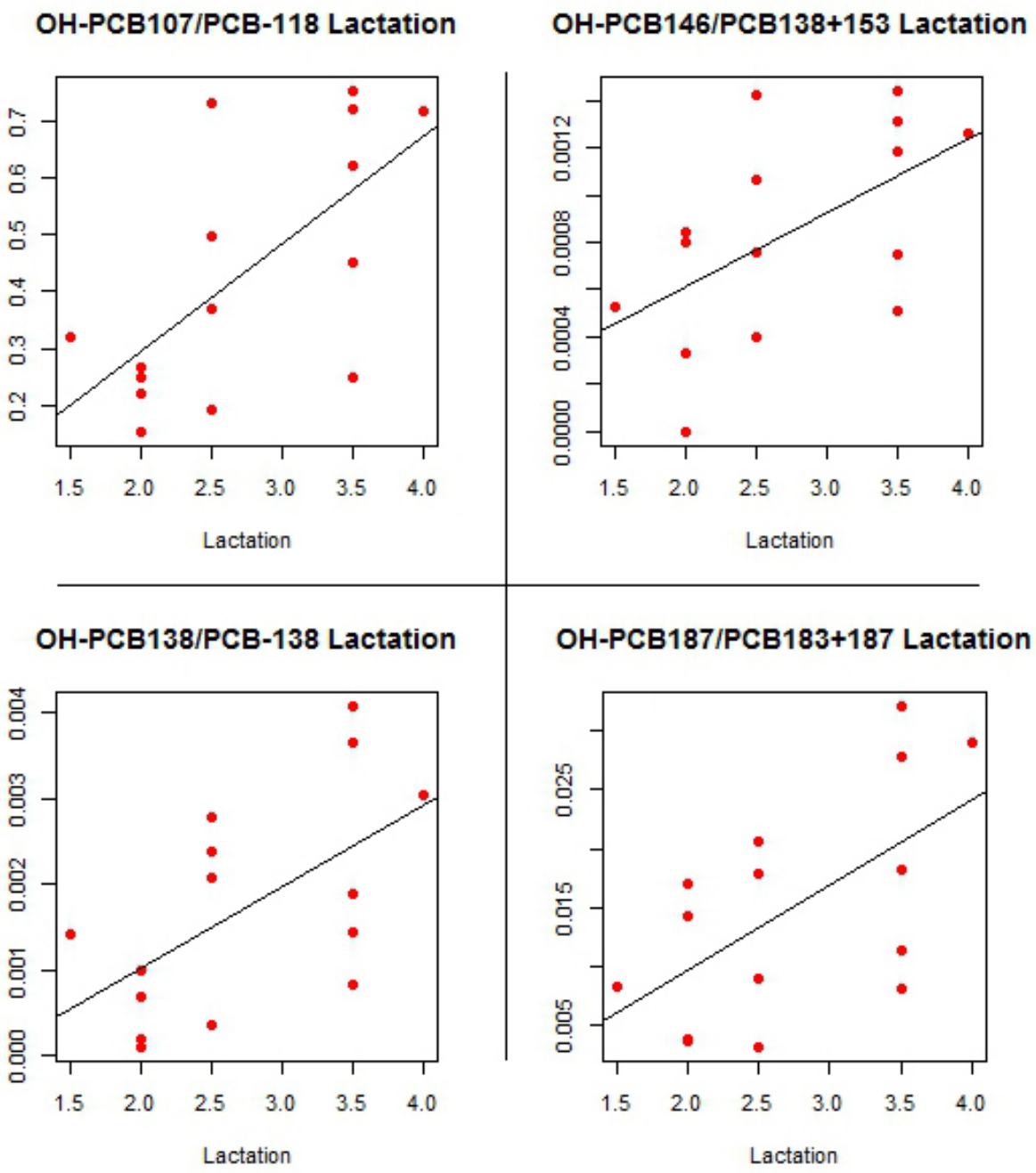

Figure 16. Significant protein normalised OH-PCB/ lipid normalised PCB ratios in pup plasma (n = 15) plotted versus lactation (mean lactation periode: 2.7 days), i.e. the number of days the pups have been nursed. Highest correlation was seen in OH-PCB107 (top left). The two right plots have both 2 precursors.

4 Discussion

Quantification of levels and patterns of brominated and chlorinated contaminants and their metabolites in milk and were performed with the purpose of investigating the importance of placental- and lactational transfer of contaminants from the mother to the pup in hooded seal. This study have for the first time analysed OH-metabolites of PCBs and PBDEs in milk of hooded seal from the "West Ice" in the Greenland Sea.

4.1 General levels and patterns

4.1.1 Contaminant levels in milk (lw)

Many studies have investigated the levels of PCBs, PBDEs and pesticides of arctic pinnipeds and cetaceans, whereas relatively few studies have analysed levels of contaminants- and especially OH- metabolites in milk.

The ∑PCB milk levels (lw) in the present study were similar to those found in harbour seals (*Phoca vitulina*) from Svalbard (Wolkers *et al.* 2004), whereas they were lower than reported for grey seals (*Halichoerus grypus*) from the Scottish Isle of May (Vanden Berghe *et al.* 2012) and higher than for harp seals (*Phoca groenlandica*) from the Canadian Gulf of St. Lawrence (Wolkers *et al.* 2002). The levels reported for ∑PBDE in grey seal milk in Vanden Berghe *et al.* (2012) were approximately one order of magnitude higher compared to this study, whereas the ∑PBDE levels reported in human milk from the Faroe Island (Fängström *et al.* 2004) were in the same range as reported in the present study. The pesticide levels in milk in the current study were lower than previously observed in grey- and harbour seals, whereas *p,p'*- DDE levels in the current study were approximately 4 times higher (Wolkers *et al.* 2002; Wolkers *et al.* 2004). The especially high fat percent in hooded seal milk (> 60%) compared to other seal species (Oftedal *et al.* 1988; Lydersen *et al.* 1997) might be part of the explanation for this.

The elevated PCB levels seen in grey seals compared to the hooded seals herein could partly be explained by the presence of this contaminant group in significant amounts in the northern United Kingdom (UK) environment, thereby accumulating in biota, whereas the higher levels in hooded seals compared to the harp seals might be because of the harp seal's short residence in the St. Lawrence estuary (Wolkers *et al.* 2002).

A possible explanation for difference in levels is partly due to their trophic position. Harbour- and hooded seals feed at a higher trophic level than grey- and harp seals (Bernt *et al.* 1999; Lesage *et al.* 2001) thereby increasing their potential contaminant levels as a result of biomagnification. In addition, also differences in metabolic capacity may influence the different levels found
(Wolkers *et al.* 2002). However, comparison between species are complicated because of differences in i.e. sampling size and which contaminants that are included in ∑Contaminant groups, thereby affecting the mean and variance between studies (Bernt *et al.* 1999).

The current study did not detect any OH- metabolites of either PCBs or PBDEs in milk of hooded seals. To my knowledge, only one study has previously reported levels of OH-PCBs in milk of pinnipeds: grey seals (29 ± 10 pg/g lw, only 4-OH-PCB107 detected) (Vanden Berghe *et al.* 2012) (LOD: 0.580 ng/g w.w. in current study), whereas three other studies have reported levels in human breast milk from Canada (Newsome & Davies 1996), Sweden (Guvenius *et al.* 2003) and the Faroe Islands (Fängström *et al.* 2004), and generally the levels appear to be low.
For OH-PBDEs, the current results corresponds well with the lack of or low levels reported in earlier studies of harbour- , ringed- and grey seals (Weijs *et al.* 2009; Routti *et al.* 2009a; Vanden Berghe *et al.* 2012).

4.1.2 Contaminant patterns

The PCB patterns in milk and plasma were dominated by pentachloro-, heptachloro- and heptachloro PCBs, mainly PCB-153, -138 and -180. A likely reason for this is that the persistent congeners, like PCB-153 (hexachloro), are more easily accumulated with age, whereas the less persistent are eliminated more easily from the organism (Ruus *et al.* 2002). PBDE-47 represented the major PBDE congener and *p,p'*- DDE was the dominant pesticide. Similar patterns are reported in seal species around the world (Espeland *et al.* 1997; Sørmo *et al.* 2003; Wolkers *et al.* 2006; Miranda Filho *et al.* 2009).
In general, the pesticide concentrations increased with increased log K_{OW} in milk and plasma, with the exception of the two chlordane congeners, *trans*-Nonachlor and Oxychlordane, which were both higher in both milk and plasma than expected based on their log K_{OW}. This pattern is different from previous reported in harp seals from the Gulf of St Lawrence with higher chlordane levels followed by *p,p'*- DDE (Wolkers *et al.* 2002).

The results demonstrated that OH-PCBs were below LOD in milk and hence the contribution of lactational transfer of OH-PCBs in explaining the OH-PCB levels reported in plasma of hooded seal pups (Gabrielsen *et al.* 2011) is small or insignificant. Thus, the OH-PCBs in levels reported in the pups are most likely due to prenatally exposure by placental transfer or by endogenous biotransformation of PCBs in the pups.

4.2 Maternal transfer to pup

4.2.1 Maternal transfer of contaminants

All the contaminants detected in maternal plasma were also found in the pup plasma indicating a maternal transfer from mother to pup. Maternal transfer is the only source of contaminant transfer to the pups, as the pups, during their lactation period and for another 4-5 weeks when they are weaned, spend all their time on the pack ice, fasting, without entering the water (Lydersen *et al.* 1997).

The results demonstrated in general a contaminant ratio > 1 between $plasma_{mother}$:milk, implying that contaminants are "retained" in the blood. The ratio increased with increasing log K_{OW} values for PCBs, PBDEs and pesticides. The lower log K_{OW} contaminants demonstrated ratios > 1 implying that milk is a poor vector of transfer (lower levels in milk than plasma) for these compounds. The $plasma_{pup}$:milk ratios is similar to $plasma_{mother}$:milk in PCBs, with a slightly higher $plasma_{pup}$:milk than $plasma_{mother}$:milk. Based on the higher $plasma_{pup}$:milk ratio for PCBs, the current study could suggest that the higher chlorinated PCBs in pups mainly originate from transplacental transfer rather than through milk, whereas a transfer from maternal plasma via milk to their pups might be a more important transfer route for the lower chlorinated PCBs.

Studies have reported contaminant transfer from females to pups to mainly occur through lactation (Gallenberg & Vodicnik 1987; Nakashima *et al.* 1997; You *et al.* 1999) where a selective transfer of especially the lower halogenated PCBs and PBDEs from maternal blubber into milk was favoured (Addison & Brodie 1987; Pomeroy *et al.* 1996; Wolkers *et al.* 2002; Wolkers *et al.* 2006) attributed by their lipophilic nature (Wolkers *et al.* 2004). This supports the current data. Also, PCB-74, -105 and -128 were all detected in milk only (not included in Fig. 11 ratios), supporting elimination through milk as an efficient transfer of

lower chlorinated contaminants to their pups. The lipophilic nature of POPs seemed driven by their log K_{OW} between mothers and pups (Frouin *et al.* 2012).

Considering the $plasma_{pup}$:$plasma_{mother}$ ratios, these were all quite similar and with ratios > 1, meaning that there is an equally efficient transfer of contaminants from mother to pups, regardless of log K_{OW}. When these results are combined with the plasma:milk ratios for pups and mothers, showing increasing ratios with log K_{OW}, this suggests that higher halogenated contaminants (i.e. more lipid soluble) are transferred predominantly placentally, and not via the milk from mother to pup in hooded seal.

The PBDE finding was similar to PCBs where higher ratios with increased halogenation were seen. PBDE-99 and -100 were both included in the results for comparison reasons and had higher ratios than PBDE-153 and -154. Having a very high variance though, their ratios should be considered with care.
There are only three halogenated groups in the PBDEs, less than for the PCBs represented by six halogenated groups, but the overall trend is decreased lactational transfer with increased halogenation.

The result provides evidence for placental transfer of higher halogenated PCBs, PBDEs and pesticides, and less for lactational transfer. Also, it has been shown that the higher halogenated contaminants are poorly transferred with the milk, favouring lactational transfer of low chlorinated contaminants.

4.2.2 Maternal transfer of the hydroxylated metabolites

In contrast to their precursors, the OH-PCBs are not bound to or dissolved in lipids, but will instead bind to serum or plasma proteins, e.g. albumin, whereas retention in blood might occur due to their affinity for transthyretin (TTR) (Malmberg *et al.* 2004).
Plasma levels of OH-PCBs from the same mother-pup pairs as used for analysing milk in the current study have previously been reported in Gabrielsen *et al.* (2011), suggesting a transfer of OH-PCBs after parturition via milk and to some degree by endogenous biotransformation of PCBs in the pups. This partly disproves with the current study where no hydroxylated metabolites of PCBs or PBDEs were detected in milk.

Rather than lactational transfer of OH-PCBs, placental transfer is a more likely route of excretion from mother and thereby exposure to the offspring (Sinjari & Darnerud 1998;

Meerts *et al.* 2002; Guvenius *et al.* 2003; Park *et al.* 2008; Gabrielsen *et al.* 2011) when the lowered ability of biotransformation in the new born pups are considered (Wolkers *et al.* 2009).

For the OH-PBDEs, previous studies have not detected metabolites in plasma of either mother or pup and as discussed in Villanger *et al.* (2013) and references therein, the OH-PBDE might not originate from endogenous metabolisation of PBDEs, but rather a dietary input from non-PBDE sources (Wiseman *et al.* 2011).
The PBDEs have been in the environment for a much shorter time than the PCBs, as the manufacturing was not initiated before the late 1970s, when the production of PCBs seized (Ross *et al.* 2009).
OH-PBDE levels have been reported as very low in previous studies. In Baltic and Svalbard ringed seals the concentration of the five major metabolites were close to the minimum level of quantification (Routti *et al.* 2009a), making it plausible that the non-detection OH-PBDEs in plasma of hooded seal mothers and pups (Villanger *et al.* 2013) was due to very low concentrations and thereby not getting detected as they fell under the LOD when measured.

The relationship in the pups between increasing protein normalised OH-PCBs and the lipid normalised PCBs over the 4 days of lactation (Fig. 16) either suggests a higher placental transfer rate or a greater metabolism in the foetus compared to the maternal compartment (Park *et al.* 2008). It is important to be aware that the measurement of plasma concentrations in the pups through the 4 days they are nursed does not represent the same individual sampled over multiple days, but multiple individuals calculated to their nearest age (half days).

4.3 Biotransformation in pup

The process of biotransformation is critical for levels and patterns of both contaminants and their hydroxylated metabolites in marine mammals.

In the following sections I have chosen to focus on the contaminant groups where metabolites have been analysed, namely PCBs and PBDEs, excluding the pesticides.

4.3.1 Biotransformation of PCBs

The $plasma_{pup}$:$plasma_{mother}$ levels (Fig. 11C) demonstrated higher PCB levels in pups compared to their mothers. It was therefore expected to find the same result when comparing OH-PCBs in mother and pup plasma as well. Instead, the opposite was found; OH-PCBs were higher in mothers compared to their pups (Fig. 15). The expectations of an equal pattern in mothers and pups might have been true if their chemical properties were the same, but as the PCBs are lipid soluble, they are capable of being transferred through milk. This is not the case for OH-PCBs which are protein associated and the current study has not found any metabolites of PCB in milk, meaning that the OH-metabolites found in pup plasma originate from placental transfer (Meerts *et al*. 2002), biotransformation, or more likely, a combination of both.

The current study found a weak increase in the ratio between metabolite and precursors (OH-PCB/PCB) and the lactational period (Fig. 16). This period equals the age of the pups, e.g. the days the pups have been nursed. The result presented in the figure demonstrates a ratio > 1 for normalised values of OH-PCB/PCB, indicating higher parental compound levels than OH-PCBs. A linear curve has been added, fitting the data points illustrating the trend. As this curve is increasing quite steep, the current study suggests a biotransformation for most of the 5 OH-PCBs detected in pup plasma; OH-PCB107, OH-PCB138, OH-PCB146 and OH-PCB187, although the rate of biotransformation is quite weak. It is important to be aware that the current data is not taken from one individual during multiple days, but are taken from multiple pups at one specific time. Previous studies have reported low CYP activity in neonates and will increase during the first months of life (Milsap & Jusko 1994), supporting the present findings.

Also, the congeners PCB-183 and -187 are grouped into the persistent group I where CYP activity is low due to a three dimensional structure inhibiting enzymatic activity (Fig. 14). This might explain the lower levels of 4-OH-PCB187 compared to 4-OH-PCB107. The latter metabolite is dominant in the plasma samples. Its two suggested precursors, PCB-105 and -118 have both lower relative contribution compared to PCB-153. Group III is metabolised by the CYP1A enzyme with affinity for co-planar PCBs.

4.3.2 Biotransformation of PBDEs

The current study did not detect OH-PBDEs in milk of hooded seals and previous studies in the same individuals have not detected levels in maternal or pup plasma either (Villanger *et al.* 2013). These findings are in accordance with earlier reported, low or not detected concentrations of OH-PBDEs in arctic fauna (Letcher *et al.* 2009; Routti *et al.* 2009a; Letcher *et al.* 2010). A slow biotransformation of PBDEs to OH-PBDEs has been reported in *in vitro* studies, whereas other studies have found no correlations between the same contaminants in marine organisms (Verreault *et al.* 2005; Wolkers *et al.* 2009; Wiseman *et al.* 2011).
A dietary exposure of OH-PBDEs from a non-PBDE source to the organisms are therefore suggested (Wiseman *et al.* 2011).

Endogenous biotransformation is regarded the major source of OH-metabolites in PCBs in marine mammals (Routti *et al.* 2008). Fish is the primarily diet of hooded seals, together with squid (Haug *et al.* 2007) having a very low capacity to metabolise PCB to OH-PCB (Murk *et al.* 1994).

5 Conclusions

The current study confirms a maternal transfer of PCBs, PBDEs and pesticides from hooded seal mothers to the pups via milk. Dominant parental congeners in milk detected in the present study were PCB-153, PBDE-47 and *p,p'* -DDE which were in accordance with previous studies of seals.

The study suggests a lower efficiency in maternal transfer to their pups of higher halogenated PCBs and PBDEs via milk, and for pesticides a similar pattern was observed for compounds with higher lipid affinity (high log K_{OW}). This coincides with previous studies showing that placental transfer of contaminants is more important for the higher halogenated POPs, whereas the milk functions as a more dominant vector of maternal transfer for the lower halogenated compounds. The current study has for the first time analysed OH-metabolites of PCBs and PBDEs in milk of hooded seals. These OH-metabolites were not detected in the present hooded seal milk samples, which suggest that placental transfer might be the major source of OH-PCBs that detected in pup plasma. However, a small increase in OH-PCBs relative to PCBs observed in the in the present hooded seals pups suggests a minor, but not neglectable, biotransformation activity in the pups.

For further analyses, umbilical cord plasma from neonates should be included in the study as it provides a direct indication of the *in utero* exposure to toxicants during development. If possible, a higher sample size should be attained.

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