# PBDEs, chlorinated POPs and their metabolites in mother and pup seals: influence of the lactation period, relationships with vitamin A status and potential impact on the immune system

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# Summary

Oceans are global sinks for persistent organic pollutants (POPs). Those lipophilic compounds tend to biomagnify in the marine trophic chain. As a result, top predators such as marine mammals accumulate huge levels of POPs over a long lifetime and are thus more vulnerable to POP-related health effects.

The aim of this thesis was to evaluate the lactational transfer of PCBs, PBDEs, OCPs and their metabolites in grey seal (*Halichoerus grypus*). Their dynamics were compared with those of vitamin A, a non-specific biomarker of effect for POP contamination. The possible interactions of those pollutants with the vitamin A status of the mothers and their pups were also examined. Mother-pup pairs were captured 2 to 3 times during the lactation period in order to collect maternal blubber, maternal serum, milk and pup serum for POP and vitamin A measurements.

POPs and vitamin A accumulated in maternal inner blubber throughout lactation, probably due to a higher affinity of those lipophilic molecules for the remaining triacylglycerols. Moreover, POPs appeared to be selectively mobilised from maternal inner blubber: lower halogenated molecules tended to be more efficiently mobilised from inner blubber to maternal serum. Additionally, we demonstrated that only low amounts of metabolites, HO-PCBs, were transferred via the milk. Interestingly, circulating HO-PCBs accumulated in both mothers and their pups at late lactation, suggesting among others, an increased biotransformation during this period.

Negative relationships between PCBs in milk and pup serum and circulating vitamin A in pups were revealed. By contrast, in mothers, POPs such as PCBs and PBDEs were positively correlated to vitamin A in both inner blubber and serum, suggesting an enhanced mobilisation hepatic vitamin A stores and redistribution in the inner blubber.

## Summary

Since vitamin A is essential for the immune system homeostasis, we attempted to assess the effects of this nutrient as well as environmentally relevant PCB and PBDE concentrations on the apoptosis, proliferation and cell cycle of mouse splenocytes and a harbour seal lymphoma B cell line. No modulation of the investigated parameters could be observed after PCB or PDBE treatment in the 2 models. Finally, we confirmed that vitamin A modulated the cell cycle machinery in LPS-induced mouse splenocytes but not in the harbour seal cell line.

# List of abbreviations

| | |
|---|---|
| AhR | Aryl hydrocarbon receptor |
| ANOVA | Analysis of variance |
| ARAT | Acyl CoA:retinol acyl transferase |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| ATRA | All-*trans* retinoic acid |
| BADP | Bisphenol-A diphenyl phosphate |
| BEHTBP or TBPH | Bis(2-ethylhexyl)-3,4,5,6-tetrabromo-phthalate |
| BFR | Brominated flame retardant |
| BHT | Butyl-4-methylphenol |
| BSEF | Bromine Science and Environmental Forum |
| BTBPE | 1,2-bis(2,4,6-tribromophenoxy)ethane |
| CAR | Consitutive Androstane Receptor |
| CCRP | CAR Cytoplasmic Retention Protein |
| CDV | Canine distemper virus |
| CHOL | Total cholesterol |
| Con A | Concanavalin A |
| CRBP | Cellular retinol binding-protein |
| CYPs | Cytochrome P450 enzymes |
| DAD | Diode-array detection |
| DBDPE | Decabromodiphenyl ethane |
| DCM | Dichloromethane |
| DDD | Dichlorodiphenyldichloroethane |
| DDE | Dichlorodiphenyldichloroethylene |
| DDT | Dichlorodiphenyltrichloroethane |
| DDXs | DDT isomers |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DRE | Dioxin-responsive elements |
| DTH | Delayed-type hypersensitivity |
| ECD | Electron capture detector |
| ECNI | Electron capture negative ion |
| EHTBB or TBB | 2-ethylhexyl-2,3,4,5-tetrabromobenzoate |
| EI | Electron ionisation |
| EPA | Environmental Protection Agency |
| EROD | Ethoxyresorufin-O-deethylase |
| EtOH | Ethanol |
| FBS | Fetal bovine serum |

## List of abbreviations

| | |
|---|---|
| GC-MS | Gas chromatography-mass spectrometry |
| GST | Glutathione-S-transferase |
| HCB | Hexachlorobenzene |
| HCDBCO | Hexachlorocyclopentadienyldibromo-cyclooctane |
| HCH | Hexachlorocyclohexane |
| HIV | Human immunodeficiency virus |
| HO | Hydroxylated metabolite |
| HPLC | High performance liquid chromatography |
| HSC | Hepatic stellate cell |
| HSP90 | Heat shock protein 90 |
| IG | Immunoglobulin |
| IL | Interleukine |
| IUPAC | International union of pure and applied chemistry |
| LMM | Linear mixed model |
| Log $K_{ow}$ | Octanol-water partition coefficient |
| LOQ | Limit of quantification |
| LPS | Lipopolysaccharide |
| LRAT | Lecithin:retinol acyltransferase |
| LW | Lipid weight |
| MeO-PBDE | Methoxylated polybrominated diphenyl ethers |
| $MeSO_2$ | Methyl sulfone metabolite |
| NEFA | Non-esterified fatty acids |
| NIST | National institute of standards and technology |
| NK | Natural killer |
| OCP | Organochlorine pesticide |
| OECD | Economic Co-Operation and Development |
| PBDE | Polybrominated diphenyl ethers |
| PBREM | Phenobarbital responsive enhancer module |
| PBS | Phosphate buffered saline |
| PCB | Polychlorinated biphenyls |
| PCDD | Polychlorinated dibenzodioxins |
| PCDF | Polychlorinated dibenzofurans |
| PHA | Phytohaemagglutinin |
| POP | Persitent organic pollutant |
| PPL | Phospholipid |
| pRB | retinoblastoma protein |
| PWM | Pokeweed mitogen |

## List of abbreviations

| | |
|---|---|
| PXR | Pregnan X receptor |
| RAL | Retinaldehyde |
| RALDH | Retinal dehydrogenases |
| RAR | Retinoic acid receptor |
| RBP | Retinol binding protein |
| RDP | Resorcinol-bis(diphenylphosphate) |
| RE | Retinyl ester |
| REACH | Registration, evaluation, authorisation and restriction of chemical substances |
| REH | Retinyl ester hydrolase |
| ReOH or ROL | Retinol |
| REP | Relative effect potency |
| ROLDH | Retinol dehydrogenases |
| ROS | Reactive oxygen species |
| RXR | Retinoid X receptor |
| SCOS | Special Committee on Seals |
| SIM | Selective Ion Monitoring |
| SPE | Solid Phase Extraction |
| SRBC | Sheep Red Blood Cell |
| $T_4$ | Thyroxine |
| TBBPA-DBPE | tetrabromobisphenol A-bis(2,3-dibromopropylether) |
| TCDD | 2,3,7,8-Tetrachlorodibenzodioxin |
| TEF | Toxic equivalency factor |
| TEQ | Toxic equivalent |
| TG | Triacylglycerol |
| TPA | 12-O-tetradecanoyl phorbol 13-acetate |
| TTR | Transthyretin |
| UDPGT | Uridine 5'-diphospho-glucuronosyltransferase |
| UNICEF | United Nation Children's fund |
| WHO | World Health Organization |
| WW | Wet weight |
| XAP2 | X-associated protein 2 |

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# Chapter 1

# General introduction

## 1.1. Persistent Organic Pollutants (POPs)

Persistent organic pollutants (POPs) are organic chemical substances that have the particularity to remain for many years in the environment, accumulate in fatty tissues of living organisms, biomagnify through the food chain and adversely affect human and wildlife health (Covaci *et al.*, 2008; Diamond and Harrad, 2009; Weijs *et al.*, 2010). Although some POPs such as dioxins may originate from natural sources, most of them result from human activity. POPs are still or were originally synthesised and used around the world because of their beneficial properties, such as their low flammability or their pesticide properties. Because POPs can be transported by air and water, they can be found far from where they were initially used and released (Jones and de Voogt, 1999), which makes international action indispensable. The Stockholm Convention on Persistent Organic Pollutants was adopted on 22 May 2001 and entered into force on 17 May 2004. Up to now, the Convention has been ratified by 151 countries. Yet, United States of America (USA) is not a Party of the Stockholm Convention. The objective of this Convention is to define the substances in question and restrict or eliminate their production and use. Nowadays, there are 21 chemicals targeted by the Stockholm Convention. Polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and some polybrominated diphenyl ethers (PBDEs), such as tetra-BDEs and penta-BDEs which are the main components of the commercial mixture of Penta-BDEs or hexa-BDEs and hepta-BDEs which are the main components of the commercial mixture of Octa-BDEs, are part of the targeted chemicals and are discussed individually in the following sections.

### 1.1.1. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) represent a family of 209 individual congeners (Ballschmiter and Zell, 1980; Borja *et al.*, 2005). They are made of two benzene rings carrying 1 to 10 chlorine (Cl) atoms that can occupy the *ortho* (2, 2', 6, 6'), *meta* (3, 3', 5, 5') and/or *para* (4, 4') position, as illustrated in Figure 1.1. PCBs have low water solubilities. In general, the water solubility decreases and lipophilicity increases with increasing number of chlorines in the PCB molecule. The lipophilicity of PCBs and other lipophilic pollutants can be characterised by the octanol-water partition coefficient (log $K_{ow}$) (Smith *et al.*, 1999). The log $K_{ow}$ indicates the partitioning of a chemical between water and octanol and is expressed as a ratio of the concentration of the chemical in octanol relative to that in water. The log $K_{ow}$ is related with aquatic solubility and bioaccumulation in organisms (Smith *et al.*, 1999). It increases with the size of a molecule (in the present case, with the number of chlorines) and its lipophilicity (Smith *et al.*, 1999). Log $K_{ow}$ of PCBs range from ~4 to ~8 (Hansen *et al.*, 1999).

Twelve congeners that are non *ortho*-substituted (CB-77, -81, -126, -169) or mono *ortho*-substituted (CB-105, -114, -118, -123, -156, -157, -167 and -189) adopt a relative coplanar structure and are considered as "dioxin-like" because of the similar structure and toxicity to polychlorinated dibenzo-*p*-dioxins (PCDD) (Crinnion *et al.*, 2011). As the highly toxic 2,3,7,8-tetrachlorinated dibenzo-*p*-dioxins (2,3,7,8-TCDD), these coplanar PCBs mediate their biological and toxic effets through the aryl hydrocarbon receptor (AhR), a cytosolic receptor protein present in most vertebrate tissues (Safe *et al.*, 1985; Van den Berg *et al.*, 2006). Therefore, their toxicity may be related to the toxicity of 2,3,7,8-TCDD. The toxic equivalent concept uses the relative effect potency (REP) determined for dioxin-like PCB congeners for producing toxic or biological effects relative to 2,3,7,8-TCDD. The total toxic equivalent (TEQ) is defined by the sum of the concentration of each compound multiplied by its toxic equivalency factor (TEF) value and is an estimate of the total 2,3,7,8-TCDD-like activity of the mixture (Van den Berg *et al.*, 2006). Other PCB

congeners are considered as “non dioxin-like” and, although they do not contribute to dioxin toxicity, they can have their own toxic effects (Crinnion *et al.*, 2011).

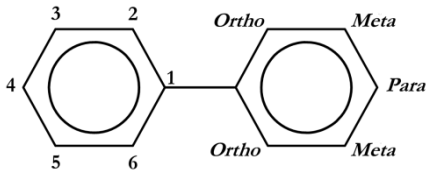


**Figure 1.1**. Chemical structure of PCBs.

Toxic effects of PCBs are numerous and include immuno- and neurotoxicity, teratogenicity, endocrine disruption, reproduction impairment and increase of the incidence of cancer in humans, laboratory animals and widlife (Borja *et al.*, 2005; Fonnum *et al.*, 2006; Ross and De Guise, 2007; Routti *et al.*, 2010; Crinnion *et al.*, 2011).

PCBs have been extensively manufactured and used since 1920s. Generally, they were manufactured as mixtures of several congeners under the names of Aroclor® (USA), Clophen® (Germany), Phenoclor® and Pyralene® (France), Fenchlor® (Italy), Sovol® (Russia), Chlorfen® (Poland) and Delor® (the former Czechoslovakia) (Borja *et al.*, 2005; Erickson and Kaley, 2011). PCBs were used as electrical insulating fluids in capacitors and transformers and also as hydraulic, heat transfer and lubricating fluids. They were also applicated as plasticisers and fire retardants and used in a range of products including caulks, adhesives, plastics, and carbonless copy paper (Erickson and Kaley, 2011). In 1977, due to the important concentration of PCBs in the environment and their association with severe health problems, Monsanto stopped their production in USA. However, in Europe and Asia, the manufacture continued into the 1980s and probably later. Today, the intentional manufacture of PCBs no longer occurs, except for the synthesis of small amounts for research purposes (Erickson and Kaley, 2011).

**Table 1.1**. European regulations and PCBs

| Legal basis | Content | Deadline |
|---|---|---|
| Directive 76/769* (27 July 1976) | • Ban of the use of PCBs in open application if > 1% | • January 1978 |
| Directive 85/467* (1st October 1985) | • Prohibition of the use of PCBs in certain closed systems | • July 1986 |
| Directive 89/677* (21 December 1989) | • Prohibition of the use and reuse of PCBs and any mixture containing them in > 0.005% by weight | • June 1991 |
| Directive 96/59/EC (16 September 1996) | • Disposal and decontamination of PCBs wastes and equipement with PCB volumes > 5 $dm^3$ | • December 2010 |
| Regulation 850/2004 (29 April 2004) | • Provisions regarding production, placing on the market, use, management of stockpiles and wastes and measures to reduce unintentional releases of POPs, including PCBs | • May 2004 |
| Regulation 199/2006 (3 February 2006) | • Maximum levels for certain contaminants in foodstuffs as regards dioxins and dioxin-like PCBs | • November 2006 |
| Regulation 596/2009 (18 June 2009) | • Amendment of Directive 96/59/EC (Art 10) → amendment of the regulatory procedure | • August 2009 |

* repealed by the REACh regulation

The first international governmental action took place in 1973 when the Organisation for Economic Co-Operation and Development (OECD) adopted a decision which recommended the use in new open products to be banned in OECD member contries. In 1987, OECD adopted a further decision on PCBs which recommended that member countries should cease the manufacture, import, export and sale of PCBs by 1 January 1989. In addition, PCBs are listed in Annex A of

the Stockholm Convention which aims at eliminating the production and use of all intentionally produced POPs. Besides, the Convention requires the signatory parties to take actions for phasing out the use of equipment containing PCBs by 2025 and the treatment and elimination of the recovered PCBs by 2028. At the European level, several Directives and Regulations have been published in order to limit the placing of PCBs on the market and to define methods of elimination. These European legislations regulating PCBs are presented in Table 1.1.

Although they have been banned for more than 3 decades and that their concentrations are declining slowly in Europe, North America, and the Arctic (Nizetto *et al.*, 2010), PCBs are still some of the principal POPs found in the environment.

While the expression and the regulation of enzymes involved in the biotransformation of PCBs vary among species and sometimes also between individuals, some generalisations can be made (Letcher *et al.*, 2000; James, 2001). The biotransformation of PCBs is a complex mechanism involving Phase I and Phase II enzymes. Phase I is catalysed by cytochrome P450 enzymes (CYPs) and consists in the oxidation of the molecule either by the direct insertion of hydroxy (HO)-group or via the formation of an epoxide that subsequently rearranges to a HO-group (Letcher *et al.*, 2000; James, 2001). Phase II consists in the conjugation of HO metabolites formed during Phase I with polar molecules such as glutathione, sulphates and glucuronic acid. Enzymes catalysing the reaction are the uridine 5'-diphospho-glucuronosyltransferase (UDPGT), gluthatione S-transferase (GST) and sulphotransferase (James, 2001).

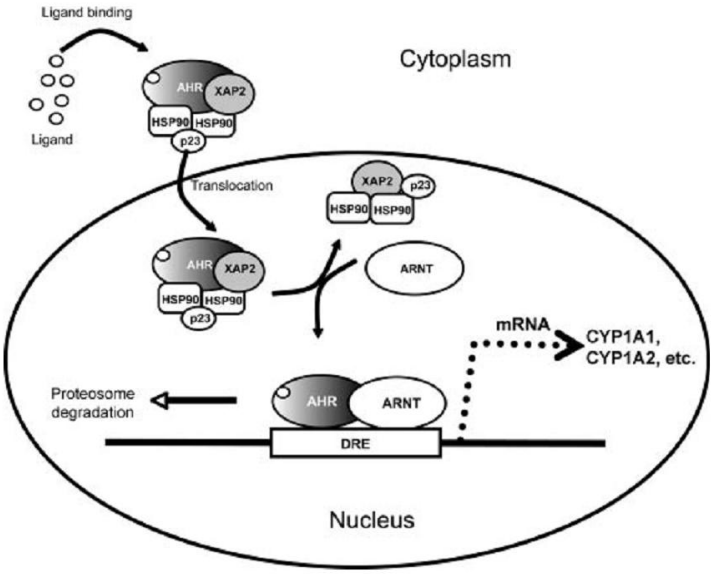


**Figure 1.2**. Model of ligand-mediated translocation of the AhR complex into the nucleus (Murray and Perdew, 2012).

The induction of a particular type of CYPs depends on the structure and substitution of PCB congeners. Non-*ortho* coplanar PCBs can induce the synthesis of CYP1A via the interaction with AhR and are considered as the most toxic congeners. Mono-*ortho* coplanar PCBs bind AhR with less affinity than non-*ortho* congeners but are also able to induce CYP1A as well as other CYPs (Letcher *et al.*, 2000). In the absence of ligand, AhR is maintained in the cytoplasm in a complex containing 2 molecules of the chaperone heat shock protein 90 (Hsp90) and a single molecule of each of the co-chaperones p23 and X-associated protein 2 (XAP2) (Murray and Perdew, 2012). Ligand binding to AhR leads to the translocation of AhR to the nucleus where AhR replaces HSP90 with the aryl hydrocarbon receptor nuclear translocator (ARNT), resulting in the formation of the ARNT/AhR heterodimer which binds to dioxin-responsive elements (DRE) of AhR battery genes activating transcription (Figure 1.2).

In contrast, non-coplanar PCBs have low affinity for AhR and induce preferentially CYP2B and CYP3A enzymes via interaction with the Consitutive Androstane Receptor (CAR) and the Pregnane X Receptor (PXR), respectively (Bandiera *et al.*, 2001). CAR and PXR are nuclear receptors that are activated by diverse endogenous (e.g. steroids) and exogenous ligands including drugs, environmental pollutants such as pesticides or non-dioxin-like PCBs and nutritional compounds (Timsit and Negishi, 2007; di Masi *et al.*, 2009). Their activation leads to the expression of CYPs and other proteins involved in the metabolism and excretion of xenobiotic and are therefore key receptors for the regulation of the metabolism and detoxification of chemicals in the organism (Timsit and Negishi, 2007; di Masi *et al.*, 2009). In the cytoplasm, CAR forms a protein complex with CAR Cytoplasmic Retention Protein (CCRP) and HSP90 (Figure 1.3). In contrast to AhR, its translocation into the nucleus does not require necessarily the direct binding of a ligand (di Masi *et al.*, 2009). In the nucleus, CAR forms a heterodimer with the retinoid X receptor (RXR) and binds to the phenobarbital responsive enhancer module (PBREM) which controls the expression of target genes of Phase I enzymes such as CYP2B and CYP3A and Phase II enzymes such as UDPGT and sulfotransferases (Klaassen and Hong Lu, 2010). Like CAR, PXR is sequestrated in the cytoplasm by CCRP and HSP90. However, the translocation of PXR into the nucleus requires a direct ligand binding (Timsit and Negishi, 2007; di Masi *et al.*, 2009). Once in the nucleus, PXR binds the PXR responsive element (PXRRE) as a heterodimer with RXR and activates the gene transcription of Phase I enzymes such as CYP2B, CYP2C, CYP3A and Phase II enzymes such as GST, UDPGT and sulfotransferases (Klaassen and Hong Lu, 2010). CAR and PXR share many DNA-binding sites and target genes as well as several ligands (Klaassen *et al.*, 2010).

The global biotransformation process generally results in the formation of polar metabolites, such as OH- and methyl sulfone ($MeSO_2$)-PCB metabolites that are more water soluble and thus easier to eliminate than the parent compounds.

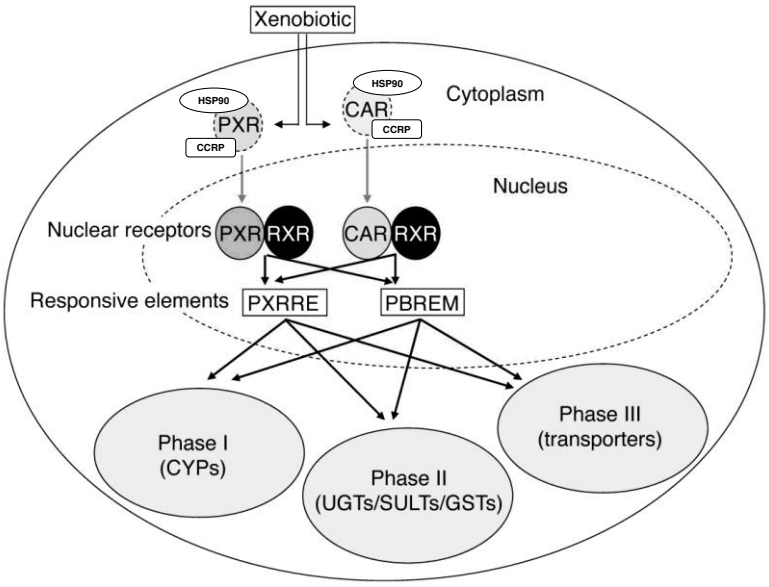


**Figure 1.3**. Illustration of the activation mechanisms of CAR and PXR (adapted from Scheer *et al.*, 2008).

However, due to their high affinity for blood proteins (Letcher *et al.*, 2000), some of these metabolites have been found to be retained in human (Dirtu *et al.*, 2010) and wildlife species (Weijs *et al.*, 2009a; Gabrielsen *et al.*, 2011). These HO- and $MeSO_2$-PCBs may have comparable or even higher toxic effects than their parent compounds in mammals, especially on thyroid and vitamin A metabolism (Meerts *et al.*, 2000 and 2002). Consideration of PCB biotransformation is therefore important in understanding the overall toxicity of PCBs (James, 2001).

### 1.1.2. Organochlorine pesticides (OCPs)

#### Dichlorodiphenyltrichloroethane (DDT)

Originally synthesised in 1874, the pesticide properties of dichlorodiphenyltrichloroethane (DDT) were discovered only in 1939

by Paul Muller who was awarded in 1948 with Nobel Prize in medicine (Maguire and Hardy, 2009). DDT was therefore extensively used to protect from disease-carrying insects (e.g., malaria, thypus) and commercialised as an agricultural pesticide from 1945. Although, from 1950s concerns had been raised regarding the accumulation of DDT in humans, wildlife and the environment, DDT remained the top-selling pesticide until the early 1960s (Maguire and Hardy, 2009). It is only after the publication of the Rachel Carson's book *Silent Spring* in 1962 describing the problematic of DDT in details that governments began to take action against DDT. In 1972, the Environmental Protection Agency (EPA) decided to ban all DDT use from USA and similar bans were instituted in most of the developed countries (Maguire and Hardy, 2009). The placing on the market or use of DDT has been restricted in Europe since 1981 (Directive 79/117/EEC) and they are listed in Annexe A of the Stockholm Convention. The production and use of DDT is however still authorised in some specific cases in Europe. For example, its production and utilisation as an intermediate product for the fabrication of dicofol, an acaricide, is allowed until 2014 (Regulation (EC) N° 850/2004). In addition, DDT is currently being produced in India to control malaria and leishmaniasis, in China for export and in the Democratic People's Republic of Korea for use in agriculture (van den Berg, 2009). It is still used extensively in tropical countries to prevent malaria transmission and has allowed protecting the lives of millions of people across the globe (Bouwman *et al.*, 2011).

The 2 main metabolites of DDT are dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE). They can be either impurities found in commercial DDT formulations, environmental degradation products or biotransformation products of DDT (Robert and Hutson 1999). The different DDT isomers are presented in Figure 1.4. DDE is the major DDT product and it has been demonstrated to be more persistent than DDT. DDE is generally found in human and animal tissues at the highest concentration (Robert and Hutson 1999; Maguire and Hardy, 2009).

The log $K_{ow}$ values of DDT and its isomers are comprised between 6.91 and 6.96 (De Bruijn *et al.*, 1989). In soils, following the action of

microorganisms including bacteria, fungi and algae, DDT is quickly biodegraded into DDD under anaerobic condition and very slowly in DDE under aerobic condition (Robert and Hutson 1999). In addition DDT can be converted in DDE and DDD following abiotic dehydrochlorination and photochemical decomposition (Robert and Hutson 1999).

Regarding DDT biotransformation, experiments on rats have reported that DDT may be converted into DDD by reductive dechlorination and into DDE by dehydrochlorination.

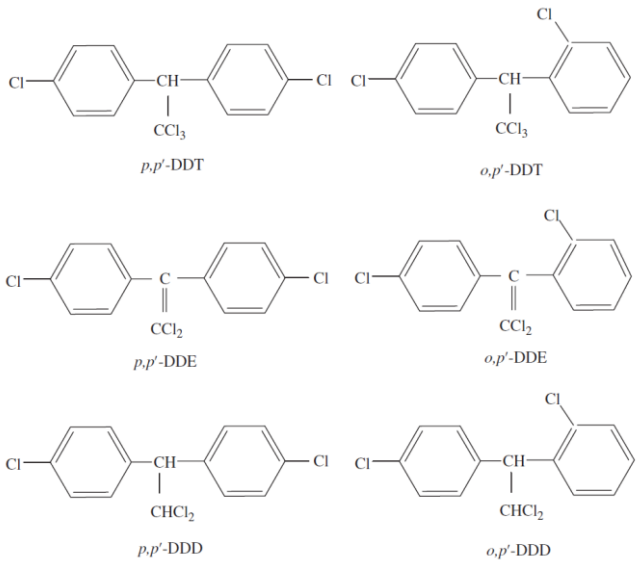


**Figure 1.4**. Chemical structures of DDT isomers (Wilson and Naidu, 2008).

An intermediate pathway converting DDD into DDE has also been demonstrated in rats (Fox *et al.*, 1998). DDT, DDE and DDD have been reported to be each potent inducer of hepatic CYP2B and CYP3A, but not CYP1A in rat hepatocytes (Nims *et al.*, 1998). Moreover, Kitamura *et al.* (2004) have demonstrated that the

reductive dechlorination of *p,p'*-DDT to *p,p'*-DDD in rat liver was catalyzed by CYP2B and CYP3A subfamily.

DDT and its metabolites are reported to have neurotoxic, carcinogenic, immunotoxic, endocrine and reproductive effects in humans, laboratory and wild animals (van den Berg, 2009).

## Hexachlorobenzene (HCB)

Hexachlorobenzene (HCB) (Figure 1.5) was first introduced in 1933 as a fungicide and was extensively used until the 1960s. Because of concerns about negative effects on human health and environment, the agricultural use of HCB was banned in the 1970s in most European countries and consequently, emissions of HCB fell sharply in the 1980s, followed by a steadier decline throughout the 1990s (Barber *et al.*, 2005). HCB was 1 of the 12 initial POPs listed under Annex A of the Stockhlom Convention.

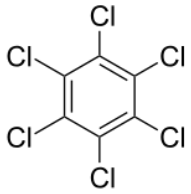


**Figure 1.5**. Chemical structure of hexachlorobenzene.

Nevertheless, HCB continues to be produced as a by-product during the manufacture of chlorinated organic chemicals (Barber *et al.*, 2005; Reed *et al.*, 2007). HCB is slightly soluble in water and has a log $K_{ow}$ value of ~5 (Reed *et al.*, 2007).

HCB is slowly metabolised and most is excreted in feces as the parent compound, with a small fraction being excreted in the urine as hydrophile metabolites, such as pentachlorothiophenol (PCBT) (Reed *et al.*, 2007). Although it has been suggested that HCB exerts its toxicity via AhR, it has not been certain whether the interaction with

AhR was due to HCB itself or caused by impurities or contamination with PCDD and/or polychlorinated dibenzofurans (PCDF) (Van den Berg *et al.*, 2006). Nevetheless, a recent study has reported that highly purified HCB weakly induced both ethoxyresorufin-O-deethylase (EROD) activity and CYP1A4/5 mRNA expression in primary cultures of chicken embryo hepatocytes, two biomarkers of AhR activation (Mundy *et al.*, 2010). HCB has been reported to induce reproduction and development impairments, endocrine disruption, immune system modulation, neurologic effects and carcinogenicity (Reed *et al.*, 2007).

### 1.1.3. Polybrominated diphenyl ethers (PBDEs)

Polybrominated diphenyl ethers (PBDEs) belong to the class of brominated flame retardants (BFRs). As PCBs, PBDEs consist of a familiy of 209 different congeners. Their chemical structure, also similar to the one of PCBs, consists of 2 benzene rings linked with an oxygen atom (Figure 1.6). Each benzene can carry 1 to 10 bromine (Br) atoms in the *ortho*, *meta* and/or *para* position. PBDEs are lipophilic substances of which the log $K_{ow}$ increases with increasing bromine content and ranges from ~6 to ~10 (de Boer *et al.*, 2000).

PBDEs have been manufactured as 3 mixtures known as Penta-, Octa- and Deca-BDEs (Costa *et al.*, 2008). Penta-BDE mixtures such as DE-71 and Bromkal 70-5DE contain 5 major congeners (BDE-99 > -47 > -100 > -153 > -154 > -85) which are also those detected the most abundantly in human and animal tissues as well as in the environment. The major congeners detected in Octa-BDE mixtures are BDE-183, -197 and -209. BDE-209 is the main congener in deca-formulations (La Guardia *et al.*, 2006). Deca-mixtures have been the most produced and used formulation (Law, 2009). BDE-209 may degrade to lower brominated PBDEs that are more persistent, bioaccumulative and toxic following photolysis, thermal degradation and biotransformation (Costa *et al.*, 2008; Eljarrat *et al.*, 2011).

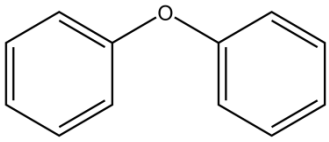

**Figure 1.6**. Chemical structure of PBDEs.

PBDEs have been introduced in the late 1970s as flame retardants in wire and cable insulation, electronics and computers as well as in upholstery. Since PBDEs are simply mixed with the material during its formation, they may migrate from products more readily than BFRs incorporated reactively (Costa *et al.*, 2008; Law, 2009).

Due to their accumulation in the environment and in organisms, the placing on the market and use of penta-BDE and octa-BDE mixtures has been strongly restricted in the European Union since 2004 (Table 1.2). Additionnaly, since May 2009, Penta- and Octa-BDE mixtures have been added to Annex A of the Stockholm Convention which requires the elimination of the production and use of all intentionally produced POPs in all signatory countries. In Europe, Deca-BDE can no longer be used in electronics and electrical applications since 2008 (Table 1.2). Besides, Deca-BDE is currently under evaluation phase regarding the Regulation (EC) n°1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). In USA, companies which are members of the Bromine Science and Environmental Forum (BSEF) have voluntarily phased out the production and use of the Penta-BDE and Octa-BDE mixtures since 2004 (BSEF, www.bsef.com). Ten states have adopted legislation banning Penta-BDE and Octa-BDE mixtures (California, Hawaii, Illinois, Maine, Maryland, Michigan, New York, Oregon, Rhode Island and Washington). Moreover, BSEF incites its members to ban the production and use of Deca-BDE by 2012. To date, only Maine, Oregon and Washington have enacted limited bans on deca-BDE mixture. Vermont has banned only deca-BDE mixture (BSEF, www.bsef.com). Regarding Asia, China published a legislation on waste of electrical and electronic equipment (similar to the WEEE

Directive) in February 2009. The legislation came into effect in January 2011 (BSEF, www.bsef.com).

Although severe restrictions have been taken by goverments, products containing PBDEs are still in use everywhere and their release into the environment will continue throughout product lifecycle, possibly for several more decades. Besides, alternate BFR compounds are introduced to respect consumer product fire safety regulations and include decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy)-ethane (BTBPE), 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB or EHTBB), bis(2-ethylhexyl)-3,4,5,6-tetrabromophthalate (TBPH or BEHTBP), tetrabromobisphenol A-bis(2,3-dibromopropylether) (TBBPA-DBPE) and hexachlorocyclopentadienyldibromo-cyclooctane (HCDBCO) (Covaci *et al.*, 2011; Wu *et al.*, 2012). Since these alternatives share physico-chemical properties comparable to those of the Penta- and Octa-BDE mixtures and that most are simply mixed with the material and not chemically bound, environmental fates similar to PBDEs may be expected (Wu *et al.*, 2012). Substitutes for BFRs include phosphorus flame retardants such as resorcinol-bis(diphenylphosphate) (RDP) or bisphenol-A diphenyl phosphate (BADP) which are considered safer than Deca-BDE by EPA (van der Veen and de Boer, 2012).

Numerous evidences suggested that PBDEs are metabolised in mammals. HO-PBDEs have been detected in human blood (Qiu *et al.*, 2009), tissues or plasma of several wildlife mammal species (McKinney *et al.*, 2006a; Letcher *et al.*, 2009; Routti *et al.*, 2009) and some PBDE conjugates were detected in bile, urine and feces of mice and rats exposed to BDE-47 and -99 (Chen *et al.*, 2006; Sanders *et al.*, 2006). In addition, recents studies have reported the formation of HO-PBDEs in human and rat hepatocytes *in vitro* (Stapleton *et al.*, 2009; Erratico *et al.*, 2011). However, the mechanisms involved in the metabolite formation are still not fully understood. Biotransformation of PBDEs may involve oxidation mediated by Phase I enzymes, which seems to be the primarily biotransformation pathway in mammals (Chen *et al.*, 2006; Stapleton *et al.*, 2009). It has been shown in mice and rat that PBDEs were weak inducers of CYP1A, a biomarker for activation of the AhR. In contrast, DE-71, prepared mixture of BDE-

47, -99, and -153 or individual congeners such as BDE-47, -99, and -209 appeared to be more able to induce CYP2B and CYP3A, both biomarkers for activation of CAR and PXR (Sanders *et al.*, 2005; Pacyniak *et al.*, 2007). In a recent study investigating the biotransformation of BDE-47 and BDE-99 in rat hepatic microsomes, Erratico *et al.*, 2011 reported that the metabolism was mediated by distinct but overlapping sets of CYPs. While CYP2A2 and CYP3A1 were the most active in BDE-47 and BDE-99 biotransformation, respectively, CYP1A1 exhibited the highest activity for 4-OH-BDE-49 and 6'-OH-BDE-99 formation (Erratico *et al.*, 2011). To our knowledge, only 2 studies have highlighted a biotransformation of PBDEs via Phase II pathways in rats. Sanders *et al.* (2006) have shown that $^{14}$C-labelled BDE-47 could be transformed *in vivo* into a glucuronide and a sulfate conjugate of 2,4-dibromophenol and into glutathione conjugates of BDE-47. A similar study using $^{14}$C-labelled BDE-99 found glutathione conjugates of BDE-99 as well as glucuronides conjugates of mono-hydroxylated BDE99 and mono-hydroxylated tetra-BDE in bile of rats (Chen *et al.*, 2006). Besides, PBDEs can also undergo debromitation but the process remains poorly understood (Staskal *et al.*, 2006; Roberts *et al.*, 2011).

In addition to be biotransformation products of synthetic PBDEs, HO-PBDEs are also produced naturally by marine organisms such as sponges and algae (Vetter *et al.*, 2002; Malmvärn *et al.*, 2005; Wiseman *et al.*, 2011). Moreover, HO-PBDEs can also result from the demethylation of other naturally-produced PBDE analogs, the methoxylated polybrominated diphenyl ethers (MeO-PBDEs) (Wan *et al.*, 2009; Wiseman *et al.*, 2011). MeO-PBDEs are structural analogs to PBDEs (Figure 1.7) and are produced naturally by several aquatic organisms such as algae, sponges and cyanobacteria (Vetter *et al.*, 2002; Malmvärn *et al.*, 2005).

**Table 1.2**. European regulations and PBDEs

| Legal basis | Content | PBDE concerned | Deadline |
|---|---|---|---|
| WEEE Directive 2002/96/EC (27 January 2003) | • Removal of plastics containing BFRs from electrical and electronic equipment (E&E) prior to recovery and recycling | • All PBDEs used in E&E | • December 2006 |
| RoHS Directive 2002/95/EC (27 January 2003) | • Ban of the placing on the market of new electrical and electronic equipment containing more than agreed levels of six substances - among which PBDEs | • Penta-BDE and Octa-BDE | • July 2006 |
| | | • Deca-BDE | • July 2008 |
| Directive 2003/11/EC (6 February 2003) | • Penta-BDE and Octa-BDE may not be placed on the market or used as a substance or as a constituent of substances or of preparations in concentrations higher than 0,1 % by mass | • Penta-BDE and Octa-BDE | • August 2004 |
| | • Articles may not be placed on the market if they, or flame-retardant parts thereof, contain these substances in concentrations higher than 0,1 % by mass | | |
| Directive 2000/60/EC (23 ctobre 2000) | • Establishment of controls of emissions, discharges and losses in the Environment and water quality standards | • Octa-BDE and Deca-BDE listed among the substances to be monitored | • Not applicable |
| | • Cessation of emissions in the environment | • Penta-BDE listed as a hasardous substance | • 2020 |

As PBDEs, MeO-PBDEs can biomagnify in the food chains and significant levels have been found in marine mammals and birds all around the world (Verreault *et al.*, 2005; Weijs *et al.* 2009b and 2010; Rotander *et al.*, 2012). MeO-PBDEs were also detected in human breast milk (Lacorte *et al.*, 2009; Fujii *et al.*, 2012). To date, few studies have investigated the toxic effects of MeO-PBDEs. Some *in vitro* studies on human cell lines observed alterations of enzyme activity such as CYP17 and aromatase (Cantón *et al.* 2005 and 2006; Song *et al.*, 2008). Nevertheless, one major concern is likely the demethylation of MeO-PBDEs which produces HO-PBDEs, a process that has been observed in some fish species but not in mammals yet (Wan *et al.*, 2009). Since HO-PBDEs have been shown to impair thyroid and vitamin A homeostasis (Meerts *et al.*, 2000; Cesh *et al.*, 2010), the presence of MeO-PBDEs in organisms may have toxicological implications.

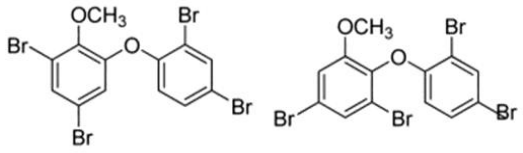


**Figure 1.7**. Chemical structure of 2'-MeO-BDE68 and 6-MeO-BDE47, the 2 main MeO-PBDE metabolites.

PBDEs are involved in various adverse effects such as endocrine disruption, immunotoxicity and alteration of neurological functions (Costa *et al.*, 2008; Fair *et al.*, 2012). Additionally, HO-PBDEs have shown higher affinity to human transthyretin (TTR) *in vitro* than its natural ligand, thyroxine ($T_4$) (Meerts *et al.*, 2000) and negative relationships between ΣHO-PBDEs and circulating retinol in bald eagles have been observed (Cesh *et al.*, 2010).

### 1.1.4. POPs in marine mammals

Because most marine mammals are top predators, have a long life span and are characterised by high lipid content (blubber), large concentrations of POPs are found in their tissues, especially in the blubber (Debier *et al.*, 2003a; Sørmo *et al.*, 2003; Weijs *et al.*, 2009c; Letcher *et al.*, 2010; Yordy *et al.*, 2010). In marine mammals, uptake of POPs occurs mainly through their diet (Borgå *et al.*, 2004). Although the concentrations of POPs are strongly influenced by the diet and the trophic position, the bioaccumulation rate also depends on the metabolic capacity of individuals and the physico-chemical properties of the molecule in question. In addition, other important factors such as the age, gender, condition, nutritional and reproductive status must be considered when studying the accumulation of POPs in individuals.

**Table 1.3.** Mean levels of PCBs and PBDEs (ng/g lw) measured in tissues of Inuit and Faroe populations.

| Population | Tissue | PCBs | PBDEs | References |
|---|---|---|---|---|
| **Inuits (Greenland)** | Blood | 2109 | N.A | Dewailly *et al.*, 1999 |
| | Fat | 5632 | N.A | |
| | Brain | 730 | N.A | |
| | Liver | 4221 | N.A | |
| **Faroe Island** | Milk | 1800 | 7.2 | Fangström *et al.*, 2005 |

Although marine mammals are generally reported as the most contaminated animals, some human populations may have similar or even higher POP levels in their tissues. For example, due to their traditional diet including large amounts of fatty tissues from sea mammal species, Inuit or Faroe populations are exposed to relatively high concentrations of POPs (Dewailly *et al.*, 1999; Fangström *et al.*, 2005). Concentrations of PCBs and/or PBDEs measured in different tissues of Inuit and Faroe populations are presented in Table 1.3.

### Distribution between tissues

Due to differences in the lipid composition of tissues, their function and the biochemical properties of the pollutants, the distribution of the chemicals may vary significantly within the animal body (Wolkers *et al.*, 2006). Table 1.4 summarises levels of PCBs and PBDEs measured in tissues of several marine mammal species from different aeras. Because POPs are highly lipophilic molecules, they are therefore found at high concentrations in blubber. However, when expressed in lipid weight, concentrations in the liver may reach similar or even higher levels than those measured in blubber (Sørmo *et al.*, 2003; Wolkers *et al.*, 2006; Yordy *et al.*, 2010; Weijs *et al.*, 2010). Blood plasma or serum contaminant levels are generally the lowest among the measured tissues, even when concentrations are expressed on a lipid weight basis (Debier *et al.*, 2003a; Sørmo *et al.*, 2003; Wolker *et al.*, 2006). This difference may be partly explained by a lower affinity of lipophilic pollutants for the more polar lipoproteins present in blood (Debier *et al.*, 2003a, 2006; Wolker *et al.*, 2006). Although brain contains higher amounts of lipids than other tissues such as liver, levels of POPs are also usually low. This may be due to the unique lipid composition of brain which is dominated by more polar phospholipids or the presence of a blood–brain barrier (Yordy *et al.*, 2010, Weijs *et al.*, 2010).

### Biotransformation

Biotransformation is an important process in the fate of POPs that results in the formation of more or less toxic molecules. This process occurs mainly in the liver and is expected to lead to the excretion and depuration of more polar metabolites (Letcher *et al.*, 2010). Biotransformation capacity varies greatly between species and depends on the xenobiotic characteristics, such as molecular structure and physico-chemical properties (Letcher *et al.*, 2010).

**Table 1.4.** Mean levels of PCBs and PBDEs (ng/g lw) measured in tissues of several marine mammal species from different areas.

| Species | Year of sampling | Tissue | PCBs | PBDEs | References |
|---|---|---|---|---|---|
| **Grey seal** | | | | | |
| Adult ♀ (Amet Island, CA) | 1995 | Blubber | 6813 | N.A | Sørmo *et al.*, 2003a |
| | | Blood | 1537 | N.A | |
| | | Milk | 1876 | N.A | |
| Pups (Amet Island, CA) | 1995 | Blubber | 1505 | N.A | |
| ♂ (Baltic) | 1996-1998 | Liver | 17000 – 132000* | N.A | Nyman *et al.*, 2002 |
| Pups (Baltic) | 1998 | Blubber | 7522 | N.A | Sørmo *et al.*, 2003b |
| **Hooded seal** | | | | | |
| Adult ♀ (St-Lawrence, CA) | 2001 | Blubber | 3130 | 44 | Wolkers *et al.*, 2006 |
| | | Milk | 1090 | 45 | |
| | | Liver | 3420 | 83 | |
| Pups (St-Lawrence, CA) | 2001 | Blubber | 1190 | 32 | |
| | | Liver | 2540 | 82 | |
| **Ringed seal** | | | | | |
| ♂ (Baltic) | 1996-1998 | Liver | 17000 – 152000* | N.A | Nyman *et al.*, 2002 |
| **Harbour Porpoise** | | | | | |
| Adult ♀-♂ (Black sea) | 1998 | Kidney | 4044 | 16 | Weijs *et al.*, 2010 |
| | | Brain | 1614 | 3 | |
| | | Liver | 8450 | 45 | |
| | | Muscle | 17420 | 81 | |
| | | Blubber | 13215 | 66 | |

* Ranges; N.A = Not applied

The presence of CYPs such as CYP1A, 2B and 3A (Goksøyr *et al.*, 1995; Wolkers *et al.*, 1999, Houde *et al.*, 2005; Wolkers *et al.*, 2009) and POP metabolites (Hoekstra *et al.*, 2003; Weijs *et al.*, 2009a; Gabrielsen *et al.*, 2011, Ochiai *et al.*, 2011) in seals and cetaceans suggest that marine mammals are able to biotransform POPs. However, although HO-PCBs, $MeSO_2$-PCBs and DDT metabolites were detected in significant amounts in several marine mammal species (Hoekstra *et al.*, 2003; Routti *et al.*, 2008; Weijs *et al.*, 2009a; Gabrielsen *et al.*, 2011, Ochiai *et al.*, 2011), no or only low levels of HO-PBDEs were found in serum or plasma of harbour (*Phoca vitulina*) and ringed (*Phoca hispida*) seals as well as polar bear (*Ursus maritimus*) (Letcher *et al.*, 2009; Routti *et al.*, 2009; Weijs *et al.*, 2009a). In addition, biotransformation of PBDEs was observed *in vitro* in hepatic microsomes of beluga whales (*Delphinapterus leucas*), polar bears and ringed seals (McKinney *et al.*, 2011). Nevertheless, only low concentrations of HO-PBDEs were measured in liver of beluga whales (McKinney *et al.*, 2006c). Those findings suggest thus that formation or retention of HO-PBDEs is low in marine mammals. Besides, the presence of HO-PBDEs in marine mammals may also result from the bioaccumulation via natural sources, as already explained in section 1.4.

Pinnipeds have a higher capacity for hepatic metabolism than cetaceans (Tanabe *et al.*, 1988; Weijs *et al.* 2009a). In general, marine mammals appear to be efficient at CYP1A-type biotransformation (dioxin-like compounds). While polar bears are proficient at CYP2B transformations, the activity of CYP2B enzymes is lower in seals and limited or absent in cetaceans (Borgå *et al.*, 2004; Houde *et al.*, 2005). The age at which biotransformation enzymes start to be efficient is not known. Gabrielsen *et al.*, 2011 found HO-PCBs metabolites in pup harbour seal and suggested some degree of endogenous biotransformation, although a maternal transfer may also be another possibility. CYP activities were detected in neonate hooded seals but they were relatively low compared to the levels in adults (Wolkers *et al.*, 2009).

### Transfer to the offspring

Sex and age are 2 factors that greatly influence POP burden in marine mammals. Generally, POP levels increase in tissues of males and immature individuals, while they decrease or remain stable in mature females (Wolkers *et al.*, 2004; Ross *et al.*, 2000). In addition, visible differences in contamination composition between adult males and females as well as juveniles are also generally reported. This is due to the fact that adult females are able to eliminate large amounts of POPs during gestation and primarily during lactation and that the transfer via milk appears to be selective (Debier *et al.* 2003b; Sørmo *et al.*, 2003; Wolkers *et al.*, 2006; Greig *et al.*, 2007; Yordy *et al.*, 2010; Desforges *et al.*, 2012; Frouin *et al.*, 2012). The lactational transfer of POPs and particularly PCBs has been extensively studied in seals because samples are more easily obtained than on cetaceans (Pomeroy *et al.*, 1996; Debier *et al.* 2003a; Sørmo *et al.*, 2003; Wolkers *et al.*, 2006; Miranda Filho *et al.*, 2009; Debier *et al.* 2012; Frouin *et al.*, 2012). It has been shown in grey seals that the maternal transfer of PCBs seems to be more important at late lactation (Debier *et al.* 2003 and 2004). One hypothesis is that the greatest part of POP mobilisation from maternal blubber may occur at late lactation, resulting in increasing POP levels in maternal serum and then in milk at that same period. In addition, the transfer from maternal blubber into milk involves diffusion of POPs through the mammary gland, a relatively aqueous layer. Therefore, there may be a selective barrier at that level favouring more hydrophilic compounds (Sørmo *et al.*, 2003). Studies on humans and rodents reported that HO-PCBs are transferred during lactation, although the greatest part of the transfer occurs during gestation (Meerts *et al.*, 2002; Guvenius *et al.*, 2003; Fangström *et al.*, 2005). Such an observation has never been demonstrated in marine mammals. Gabrielsen *et al.* (2011) reported positive relationships between serum HO-PCB levels and the age and body mass of hooded seal pups and the authors suggested thus a bioaccumulation of HO-PCBs via the milk or an endogenous biotransformation of PCBs in pups. Nevertheless, that study did not investigate the presence of HO-PCBs in milk.

The important daily ingestion of contaminated milk, with higher POP concentrations at late lactation, leads to the accumulation of POPs in suckling newborns as reported by the higher POP concentrations in their serum and blubber at late lactation (Debier *et al.*, 2003 and 2012). Since newborns are exposed to high amounts of toxic contaminants while being in a critical developmental period of their life, they may be more at risk than adults. Previous studies have shown that pup or juvenile animals exposed to environmental contaminants, even to low concentrations, had reduced immune competence and impairment of thyroid hormone and vitamin A homeostasis (Simms *et al.*, 2000; Hall *et al.*, 2003; Mos *et al.*, 2007; Sørmo *et al.*, 2009b).

## Immunotoxicity

The interaction of chemical compounds with the immune system may result in immunosuppression or an overstimulation of immune responses (Ladics and Woolhiser, 2007). Xenobiotics may affect the immune system both directly or indirectly. For example, they may directly suppress the immune system by reducing the cell number or organ weights, altering specific immune functions, inhibiting the proliferation of lymphocytes and/or reducing the production or release of cytokines (Ladics and Woolhiser, 2007). On the other hand, immunomodulation may also occur indirectly by affecting other physiological systems such as the neuroendocrine system or via the metabolic activation of the xenobitotic into toxic metabolites (Ladics and Woolhiser, 2007). In addition, vitamin A which is an important regulator of the immune system has been shown to be negatively affected by environmental pollutants such as PCBs and OCs. Therefore, these POPs may also indireclty alter the immune system through a modulation of vitamin A homeostasis.

Disease outbreaks with mass mortalities of seals, dolphins and porpoises in the late 1980s and early 1990s have generated an interest in marine mammal immunotoxicology. Although the main cause of those outbreaks was morbillivirus infection, it is suspected that environmental pollutants may have contributed to an increased

vulnerability to infection in contaminated animals. Indeed, individuals that died from infectious disease had generally higher concentrations of POPs in their tissues than those that survived or were sampled before the outbreak (Ross and De Guise, 2007). However, such data should be interpreted with cautious since the condition of the sick or dying animals could have led to elevated concentrations of POPs in their lower amount of blubber.

Both field and semi-field studies generally reported negative relationships between the investigated immune functions and POP concentrations measured in animals (Table 1.5a) (Ross *et al.*, 1995 and 1996; De Swart *et al.*, 1994 and 1995; Lahvis *et al.*, 1995; Bernhoft *et al.*, 2000; Mos *et al.*, 2006). However, some immune parameters such as B lymphocyte proliferation were apparently not affected (De Swart *et al.*, 1994 and 1995; Mos *et al.*, 2006). In addition, Levin *et al.* (2005a) reported that, in some cases, PCBs may induce the proliferative response of B and T lymphocytes in harbour seal pups. In parallel to (semi-) field studies, a lot of *in vitro* experiments have been performed on several marine mammal species. The use of *in vitro* studies has allowed major advancements in the field of immunotoxicology and is very useful to study the direct characterisation of the toxic effects of specific contaminants on immune cells and the detection of interactions between several contaminants. Most of those *in vitro* studies have investigated the impact of PCBs on various immune parameters of several marine mammal species and are summarised in Table 1.5b. Marine mammals appeared to be sensitive to POP *in vitro* exposure but the direction of the modulation varied among species and congeners and/or mixtures used during the exposure (Table 1.5b). Additionally, interactions between contaminants in a mixture have been reported in several studies (De Guise *et al.*, 1998; Levin *et al.*, 2006; Mori *et al.*, 2006; Levin *et al.*, 2007; Mori *et al.*, 2008). Those previous works demonstrated that the TEQ approach should be used carefully when assessing the risk of contaminant-mediated immunotoxicity in marine mammals, since non-coplanar molecules are not taken into account.

**Table 1.5a (to be continued).** Effects of different POPs on several immune parameters of marine mammals observed during field or semi-field studies.

| Immune parameters | Measured chemicals | Concentrations (mean or range) | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| **T lymphocyte proliferation** | PCBs, DDT, Dieldrin, HCB, ß-HCH, PCDFs and PCDDs | PCBs: 1460, DTT: 487, Dieldrin: 491, HCB: 42, ß-HCH: 17, PCDFs: 0.4 and PCDDs: 0.07* | Harbour seal | ↓ | De Swart *et al.*, 1994 and 1995 |
| | PCBs, DDTs | PCBs: 26–752 ng/g ww blood, DDT: 13–563 ng/g ww blood | Bottlenose dolphins | ↓ | Lahvis *et al.*, 1995 |
| | PCBs | 7624 ng/g lw blubber | Grey seal | ↓ | Sørmo *et al.*, 2009b |
| | PCBs | 396–6587 ng/g lw blubber | Harbour seal | ↓ | Mos *et al.*, 2006, |
| | PCBs | 2517 ng/g lw blubber | Harbour seal | ↑ | Levin *et al.*, 2005a |
| **B lymphocyte proliferation** | PCBs, DDT, Dieldrin, HCB, ß-HCH, PCDFs and PCDDs | PCBs: 1460, DTT: 487, Dieldrin: 491, HCB: 42, ß-HCH: 17, PCDFs: 0.4 and PCDDs: 0.07* | Harbour seal | = | De Swart *et al.*, 1994 and 1995 |
| | PCBs | 396–6587 ng/g lw blubber | Harbour seal | = | Mos *et al.*, 2006 |
| | PCBs | 2517 ng/g lw blubber | Harbour seal | ↑ | Levin *et al.*, 2005a |
| **Delayed-type hypersensitivity (DTH)** | PCBs, DDT, Dieldrin, HCB, ß-HCH, PCDFs and PCDDs | PCBs: 1460, DTT: 487, Dieldrin: 491, HCB: 42, ß-HCH: 17, PCDFs: 0.4 and PCDDs: 0.07* | Harbour seal | ↓ | Ross *et al.*, 1995 |

| Immune parameters | Measured chemicals | Concentrations (mean or range) | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| **NK cell activity** | PCBs, DDT, Dieldrin, HCB, ß-HCH, PCDFs and PCDDs | PCBs: 1460, DTT: 487, Dieldrin: 491, HCB: 42, ß-HCH: 17, PCDFs: 0.4 and PCDDs: 0.07* | Harbour seal | ↓ | Ross *et al.*, 1996 |
| **IgG levels** | PCBs, HCB | 20.5–228 ng/g ww serum | Polar bear | ↓ | Bernhoft *et al.*, 2000 |

↓: decrease; ↑: increase; =: no effect; * Estimated daily intakes of organochlorines in µg/day by seal feeding on herring from the Baltic sea

**Table 1.5b (to be continued).** Effects of different POPs on several immune parameters of marine mammals observed during *in vitro* studies.

| Immune parameters | Chemicals | Concentrations | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| **T lymphocyte proliferation** | CB153, 169, 180, mixture (CB 138+CB153+CB 180), TCDD and DDE | PCBs: 5 to 25 ppm each<br>DDE: 10 to 100 ppm<br>TCDD: 1 to 15 pg/ml<br>Mixture: 5 ppm of each PCB | Beluga whale | = | De Guise *et al.*, 1998 |
| | CB-138, mixture (CB138+CB153+CB180) and DDT | CB-138: 20 and 25 ppm<br>DDT: 50 to 100 ppm<br>Mixture: 5 ppm of each PCB | Beluga whale | ↓ | De Guise *et al.*, 1998 |
| | CB77, 126 and 169 | 2.8 to 3400 pmol/l | Dall's porpoises and Bottlenose dolphin | = | Nakata *et al.*, 2002 |
| | Aroclor 1016, 1221, 1248, 1254, 1260, 1262 and 1268 | Aroclors 1016, 1221, 1254, 1260 and 1268: 50 and 100 ppm<br>Aroclor 1248: 12.5 ppm<br>Aroclor 1262: 25 and 100 ppm | Grey seal | ↓ | Frouin *et al.*, 2010a |
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Killer whale | ↓ or = | Mori *et al.*, 2006 |
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Beluga whale, Commerson's dolphin, Harbour seal, Northern Fur Seal, Steller sea lion and Sea otter | ↑ or = | Mori *et al.*, 2006 |

| Immune parameters | Chemicals | Concentrations | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| **T lymphocyte proliferation** | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Bottlenose dolphin | ↓, ↑ or = | Mori *et al.*, 2006 |
| | Aroclor 1254 | 15 ppm | Grey seal | ↓ | Dufresne *et al.*, 2010 |
| | CB156 and 180 | 10 µmol/l | Harbor seal | = | Neale *et al.*, 2002 |
| | benzo[a]pyrene (B[a]P) | 10 µmol/l | Harbor seal | ↓ | Neale *et al.*, 2002 |
| **B lymphocyte proliferation** | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Beluga whale and Sea otter | = | Mori *et al.*, 2008 |
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Pilot whale, Bottlenose dolphin, Harbor seal, Northern Fur Seal | ↑ or = | Mori *et al.*, 2008 |
| **NK cell activity** | Aroclor 1242 and 1260 | 3 and 30 ng/ml | Harbour and Grey seal | = | Hammond *et al.*, 2005 |
| **Phagocytosis** | CB138, 153, 180, 169, TCDD, DDT and DDE | PCBs: 5 to 25 ppm each<br>DDE: 10 to 100 ppm<br>TCDD: 1 to 15 pg/ml | Beluga whale | = | De Guise *et al.*, 1998 |

| Immune parameters | Chemicals | Concentrations | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| **Phagocytosis** | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Sea otters, Pacific white-sided dolphin, Northern fur seal, Steller sea lion, Bottlenose dolphin, Beluga whale | ↓ or = | Levin *et al.*, 2004, 2005b and 2007a |
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Killer whale | ↑ or = | Levin *et al.*, 2005b |
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Commerson's dolphin and Harbour seal | ↓, ↑ or = | Levin *et al.*, 2005b |
| | Aroclor 1242 and 1260 | 3 and 30 ng/ml | Harbour seal | ↓ | Hammond *et al.*, 2005 |
| | Aroclor 1242 and 1260 | 3 and 30 ng/ml | Grey seal | = | Hammond *et al.*, 2005 |
| | BDE47, 99 and 153 | 1.5, 3, 6 and 12 µmol/l | Harbour seal | ↓ | Frouin *et al.*, 2010b |
| **Respiratory burst activity** | CB138, 153, 169, 180, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Sea otters and Harbour seal | ↓ or = | Levin *et al.*, 2007a and 2007b |

| Immune parameters | Chemicals | Concentrations | Species | Direction of the modulation | References |
|---|---|---|---|---|---|
| | CB138, 153, 180, 169, TCDD and mixtures | PCBs: 5 ppm each<br>TCDD: 0.05 ppb<br>Mixture: 5 ppm of each PCBs and 0.05 ppb TCDD | Bottlenose dolphin, Beluga whale and Killer whale | ↑ or = | Levin *et al.*, 2007b |
| | Aroclor 1242 and 1260 | 30 ng/ml | Harbour seal | ↓ | Hammond *et al.*, 2005 |
| | Aroclor 1242 and 1260 | 3 and 30 ng/ml | Grey seal | = | Hammond *et al.*, 2005 |
| | BDE47, 99 and 153 | 1.5, 3, 6 and 12 µmol/l | Harbour seal | ↑ | Frouin *et al.*, 2010b |
| **Thiol levels** | BDE47, 99 and 153 | 1.5, 3, 6 and 12 µmol/l | Harbour seal | ↓ | Frouin *et al.*, 2010b |
| **Apoptosis** | BDE47, 99 and 153 | 1.5, 3, 6 and 12 µmol/l | Harbour seal | = | Frouin *et al.*, 2010b |

↓: decrease; ↑: increase; =: no effect

## 1.2. Vitamin A

### 1.2.1. Generalities

Vitamin A is an essential micronutrient required for several important physiological processes in the body such as normal vision, reproduction, embryonic development, cell and tissue differentiation and immune function (Ross and Harrison, 2007; D'Ambrosio *et al.*, 2011). The term vitamin A is used to define compounds with the biological activity of retinol and include the provitamin A carotenoids (ß-carotene, α-carotene and ß-cryptoxanthin) and preformed vitamin A (retinyl esters, retinol, retinal and retinoic acid). The main vitamin A metabolites are 11-cis-retinal and all-trans-retinoic acid. 11-cis-retinal is required for vision and all-trans-retinoic acid is the most bioactive form of vitamin A that regulates the transcription of hundreds genes in nearly all tissues through the binding and activation of retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Ross and Harrison, 2007; D'Ambrosio *et al.*, 2011). The different forms of vitamin A are illustrated in Figure 1.8. The carotenoids are obtained from vegetables and fruits, while retinyl esters and retinol are present in foods of animal origins such as meat, eggs and dietary products (Ross and Harrison, 2007).

Dietary retinol is directly absorbed from the intestinal lumen into the enterocytes, while dietary retinyl esters are first hydrolysed to retinol in the intestinal lumen or at the enterocyte brush border before absorption by enterocytes. Carotenoids are absorbed intact into the enterocytes where they can be converted into retinol or packed unmodified into chylomicrons (Senoo *et al.*, 2010; Ross and Harrison, 2007; D'Ambrosio *et al.*, 2011). Retinol within the enterocytes are esterified and incorporated into chylomicrons that are secreted into the lymphatic vessels. They then reach the blood circulation where they are converted into chylomicron remnants that are rapidly cleared into the liver (Debier and Larondelle, 2005; Senoo *et al.*, 2010; Ross and Harrison, 2007; D'Ambrosio *et al.*, 2011). In addition, although about 75% of the retinyl esters within chilomicrons are taken up by the liver, the remaining can be taken up by extrahepatic

tissues such as adipose tissue, lungs and kidneys (Debier and Larondelle, 2005; D'Ambrosio *et al.*, 2011).

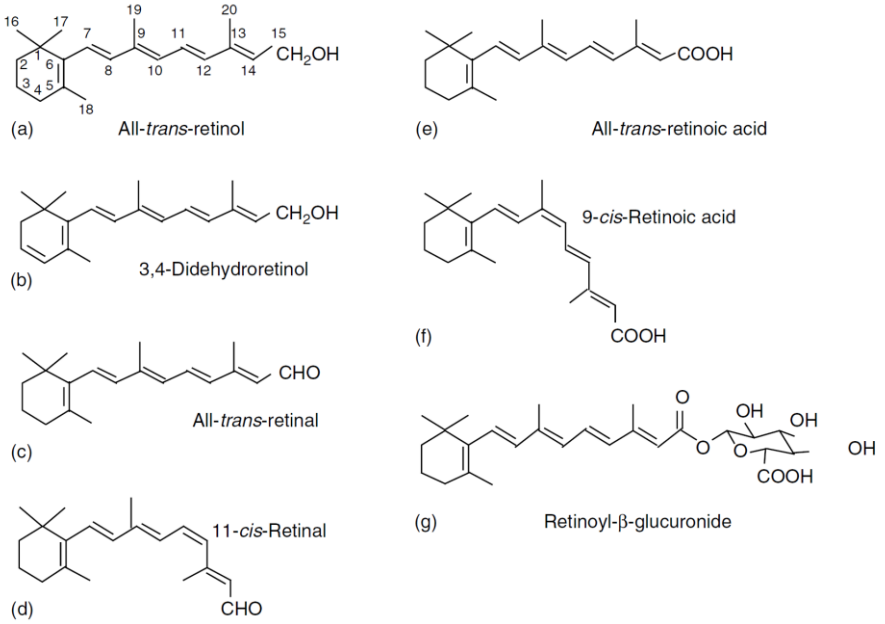


**Figure 1.8.** The different forms of vitamin A and major metabolites (Ross and Harrison, 2007).

In general, most mammals store more than 90% of their total body vitamin A in the liver. Once in hepatic parenchymal cells, retinyl esters are hydrolysed into retinol by retinyl ester hydrolases (REH) and transferred into hepatic stellate cells (HSC) where they are stored in cytoplasmic lipid droplets. The transfer into HSC is still not fully understood. Although earlier studies suggested that it was mediated by retinol-binding protein (RBP), experiments on RBP-deficient mice demonstrated that RBP was not required in the transfer of retinol from hepatocytes to HSC (D'Ambrosio *et al.*, 2011; Shirakami *et al.*, 2012). Studies on cellular retinol binding protein I (CRBPI)-deficient mice suggested that CRBPI may mediate the

transfer of retinol from hepatocytes to HSC. Indeed, retinyl ester levels in CRBPI-deficient mice were significantly reduced, indicating that CRBPI is necessary for assuring efficient retinol esterification and storage *in vivo* (D'Ambrosio *et al.*, 2011). Once in the HSC, retinol is bound to CRBPI and transferred to lecithin retinol acyltransferase (LRAT) in order to be esterified and stored in HSC lipid droplets. Acyl CoA:retinol acyl transferase (ARAT) may work in association with LRAT to form retinyl esters (D'Ambrosio *et al.*, 2011).

In case of insufficient intake of vitamin A, retinol is mobilised from hepatic stores to supply peripheral tissues. Retinyl esters stored in the HSC are converted into retinol by REH and is thought to be transferred back to the hepatocyte where it binds to its specific transport protein, RBP (Debier and Larondelle, 2005; Senoo *et al.*, 2010; D'Ambrosio *et al.*, 2011).

The complex retinol–RBP is secreted into the bloodstream and delivered to extrahepatic tissues for intracellular synthesis of retinoic acid. In blood, RBP is found in a 1:1 protein-protein complex with TTR, the $T_4$ hormone carrier. The association of the retinol-RBP complex with TTR allows reducing its glomerular filtration (Debier and Larondelle, 2005; D'Ambrosio *et al.*, 2011).

The mechanisms involved in the uptake of retinol-RBP by extrahepatic tissues have remained obscure for long. Although strong evidences accumulated since the 1970s for the existence of a specific cell-surface RBP receptor that mediates vitamin A uptake (Sun and Kawaguchi, 2011), it is only in 2007 that a high affinity RBP receptor, known as STRA6, has been identified in bovine retinal pigment epithelium cells (Kawaguchi *et al.*, 2007). STRA6 can bind RBP with high affinity and facilitate the release of retinol from RBP and its transfer into the cell (Kawaguchi *et al.*, 2007; Sun and Kawaguchi, 2011). This receptor has been characterised in many human, mouse and rat tissues, especially in blood-organ barriers such as retinal pigment epithelium, placenta and choroid plexus (Kawaguchi *et al.*, 2007; Sun and Kawaguchi, 2011).

Although liver contains the major concentrations of body vitamin A, adipose tissue can store up to 20% of the total vitamin A in the body as retinyl esters (Frey and Vogel 2011). In marine mammals, adipose tissue can contain up to 60 % of the total body vitamin A (Schweigert

*et al.*, 2002; Mos *et al.*, 2007; Rosa *et al.*, 2007; Routti *et al.*, 2010). Adipose tissue plays an important role in the vitamin A homeostasis. It can take up circulating retinol bound to RBP and/or absorb retinyl esters and carotenoids packed in chylomicrons, store vitamin A as retinyl esters, convert it to retinoic acid and, under dietary vitamin A deficiency conditions, mobilise vitamin A into the blood circulation (Frey and Vogel 2011).
After cellular uptake, retinol can be esterified and stored in lipid droplets (e.g. adipose tissue) or transformed into retinoic acid (e.g. eyes and lungs) (Debier and Larondelle, 2005). The transformation into retinoic acid requires first a reversibly oxidation of retinol into retinal by retinol dehydrogenases (RolDH). The next step is the irreversible oxidation of retinal to retinoic acid catalysed by retinaldehyde dehydrogenases (RALDH). In addition to dehydrogenases, some CYPs such as CYP1A or CYP3A are also involved in both reactions (Debier and Larondelle, 2005; Theodosiou *et al.*, 2010). Retinoic acid can then bind the RAR and RXR nuclear receptors in order to regulate the transcription of a large number of genes or may be transformed into more polar retinoids, such as 4-oxo-retinoic acid that will be excreted. The catabolism of retinoic acid occurs principally via enzymes of the CYP26 family (Debier and Larondelle, 2005; Theodosiou *et al.*, 2010).

### 1.2.2. Importance of vitamin A to the immune system

Vitamin A is an important regulator of the immune system in mammals. The importance of that nutrient in the regulation of the immune system was first reported by observations that vitamin A deficiency increased the susceptibility to infectious diseases in developing countries and in laboratory experiments (Stephensen, 2001; Mora *et al.*, 2008).

Vitamin A deficiency impairs mucosal immunity via several mechanisms including loss of cilia in the respiratory tract, loss of microvilli from small intestine and decrease in goblet cells and mucin production in the mucosal epithelia. Besides, vitamin A deficiency alters the normal development of neutrophiles and can therefore

result in a decrease of the phagocytosis activity and a reduced protection against bacterial infections (Stephensen, 2001). It has also been demonstrated that vitamin A depletion decreases the cytolytic activity of the natural killer (NK) cells by reducing considerably their number (Ross and Stephensen, 1996; Stephensen, 2001). In addition, Friedman and Sklan (1989) showed that vitamin A deficiency leads to significant impairment of T lymphocyte activity and antibody production. A supplementation of vitamin A can usually restore the normal immune cell functions (Friedman and Sklan, 1989; Ross and Stephensen, 1996; Stephensen, 2001).

As already highlighted, vitamin A deficiency observed in developing countries is thought to be an important contributing factor to child death associated with infectious diseases (Blomhoff *et al.*, 2004). It has been shown to be associated with gastrointestinal and respiratory infections, while vitamin A supplementation tends to significantly reduce mortality in HIV-infected or malnourished children (Mora *et al.*, 2008). In addition, studies in several African countries reported that treatment with vitamin A could reduce the mortality associated with measles (Sommer, 2008). As a result, the United Nation Children's fund (UNICEF) and the World Health Organization (WHO) recommended vitamin A treatment for children suffering from acute measles (Sommer, 2008).

### 1.2.3. Vitamin A metabolism and POPs

Although vitamin A physiology is strictly regulated in mammals, several POPs can interfere with the transport, storage and metabolism of vitamin A. Numerous studies have already investigated the relationships between POPs and vitamin A homeostasis in humans, laboratory animals and wildlife species (Zile *et al.*, 1992; Brouwer *et al.*, 1998; Lans *et al.*, 1993; Hallgren *et al.*, 2001; Debier *et al.*, 2005a; Ellis-Hutchings *et al.*, 2006; Novák *et al.*, 2008). Most of them focused on the effects of PCBs, and to a lesser extent of DDTs, HCB and PBDEs on vitamin A metabolism. The reported effects are generally a decrease of hepatic and adipose tissue retinoid stores and an increase of the levels in the kidneys (Zile, 1992;

Håkansson *et al.*, 1994; Hallgren *et al.*, 2001; Nyman *et al.*, 2003; Ellis-Hutchings *et al.*, 2006; Mos *et al.*, 2007; Novák *et al.*, 2008). In contrast, the effects observed in blood, plasma or serum varied between both an increase and a decrease of vitamin A levels or no change at all. Those discrepancies are likely due to the species, the physiological state and the POP concentrations at which the animals were exposed (Zile, 1992; Nyman *et al.*, 2003; Novák *et al.*, 2008; Cesh *et al.*, 2010; Delport *et al.* 2011). The mechanisms by which POPs are able to disrupt vitamin A homeostasis are still poorly understood. It has been shown that the regulation of some CYPs such as CYP1A or CYP3A that are involved in the metabolism of vitamin A can be modulated by TCDD, PCBs and pesticides in rodents, fish and frogs (Boyer *et al.*, 2000; Leiva-Presa *et al.*, 2006; Novák *et al.*, 2008). Other enzymes such as LRAT, ARAT and REH were also reported to be modulated by PCBs and TCDD in fish and laboratory animals (Chen *et al.*, 1992; Nilsson *et al.*, 1996; Ndayibagira and Spear, 1999; Nilsson *et al.*, 2000). A well-established mechanism is the competition of some HO-PCBs and HO-PBDEs with $T_4$ on the binding site of TTR, as observed in humans and rats (Brouwer *et al.*, 1998; Lans *et al.*, 1993, Meerts *et al.*, 2000). In addition to be the blood transport protein of $T_4$, TTR is also involved in the blood transport of the RBP-retinol complex. The binding of HO-PCBs or HO-PBDEs to TTR instead of $T_4$ results in a weaker affinity for the RBP-retinol complex, leading to the excretion of the RBP-retinol complex. Finally, PCBs and TCDD may affect vitamin A metabolism at the nuclear level. Indeed, TCDD appeared to prevent the binding of retinoic acid to RAR as reported in human keratinocytes *in vitro* (Lorick *et al.*, 1998). Besides, PCB levels in blubber were positively associated with the relative RARα expression of blubber in harbour seals (Mos *et al.*, 2007).

### 1.2.4. Vitamin A in marine mammals

Several differences are observed between terrestrial and marine mammals regarding the vitamin A status. For example, while the liver is the primary storage site for vitamin A in most of terrestrial mammals, marine mammals can store up to 60% of their total body

vitamin A in the blubber (Schweigert *et al.*, 2002; Mos *et al.*, 2007; Rosa *et al.*, 2007; Routti *et al.*, 2010). Concentrations have been shown to increase with age (Schweigert *et al.*, 1987; Käkelä *et al.*, 1997; Simms et Ross, 2000). Although vitamin A circulates as retinyl esters in lots of carnivorous mammal species, Schweigert *et al.* (2002) detected no retinyl esters in adult and pup grey seal plasma. Regarding lactation, Debier *et al.* (2002 and 2012) reported that seal milk contains relatively higher vitamin A concentration as compared to terrestrial mammals (Table 1.6). However, when expressed per unit of milk lipids, vitamin A levels become comparable to the ones observed in the milk of farming animals or humans (Debier *et al.*, 2005b). In terrestrial mammals, higher amounts of vitamin A are found in colostrum as compared to mature milk (Table 1.6). This allows the suckling newborn to quickly increase its vitamin A levels which are relatively low at birth, due to the limited placental transfer (Debier *et al.*, 2005b). By contrast, Debier *et al.* (2002) revealed that vitamin A levels in mature grey seal milk were higher than in colostrum (Table 1.6).

**Table 1.6**. Vitamin A concentrations in colostrum and mature milk of different mammal species (adapted from Debier *et al.*, 2005b).

| Species | Colostrum | Mature milk |
|---|---|---|
| Human[a] | 1.0 µg/ml | 0.3 µg/ml |
| Cow[b] | 2.3 – 3.7 µg/ml | 0.3 – 0.6 µg/ml |
| Sheep[b] | 6.5 µg/ml | 0.3 µg/ml |
| Grey seal[b] | 6.3 µg/g | 10.1 µg/g |

[a]Macias and Schweigert, 2001; [b]See references in Debier *et al.*, 2005b

The particular dynamics observed in seal milk may be due to the fact that females fast in most seal species and that, therefore, all the vitamin A contained in milk comes exclusively from maternal stores and especially from blubber. A similar rise at late lactation was recently observed in Northern elephant seal milk (Debier *et al.* 2012). In parallel, the authors reported an increase of vitamin A content in

maternal blubber, suggesting a less efficient mobilisation of vitamin A from blubber as compared to triacylglycerols. The authors suggested that the mobilisation of vitamin A from blubber could be more efficient at the end of the nursing period, leading to the increase observed in milk (Debier *et al.* 2012). This hypothesis is supported by the different dynamics observed for lipids and vitamin A in grey seal milk. Indeed, Debier *et al.*, 2002 have shown that levels of lipids in milk increased greatly during the first half of the lactation, while vitamin A started to increase during the second part of the lactation (Figure 1.9).

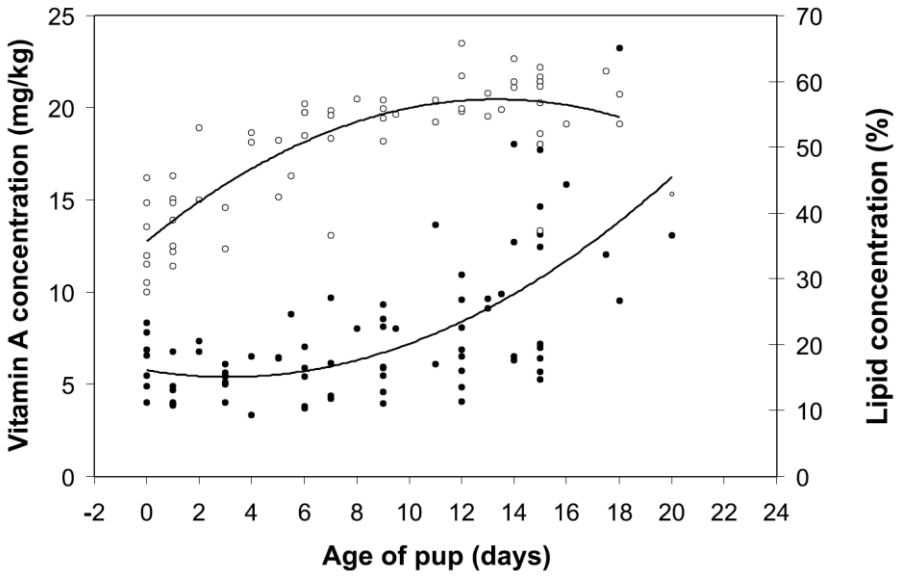


**Figure 1.9**. Evolution of vitamin A (●) and lipid (○) content in grey seal milk throughout lactation (Debier *et al.*, 2002).

As for terrestrial mammals, the placental transfer of vitamin A appeared to be limited, since grey seal newborns had very low levels of vitamin A in their serum (Debier *et al.*, 2002). But although colostrum did not contain higher vitamin A levels than mature milk, circulating vitamin A levels increased rapidly in nursing pups,

reaching levels similar to their mothers after only 3 days (Debier *et al.*, 2002).

Due to their high trophic position and their important lipid content, marine mammals accumulate large amounts of POPs in their organism (see section 1.1.5). Several studies have already investigated the effects of PCBs and DDTs on vitamin A metabolism in marine mammals and both positive and negative relationships have been reported. In blubber, a positive association between PCBs and vitamin A was found in dolphins (Tornero *et al.*, 2006), while a negative correlation was highlighted in harbour seals (Mos *et al.* 2007). Moreover, RARα expression in harbour seal blubber was positively correlated with total PCB concentrations measured in that tissue. Such an elevated RAR expression could reflect an up regulation due to reduced concentrations of vitamin A present in blubber or a direct PCB-associated effect at the molecular level (Mos *et al.* 2007). Nyman *et al.*, (2003) observed positive relationships between hepatic PCB and DDT levels and circulating vitamin A, while they were negatively related with liver and blubber vitamin A contents in grey seals. The authors suggested an enhanced mobilisation of hepatic and blubber vitamin A stores, leading to the depletion of vitamin A levels in these tissues and an increase in blood (Nyman *et al.*, 2003).

It was also reported that more contaminated harbour seal pups from Washington State (recently weaned) had significantly lower circulatory retinol levels as compared to less contaminated pups from British Columbia (nursing and fasted) (Simms *et al.*, 2000). However, when individuals were analysed in function of their nutritional status, the authors observed that total circulatory retinol levels were positively correlated with PCB levels in weaned and fasting pups, while no detectable effects of PCBs on total retinol levels could be found in nursing pups. These findings indicate thus that the nutritional status of the individual is an important parameter to take into account when using vitamin A as a biomarker (Simms *et al.*, 2000).

A part from Nyman *et al.* (2003) and Simms *et al.* (2000), other studies on marine mammals reported negative relationships between circulating vitamin A and PCB or DDT concentrations (Jenssen *et al.*,

2003; Debier *et al.*, 2005a; Mos *et al.*, 2007). Although these studies did not investigate the presence of circulating HO-PCBs, it was suggested that the decrease in retinol concentrations was probably linked to an interaction of specific HO-PCBs with the TTR-RBP-retinol complex, resulting in a greater excretion of circulating vitamin A by glomerular filtration, as observed in humans and laboratory animals (Brouwer *et al.*, 1998; Lans *et al.*, 1993).

In addition to be able to modulate the vitamin A homeostasis in marine mammals, PCBs also appear to share similar dynamics of mobilisation and transfer with vitamin A in marine mammals. Previous studies on grey seals have shown that, unlike lipids or vitamin E, vitamin A and PCBs had common dynamics in milk over the course of lactation: concentrations of both compounds remain constant during the first half of lactation and then increase at late lactation (Debier *et al.* 2004) (Figure 1.10). The authors suspected that higher amounts of vitamin A and PCBs could be mobilised from blubber at late lactation. Findings from Debier *et al.* (2012) who observed that both PCBs and vitamin A tended to increase at late lactation in maternal inner blubber and in milk of Northern elephant seal (*Mirounga angustirostris*) seem to confirm this theory.

While several studies investigated the potential effects of PCBs or DDTs on vitamin A status in marine mammals, the impacts of PBDEs on this important nutrient are lacking. Since they have been reported to alter vitamin A status in laboratory animals and birds (Hallgren *et al.*, 2001; Fernie *et al.*, 2005; Ellis-Hutchings *et al.*, 2006; Cesh *et al.*, 2010), it is possible that PBDEs may also affect vitamin A homeostasis in marine mammals.

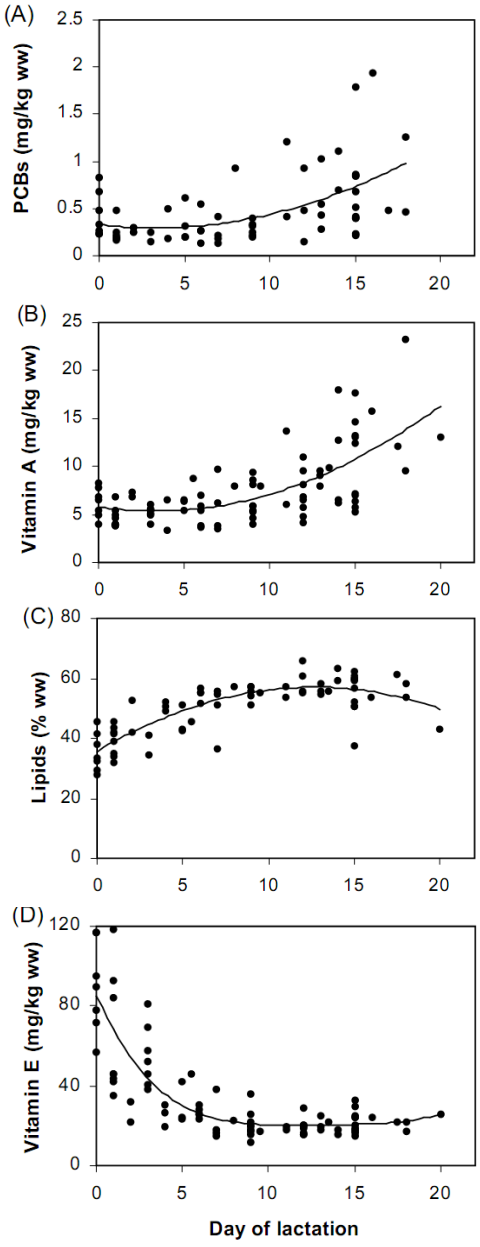


**Figure 1.10.** Global dynamics of PCBs (A), vitamin A (B), total lipids (C) and vitamin E (D), in grey seal milk throughout lactation (Debier *et al.*, 2004).

## 1.3. Lipid metabolism in marine mammals: generalities

Blubber of marine mammals is the principal site of energy storage and also ensures insulation and buoyancy. It can constitute up to 50% of the total body mass (Iverson *et al.* 1995 and 2002). The most common form of lipids stored in blubber are triacylglycerols and the fatty acid composition in the blubber of a marine mammal is strongly influenced by the fatty acid composition of its diet (Best *et al.*, 2003; Grahl-Nielsen *et al.*, 2003)

Several studies have shown that fatty acid composition of marine mammal blubber is stratified (Best *et al.*, 2003; Grahl-Nielsen *et al.*, 2003; Wheatley *et al.*, 2007). Recently, Strandberg *et al.* (2008) revealed for the first time 3 chemically distinct layers in terms of fatty acid composition in ringed seal (*Pusa hispida*). The authors have shown that superficial layers were enriched with short-chain monounsaturated fatty acids, while inner layers contained mainly saturated and long-chain monounsaturated fatty acids. The outer blubber is adapted to low temperatures and do not seem to be readily available for metabolism. This layer mainly ensures insulation and buoyancy. The relatively homogenous middle layer is apparently not influenced heavily by environmental temperature or recent metabolic activity and may therefore represent long-term averages of dietary fatty acids (Strandberg *et al.*, 2008). The inner layer is the most metabolically active and likely reflects recent lipid mobilisation and deposition (Best *et al.*, 2003; Wheatley *et al.*, 2007; Strandberg *et al.*, 2008).

A lot of marine mammal species depend on their accumulated lipid stores during periods of fasting (e.g., during moulting, reproduction, lactation, migration or post-weaning). A recent study on lactating grey seals has reported that the release of fatty acids from the blubber of lactating grey seals was selective and that the patterns of mobilisation were linked to the fatty acid molecular structure (Arriola, 2010). For a given fatty acid chain length, the mobilisation increased with the number of double bonds, and for a given double

bond, mobilisation increased as chain length decreased (Arriola, 2010). In other words, more polar fatty acids appear to be more readily released from maternal blubber. Similar patterns of fatty acid mobilisation from the adipose tissue were already shown for rodents and humans (Raclot, 2003).

It has been reported in several seal species that during lactation, lipoprotein lipase, the enzyme responsible for tissue uptake of fatty acids, was negligible in maternal blubber, while its activity in the mammary gland was elevated, facilitating the lipid uptake by the mammary gland (Iverson *et al.* 1995; Mellsih *et al.*, 1999; Champagne *et al.*, 2012). Similarly, lipoprotein lipase activity was high in the suckling pup blubber, favouring the rapid deposition of fat which is essential for the post-weaning fast.

## 1.4. Studied species: The grey seal

### 1.4.1. Morphology and distribution

The grey seal belongs to the Phocidae family. Adult animals have a grey-brown colour with darker patches, while newborn pups have a white, long-haired coat (Figure 1.11). Additionally, pelage colour also varies within sexes, males being darker than females (Jefferson *et al.*, 2008). Grey seals are characterised by an important sexual dimorphism. Adult males are larger than females and can reach up to 2.3 m in length and may weigh more than 300 kg, while females reach up to 1.9 m in length and weigh below 200 kg (Jefferson *et al.*, 2008; Special Committee on Seals (SCOS), 2011). The anatomy of the face also differs between males and females, with males having generally a broader head and a larger muzzle than females. In general, Canadian grey seals are slightly larger than European individuals (Jefferson *et al.*, 2008). Males may live for over 20 years and females often live for over 30 years (Bonner, 1981; SCOS, 2011).

The grey seal is only found in the North Atlantic. Three distinct populations of grey seals are generally observed (Figure 1.12) (Bonner, 1981; Jefferson *et al.*, 2008): the Northwest Atlantic, the

Northeast Atlantic and the Baltic Sea. The Northwest Atlantic population is found from the Northeast United States to Cape Chidley, with the main breeding colonies in Magdalene Islands, Amet Island, Sable Island and Point Michaud. In 2010, the Northwest Atlantic population size (including pups) was estimated at 348 900 individuals (Thomas *et al.*, 2011). The Northeast Atlantic population is observed around the British Isles, Iceland, France, Netherlands, Norway and the Kola Penninsula of Russia.

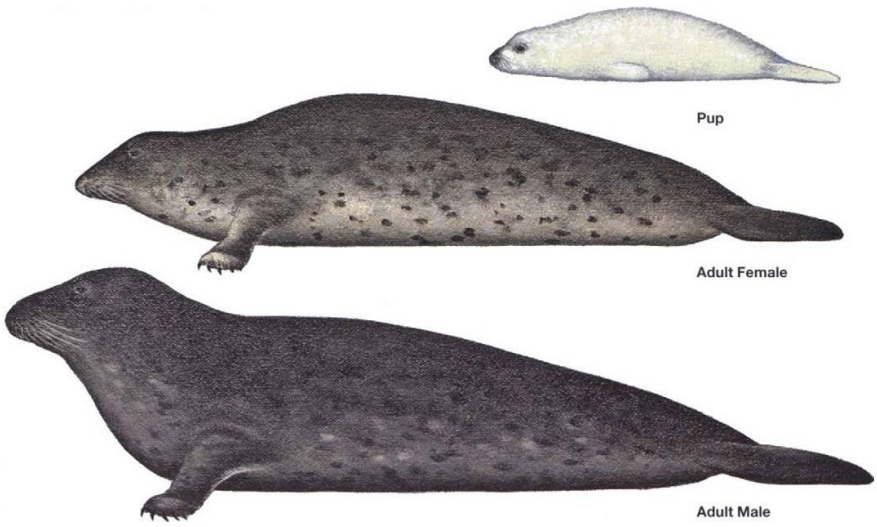


**Figure 1.11**. Morphology of the adult male, female and pup grey seal (Jefferson *et al.*, 2008).

The highest concentration of the Northeast Atlantic population is found around the British Isles where the total UK grey seal population at the start of the 2010 breeding season was estimated at 111 300 individuals (SCOS, 2011). The size of the Baltic stock has known important fluctuations. In the early 1900s, it was estimated to about 88 000 - 100 000 animals and was represented only by a few thousands of individuals in the late 1970s (Hårding and Härkönen, 1999). This dramatic decrease was likely due to intense hunting and

reproductive impairments caused by environmental contamination (Hårding and Härkönen, 1999). Nowadays, the Baltic grey seal population has recovered and in 2010, the estimated population size was about 23 000 individuals in the entire Baltic Sea (Lundström, 2012).

These populations are not only geographically separated but have also different timing in the breeding seasons (Bonner, 1981). In addition, mitochondrial DNA variations have been reported between the different stocks (Boskovic *et al.* 1996).

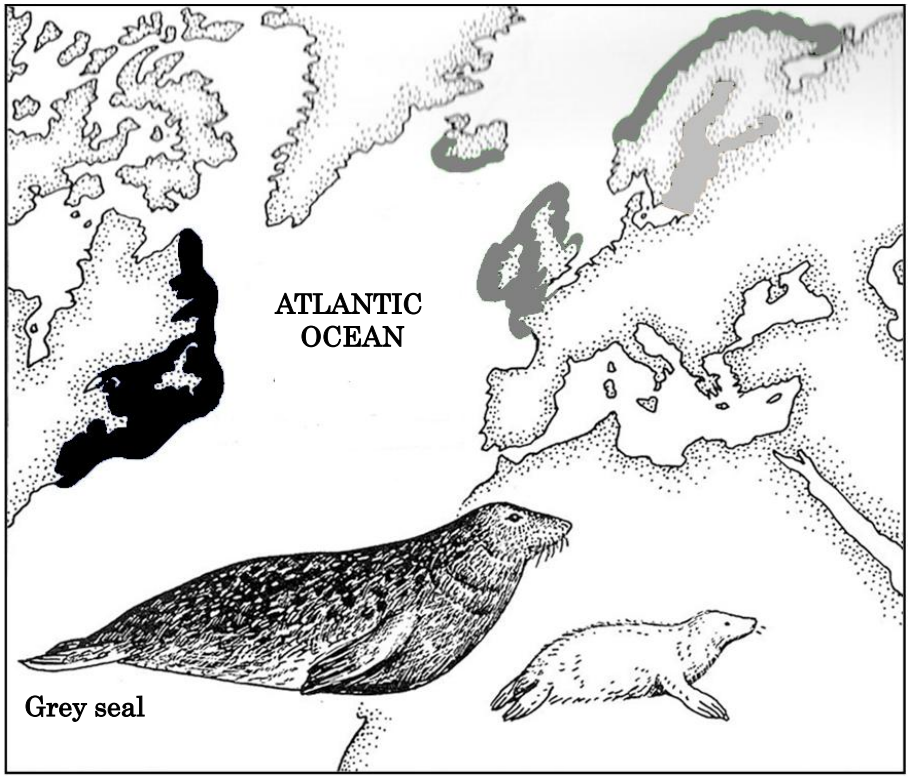


**Figure 1.12**. Geographical distribution of grey seals. In black: the Northwest Atlantic population, in dark grey: the Northeast Atlantic population and in light grey: the Baltic population. Adapted from Bonner (1990).

Grey seal primarily feed upon a wide range of fish species and diet studies indicate that their favourite are sandeel and gadoids. They generally forage along the coasts but can also perform long-distance travel (> 100 km) (Mcconnell *et al.*, 1999).

### 1.4.2. Reproduction and lactation

Female and male grey seals reach sexual maturity between 4-7 years of age but males are not socially mature before the age of 10 (Bonner, 1981).

Depending of the geographic localisation, pupping occurs ashore or on the sea-ice in the winter (e.g., Canada and the Baltic) (Bonner, 1981). The timing of the breeding season varies between the 3 distinct populations (Bonner, 1981). Breeding occurs between September and January in the Northeast Atlantic population, while parturition is observed between January and February in the Northwest Atlantic population (Bonner, 1981). Baltic seal pups are delivered in February and March (Bonner, 1981). Females usually give birth to only one pup. The first instants after delivery are important to set up the link between the mother and the newborn. After the delivery, a short lactation period (16 to 21 days) is observed during which the mother transfers large amounts of energy to its pup (Pomeroy *et al.*, 1996). Like most of phocid seals, grey seal females fast during the lactation period, while producing milk extremely rich in lipids (up to 60%) which is entirely derived from their own body energy stores (Iverson *et al.*, 1993; Pomeroy *et al.*, 1996). Female size appears to play an important role in the success of the pup weaning. Large females tend to arrive ashore to deliver earlier in the season and lose weight more rapidly than smaller females, while females with lower body mass tend to wean smaller pups or wean pups prematurely (Anderson and Fedak, 1987; Iverson *et al.* 1993; Pomeroy *et al.* 1999). Maternal expenditure during lactation is huge. During that short nursing period, females can lose between 25 to 50 % of their initial body mass (Iverson *et al.*, 1993; Pomeroy *et al.*, 1999). In contrast, due to the important daily ingestion of milk, suckling pups rapidly gain mass (between 0.8 to 2.8 kg/day) (Iverson *et al.*, 1993; Pomeroy *et al.*, 1999).

Newborns have almost no subcutaneous lipid at parturition and the ingestion of a fat-milk is therefore essential for the constitution of their own blubber. Some studies reported a higher maternal investment in male offspring compare to females (Anderson and Fedak 1987). In contrast, other data showed no evidence of differential maternal expenditure in the sexes (Pomeroy *et al.*, 1999).

Composition of the milk varies with time: the fat content increases especially during the first half of the lactation period, while the percentage of water decreases and the protein content remains relatively stable (Debier *et al.*, 2002; Lang *et al.*, 2011). The decrease in water content throughout lactation appears to be an adaptation allowing lactating females to maintain their hydric balance during the fast (Lydersen *et al.*, 1995).

Once weaned, the pup is left on its own by its mother and may stay ashore or on the sea-ice for several days or weeks, living on its own body reserves that have been accumulated during lactation before being able to forage for the first time (Jefferson *et al.*, 2008). Pups lose on average 0.5 kg of body mass daily during the post-weaning fast. The mating takes place after weaning on land or in the water. Gestation lasts about 11.5 months and includes a period of delayed implantation of about 3 months (Bonner, 1981).

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# Aims of the study

In the introduction we have seen that POPs have the potential to modulate vital parameters of the physiology such as the immune system or the homeostasis of vitamin A in a lot of mammal species. In several cases, the toxicity of metabolites may be more important than the parent compound. It has also been demonstrated that marine mammals are relatively affected by POP contamination and that the transfer of those pollutants to offspring through the milk is very high, which may be particularly deleterious for the developing organism. Significant relationships between POPs and vitamin A status have been reported in several marine mammal species. Moreover, a previous work of our research team has shown a parallelism between PCBs and vitamin A in grey seal milk, suggesting that those compounds may share common mechanisms of mobilisation from body stores and transfer into the milk. In addition to vitamin A, the immune system represents a sensitive target for many POPs and several immune functions have been shown to be altered after POP exposure in marine mammals.

Although the impacts of chlorinated pollutants, such as PCBs, on marine mammal health have been well documented, information on the toxicity of PCB metabolites as well as the more recent PBDEs is still scarce. Besides, while a lot of studies have investigated the lactational transfer of PCBs and OCPs, only a limited number have focused on PBDEs and none have examined the HO metabolites.

The **general purpose** of the present thesis is to investigate the transfer of vitamin A and POPs from the mother to its pup during the lactation period in seals.

More specifically, we aim at:

- **Comparing the dynamics of transfer of vitamin A and PCBs in grey seal, especially in maternal blubber**. The purpose is to better understand the origin of the parallelism between vitamin A and PCBs that has been previously observed in grey seal milk

- **Examining the dynamics of transfer of PBDEs, PCBs, OCPs and their metabolites** in grey seal during the lactation period. Indeed, although the lactational transfer of PCBs and OCPs in seals has been the subject of several previous studies, none has already reported in detail how PBDEs and hydroxylated metabolites of PCBs and PBDEs are transferred during lactation in these animals.

- **Highligting potential impacts of those POPs on the vitamin A status** in both mother and pup grey seal during lactation.

Vitamin A is an important regulator of the immune system. However, the homeostasis of this nutrient has been shown to be adversely affected following POP exposure. Therefore, an alteration of the vitamin A status resulting from POP contamination may indirectly affect the immune system and thus possibly increase the risk of infections. The influence of vitamin A on the immune system of marine mammals and its possible interactions with the immunotoxic effects of POPs deserve further investigation. It is the reason why our last objective was designed to

- **Study the impact of PBDEs, PCBs and vitamin A on several immune parameters** in 2 *in vitro* models in order to gain new insights on immunotoxicology, especially in marine mammals.

To achieve these goals, our work was divided in 4 experimental sections, each with a specific objective:

1. To **analyse the concentrations of vitamin A and PCBs** in the different tissues of transfer (maternal blubber → maternal serum → milk → pup serum) in free-ranging grey seals. The potential relationships between vitamin A and PCBs in the same tissue as well as between tissues were also examined.

2. To **investigate the transfer of PBDEs, PCBs, OCPs and their metabolites in mother-pup pairs throughout lactation**. Maternal blubber, milk and serum as well as pup serum samples were collected in order to measure the concentrations and evaluate the profile of the different POPs in the 4 investigated tissues.

3. To measure concentrations of PBDEs, PCBs, OCPs and their metabolites as well as levels of vitamin A in the blubber and serum of lactating grey seal females in order **to evaluate the impact of POP contamination on vitamin A status**.

4. To **evaluate the effect of several congeners of PCBs, PBDEs and vitamin A on murine splenocytes and a harbour seal cell line**. Apoptosis, proliferation and cell cycle were investigated because they are key functions in the maintenance of the immune system homeostasis.

The first 3 experimental chapters are published or in press in peer-reviewed international journals. The 4$^{th}$ experimental chapter reports preliminary results obtained from *in vitro* experiments on murine splenocytes and the harbour seal cell line. In the final part of the thesis, a general discussion has been drawn and perspectives for future projects have been proposed.

# Chapter 2

# Relationships between vitamin A and PCBs in grey seal mothers and pups during lactation

## Foreword

As discussed in the introduction, a previous study carried out in our laboratory has reported a curious parallelism between vitamin A and PCBs in grey seal milk[1]. In this first experimental chapter, we attempted to investigate the origin of this parallelism by comparing the dynamics of vitamin A and PCBs in different tissues of transfer and particularly in the maternal blubber. Similar dynamics of both compounds in maternal blubber could partly explain the dynamics observed in milk. Concentrations of vitamin A and PCBs were assessed in maternal blubber, serum, milk and pup serum. The relationships between vitamin A and PCBs were also examined. The results obtained in the present chapter have been published in *Environmental Pollution*. Some changes have been introduced in the original published version. These changes do not modify the conclusions of the published version. Therefore, this chapter is a redraft after the published version.

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[1] Debier, C., Pomeroy, P.P., Thomé, J.P., Mignolet, E., de Tillesse, T., Larondelle, Y., 2004. An unexpected parallelism between Vitamin A and PCBs in seal milk. Aquatic Toxicology 68, 179-183.

# Relationships between vitamin A and PCBs in grey seal mothers and pups during lactation

*Redrafted after*

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Authors' contributions

The work presented here was carried out in collaboration between all authors. CD defined the research theme. AA and PP participated in the sample collection. MVB, AM, SP and VS carried out the laboratory experiments. JPT, PP, YL and CD contributed to reagents/materials/analysis tools. MVB and FG performed the statistical analyses. MVB analysed the data. MVB, AM, JPT, YL and CD were engaged in active discussions. MVB wrote the paper. FG and PP provided advice on the manuscript. CD supervised the work. All authors have contributed to, seen and approved the manuscript.

# Abstract

A previous study has shown a simultaneous increase of vitamin A and PCBs in grey seal (*Halichoerus grypus*) milk at late lactation (Debier *et al.*, 2004). Here we sought to understand this unexpected relationship by comparing the dynamics of vitamin A and PCBs in the different tissue compartments of transfer. Lactating grey seals and their pups were sampled longitudinally in Scotland during the 2006 breeding season. As blubber reserves decreased, concentrations of vitamin A and PCBs increased during lactation in the inner layer of maternal blubber. A concomitant rise was observed in milk and consequently in the serum of suckling pups. The similar dynamics of vitamin A and PCBs in milk and inner blubber suggest a common mechanism of mobilisation from maternal body stores and transfer into the milk. A panel data analysis highlighted a negative impact of PCBs in milk and pup serum on vitamin A status in pup serum.

**Keywords:** grey seal, lactation, vitamin A, PCBs, blubber

## 2.1. Introduction

Vitamin A is a fat-soluble nutrient present in food in the form of retinyl ester or ß-carotene (pro-vitamin A). It is involved in several essential biological functions such as vision, reproduction, immunity, growth and development (Debier and Larondelle, 2005). As a result of its limited placental transfer, vitamin A is particularly important for newborns, which depend on its presence in the milk to form their own body stores (Debier *et al.*, 2005a; Salle *et al.*, 2005). In terrestrial mammals, the liver can contain up to 90% of the total body vitamin A. In several seal species, the blubber can also store a large proportion of vitamin A (40-66%) and its concentration in this tissue tends to increase with age (Schweigert *et al.*, 1987; Käkelä *et al.*, 1997; Mos and Ross, 2002; Schweigert *et al.*, 2002). Although blood, liver as well as kidneys play important roles in the homeostasis of vitamin A in mammals, persistent organic pollutants such as polychlorinated biphenyls (PCBs) can interfere with its metabolism in those tissues (Zile, 1992; Simms and Ross, 2000; Hallgren *et al.*, 2001, Käkelä *et al.*, 2003). Because of its predictable response to pollutant exposure, vitamin A has become a reliable biomarker of organochlorine-related toxicity in wildlife species (Rolland, 2000; Simms and Ross, 2000).

PCBs are highly lipophilic and stable molecules that can easily accumulate throughout the trophic chain (Ross *et al.*, 1996; Muir *et al.*, 2003; Borja *et al.*, 2005). As a result, top predators such as marine mammals bioaccumulate high levels of these environmental pollutants, which can alter many physiological functions including the metabolism of vitamin A (Simms and Ross, 2000; Mos *et al.*, 2007). Several studies on marine mammals have already shown the negative impact of PCBs on vitamin A concentrations resulting, among other things, from the competition between some PCB hydroxymetabolites and thyroxine ($T_4$), a thyroid hormone, for its binding site on transthyretin (TTR), the major serum carrier of $T_4$ and retinol binding protein (RBP)–retinol complex. The binding of a PCB metabolite to TTR instead of $T_4$ results in a loss of TTR affinity for the relatively small RBP–retinol complex which is then more

easily excreted via the kidneys (Rolland, 2000; Simms *et al.*, 2000; Simms and Ross, 2000; Jenssen *et al.*, 2003; Debier *et al.*, 2005b). More recently, a negative association between blubber vitamin A and PCB concentrations has been observed in harbour seals, which means that both types of compounds may also interact at the adipose tissue level (Mos *et al.*, 2007). An elevated expression of RARα (a nuclear receptor for retinoic acid, the main active form of vitamin A) was noticed in the most contaminated animals, suggesting a PCB-associated effect at the molecular level (Mos *et al.*, 2007).

Grey seals (*Halichoerus grypus*) fast throughout their short lactation period (from 16 to 21 days) (Bonner, 1981; King, 1983). During that time, large amounts of fat-soluble compounds such as vitamin A are mobilised from the blubber and transferred into the milk to satisfy the demand of the newborn (Debier *et al.*, 2002; Schweigert *et al.*, 2002). However, the phenomenon also allows the transfer of lipophilic xenobiotics such as PCBs to suckling pups (Addison and Stobo, 1993; Pomeroy *et al.*, 1996, Beckmen *et al.*, 1999; Debier *et al.*, 2003; Wolkers *et al.*, 2004).

Similar dynamics for vitamin A and PCBs in milk have been shown previously in grey seals (Debier *et al.*, 2004). Both types of compounds underwent a significant increase at the end of the nursing period. In order to understand the origin of this parallelism, the present paper focuses on how vitamin A and PCB levels change in different body compartments (serum, milk and within blubber) during lactation. It also investigates the potential relationships between vitamin A and PCBs in the same biological compartment as well as between compartments. The covariates stage of lactation and maternal body fat content were taken into account in the discussion of the results.

## 2.2. Material and methods

### 2.2.1. Seal sampling

The study was performed on 2 grey seal colonies on the Isle of May (IOM) and on the island of North Rona (NR), Scotland, in October–

November 2006. A total of 13 mother-pup pairs were captured and sampled on IOM and 11 pairs on NR (Table 2.1). In both colonies, each pair was sampled 2 or 3 times throughout the lactation period. These samples were termed early, mid or late. Longitudinal samples of maternal blubber, milk and blood serum (mother and pup) were collected as described in Debier *et al.* (2002, 2003). Vitamin A and PCBs were quantified in each of the 4 compartments. Animals were handled and weighed as described in Pomeroy *et al.* (1996) under UK Home Office licence. Measurement of female total body fat was derived from the hydrogen isotope dilution model described in Reilly and Fedak (1990).

## 2.2.2. Chemical analysis

### Milk fat content

Milk lipids were extracted into an accelerated solvent extractor at 80°C with a mixture of hexane, dichloromethane and methanol. The fat content of samples was determined gravimetrically (see Debier *et al.*, 2003 for more details).

### Vitamin A

Vitamin A extraction and analysis in serum and milk were performed as described in Debier *et al.* (2002). Briefly, three successive extractions with hexane were carried out on serum samples. The extracts were pooled, evaporated and the residue was reconstituted with 300 ml of methanol before being analysed by high performance liquid chromatography (HPLC) using a spectrofluorometric detector. Results are reported in mg/l of serum. Milk samples were saponified with ethanolic KOH under nitrogen. Two extractions with diethyl ether containing 2,6-di-tert- butyl-4-methylphenol (BHT) as stabilizer were then performed, the organic phases were pooled and washed several times with MilliQ water before being filtered and evaporated.

**Table 2.1.** Number of samples collected on the mother-pup pairs followed during the longitudinal study.

| Seal code | Location | Inner and outer blubber | | Maternal blood | | Milk | | Pup blood samples | |
|---|---|---|---|---|---|---|---|---|---|
| | | VA | PCBs | VA | PCBs | VA | PCBs | VA | PCBs |
| **Arsetho** | NR | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Cap6** | NR | 2 | 0 | 2 | 0 | 2 | 2 | 2 | 2 |
| **Fab** | NR | 2 | 0 | 3 | 0 | 3 | 0 | 3 | 0 |
| **FFN**[a] | NR | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Keyhole** | NR | 2 | 2 | 2 | 0 | 2 | 2 | 2 | 0 |
| **Laika**[a] | NR | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Mum 1**[a] | NR | 0 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| **Mum 2** | NR | 0 | 2 | 2 | 0 | 2 | 2 | 2 | 0 |
| **Nova**[a] | NR | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| **Sam**[a] | NR | 0 | 0 | 2 | 2 | 3 | 3 | 2 | 3 |
| **Splits**[a] | NR | 2 | 0 | 2 | 2 | 2 | 2 | 2 | 0 |
| **TH**[a] | NR | 2 | 2 | 2 | 3 | 2 | 3 | 2 | 2 |
| **31635**[a] | IOM | 0 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **45251**[a] | IOM | 0 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **50711**[a] | IOM | 0 | 2 | 3 | 3 | 3 | 3 | 3 | 2 |
| **2W** | IOM | 2 | 0 | 2 | 0 | 3 | 0 | 2 | 0 |
| **3B** | IOM | 2 | 2 | 3 | 0 | 3 | 3 | 3 | 2 |
| **3U** | IOM | 0 | 0 | 0 | 2 | 1 | 2 | 0 | 2 |
| **40799/800**[a] | IOM | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **4B**[a] | IOM | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 2 |
| **4H**[a] | IOM | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **5H**[a] | IOM | 2 | 2 | 2 | 3 | 3 | 3 | 2 | 3 |
| **7J**[a] | IOM | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **OH**[a] | IOM | 2 | 2 | 3 | 1 | 3 | 3 | 3 | 2 |
| **OJ**[a] | IOM | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |

[a]Animals used in the panel data analysis. We chose individuals having been sampled at least once for vitamin A and PCBs in inner blubber and/or maternal serum.

The residue was reconstituted in 10 ml of methanol before being analysed by the HPLC method as already used for serum samples. Results are expressed in mg/kg of wet weight.

Blubber biopsy samples (approximately 4 cm long and 6 mm wide) were cut into 3 equal parts in order to separate inner blubber from outer blubber. The inner and the outer parts were then weighed separately and analysed using the same protocol as for milk. Results are also reported in mg/kg of wet weight.

#### PCBs

Nineteen PCB congeners (IUPAC 44, 70, 95, 101, 105, 110, 118, 128, 138, 149, 153, 156, 180, 183, 187, 194, 195, 206, 209) were analysed in blubber, serum and milk and analysed by gas chromatography (GC) coupled with an electron capture detector (ECD) as detailed in Debier *et al.* (2003). Briefly, after fat extraction, all the samples were purified by a sulphuric acid mixture (fuming sulphuric acid 30% and concentrated sulphuric acid 95%, 1:3, V:V) and Florisil (Supelco, Envi-Florisil, Bellefonte, PA) clean-ups and the purified extracts were then analysed by GC. As for vitamin A, blubber samples were cut into 3 pieces and the inner and outer layers were analysed separately. For each of these two parts, fat was extracted from the tissue by heating it in a microwave at 650 W (Debier *et al.*, 2003). Results are expressed in mg/kg of blubber lipids, in mg/kg of wet weight for milk and in mg/l of serum.

### 2.2.3. Statistical analyses

#### Changes throughout lactation

Variations in vitamin A and PCB concentrations in serum and milk with regard to individual seals and stage of lactation in serum and milk were evaluated using a two-way ANOVA (significance level 0.10). Changes of concentrations in inner and outer blubber with time and among individuals were determined using a three-way ANOVA

(significance level 0.10). A Bonferroni post hoc test was used after each ANOVA to compare concentrations among the periods of lactation. Logarithms of the concentrations were analysed in order to decrease the variance heterogeneity observed between the beginning and the end of lactation. ANOVA tests were performed in the Statistica software (StatSoft, Inc., Tulsa, OK).

### Correlations in levels of vitamin A between body compartments and effect of PCBs

Although an ANOVA analysis is able to determine the dynamics of compounds with time within a particular compartment, it does not imply temporal cause of this change. The dynamics observed could be attributed to other explanatory variables which are fluctuating with time, such as the total body fat of the female, for example. Therefore, dynamics of vitamin A and relationships with PCBs throughout all compartments were investigated using panel data regressions. Such models allow us to evaluate the potential impact of several parameters simultaneously and take the unobserved heterogeneity between individuals into account.

Briefly, a panel is a database where data vary according to a temporal and an individual dimension. Introducing fixed effects (i.e. a different intercept for each individual) allows us to take the unobserved heterogeneity into account, namely the intrinsic and unobservable differences between complex animals like seals. This problem would be insuperable with simple cross-sectional data (one set of data for each individual with no repetition in time). It must be noted that unobserved heterogeneity, when not accounted for, introduces a severe statistical bias because it appears in the regression residual while it is also likely correlated to the regressors, jeopardising therefore the exogeneity assumption necessary for the consistency of Ordinary Least Squares (OLS) estimators. By definition, fixed effects solve this problem. For a more complete account of estimation with panel data, see Greene (2003).

The different parameters influencing the data here are the animal, the body compartment (blubber, maternal serum, milk and pup serum), the mother's total body fat and time (lactation stage) which are considered as continuous variables. Regression analysis requires the formulation of the model as the impact of independent (or "explanatory") variables on a dependent (or "explained") variable. In our case, it is of course essential to study in turn several dependent variables (vitamin A measured in each compartment of the pup-mother pair), but these regressions will all take the general form:

$$Y(i,t)=a+bX(i,t)+u(i)+e(i,t)$$

where X(i,t) and Y(i,t) are the vectors of the independent and dependent variables for each individual i and time t, u(i) represents the fixed effects for each mother-pup pair and e(i,t) contains the independently and identically distributed error terms, uncorrelated with the other vectors. The relevant explanatory variables (i.e. the substantive content of X(i,t)) change from one compartment to the other. Indeed, we should not expect to explain the vitamin A levels in milk the same way as in blubber (e.g. the relationships between levels in maternal serum and those in its previous compartment, the blubber, are not the same as the relationships between levels in pup serum and its previous compartment, the mother's milk). However, we keep some of the regressors such as the stage of lactation (time effect) and the female total body fat in all regressions for the sake of consistency and completeness in interpretations.

Correlation data were analysed using the STATA software (StataCorp LP, College Station, TX).

## 2.3. Results

### 2.4.4. Dynamics of vitamin A and PCBs throughout lactation

#### Maternal blubber

Female total body fat, most of which is blubber, decreased as lactation progressed from 56.9 ± 19.9 kg at early lactation to 35.6 ± 11.4 kg at late lactation ($p < 0.001$) and also varied among individuals ($p = 0.066$).

Vitamin A and PCB levels were different between mothers ($p = 0.047$ and $p < 0.001$, for vitamin and PCBs respectively). At early lactation, levels of vitamin A and PCBs were higher in the outer layer as compared to the inner layer ($p = 0.044$ for vitamin A and $p = 0.042$ for PCBs) (Table 2.2). The opposite situation occurred for vitamin A at late lactation ($p = 0.037$), whereas there were no differences in PCBs between the two layers ($p = 1.000$) (Table 2.2). Vitamin A and PCB concentrations increased between early and late lactation in inner blubber ($p < 0.001$ and $p < 0.001$ for vitamin A and PCBs, respectively) (Table 2.2). In the outer layer, both compounds remained stable between the two periods ($p = 1.000$ for vitamin A and $p = 0.409$ for PCBs) (Table 2.2).

#### Maternal serum

Vitamin A and PCB levels differed between individuals ($p = 0.002$ and $p < 0.001$ for vitamin A and PCBs, respectively). There were significant variations among lactation stages ($p < 0.001$ for vitamin A and $p = 0.012$ for PCBs). Vitamin A was higher at late lactation, whereas for PCBs only concentrations at mid lactation were lower than those at late lactation (Table 2.2).

**Table 2.2.** Mean (± standard deviation) of vitamin A and PCB levels in the different compartments.

| Compartments | Vitamin A | | |
|---|---|---|---|
| | Early lactation | Mid lactation | Late lactation |
| Outer blubber (mg/kg blubber) | 53.5 ± 20.7[a] | | 58.2 ± 18.9[a] |
| Inner blubber (mg/kg blubber) | 37.9 ± 15.2[b] | | 89.3 ± 53.2[c] |
| Maternal serum (µg/l serum) | 315.0 ± 100.2[a] | 281.3 ± 76.8[a] | 383.6 ± 93.6[b] |
| Milk (mg/kg milk) | 5.7 ± 1.5[a] | 6.0 ± 2.3[a] | 13.5 ± 11.5[b] |
| Pup serum (µg/l serum) | 165.2 ± 52.1[a] | 324.3 ± 98.0[b] | 425.5 ± 118.3[c] |
| **Compartments** | **PCBs** | | |
| | Early lactation | Mid lactation | Late lactation |
| Outer blubber (µg/kg lipids) | 1958.0 ± 1216.3[a] | | 2996.9 ± 2462.4[a] |
| Inner blubber (µg/kg lipids) | 1246.9 ± 850.4[b] | | 4056.5 ± 5206.6[a] |
| Maternal serum (µg/l serum) | 7.7 ± 8.0[a,b] | 5.3 ± 3.1[a] | 10.7 ± 10.5[b] |
| Milk (µg/kg milk) | 206.9 ± 95.0[a] | 277.8 ± 126.4[b] | 496.9 ± 400.5[c] |
| Pup serum (µg/l serum) | 9.7 ± 6.1[a] | 11.9 ± 6.2[a] | 20.4 ± 20.2[b] |

Values followed by different letters are significantly different ($p \leq 0.10$). For blubber statistics, consider outer and inner blubber as one compartment (e.g. for vitamin A concentration, outer blubber is significantly different than inner blubber at early but not at late lactation). All results are expressed per unit of wet weight except PCBs in blubber which are expressed per unit of lipids.

## Milk

Milk lipid content increased as lactation progressed from 38.5 ± 6.4% at early lactation to 56.7 ± 3.4% at late lactation ($p < 0.001$) and also varied among females ($p = 0.050$).

Concentrations of vitamin A and PCBs increased during the nursing period ($p < 0.001$ and $p < 0.001$ for vitamin A and PCBs, respectively) (Table 2.2). Concentrations also varied among mothers ($p = 0.016$ for vitamin A and $p < 0.001$ for PCBs).

### Pup serum

Levels of vitamin A and PCBs were different between individuals ($p = 0.027$ and $p < 0.001$ for vitamin A and PCBs, respectively) and also varied with stage of lactation ($p < 0.001$ for vitamin A and $p < 0.001$ for PCBs). Vitamin A increased constantly during the nursing period while PCBs only increased at the end (Table 2.2).

## 2.4.5. Correlations between compartments and effect of PCBs

A panel data analysis was used to investigate the potential relationships between the different compartments and the possible impacts of PCBs on vitamin A. In all the regressions performed, the unobserved heterogeneity was controlled by the fixed effects technique which incorporates a binary or dummy variable for each mother-pup pair (Greene, 2003). Only animals with complete inner blubber and/or maternal serum data for vitamin A and PCBs were chosen for all the regressions (Table 2.1) as they are the two body compartments involved in initial transfer of compounds.

### Maternal blubber

Vitamin A levels in inner blubber were investigated according to the female total body fat and the stage of lactation (Table 2.3a). In the significant and effective regression ($p < 0.001$, $R^2 = 0.899$) vitamin A levels tented to increase as maternal body fat decreased ($p = 0.090$).

### Maternal serum

Vitamin A concentrations in maternal serum were analysed according to PCB levels in maternal serum, vitamin A in inner blubber, the stage of lactation and the female total body fat (Table 2.3b). The regression was significant and effective ($p = 0.004$, $R^2 = 0.813$). Vitamin A in maternal serum was positively correlated with vitamin A contained in inner blubber ($p = 0.107$). Serum PCBs and maternal

total body fat did not show any effect on maternal serum vitamin A concentrations.

**Table 2.3**. Linear regression with fixed effects of vitamin A.

| a) in maternal inner blubber | | | | |
|---|---|---|---|---|
| Nb obs = 22, Prob>F = 0.000, R-squared = 0.899, Root MSE = 0.098 | | | | |
| VA inner blubber | Coef. | Std. Err. | t | P> \|t\| |
| Stage of lactation | 0.007 | 0.009 | 0.79 | 0.450 |
| Female tot body fat | -0.629 | 0.331 | -1.90 | 0.090 |
| b) in maternal serum | | | | |
| Nb obs = 21, Prob>F = 0.004, R-squared = 0.813, Root MSE = 0.097 | | | | |
| VA maternal serum | Coef. | Std. Err. | t | P> \|t\| |
| PCB maternal serum | 0.170 | 0.304 | 0.56 | 0.597 |
| VA inner blubber | 0.626 | 0.330 | 1.89 | 0.107 |
| Stage of lactation | -0.006 | 0.007 | -0.79 | 0.458 |
| Female tot body fat | 0.192 | 0.386 | 0.50 | 0.636 |
| c) in milk | | | | |
| Nb obs = 46, Prob>F = 0.000, R-squared = 0.823, Root MSE = 0.128 | | | | |
| VA milk | Coef. | Std. Err. | t | P> \|t\| |
| VA maternal serum | 0.566 | 0.204 | 2.78 | 0.010 |
| PCB maternal serum | 0.210 | 0.142 | 1.47 | 0.153 |
| Stage of lactation | 0.023 | 0.009 | 2.51 | 0.019 |
| % lipid milk | -0.007 | 0.005 | -1.40 | 0.174 |
| d) in pup serum | | | | |
| Nb obs = 26, Prob>F = 0.000, R-squared = 0.995, Root MSE = 0.040 | | | | |
| VA pup serum | Coef. | Std. Err. | t | P> \|t\| |
| PCB pup serum | -0.460 | 0.188 | -2.45 | 0.058 |
| VA milk | 0.790 | 0.243 | 3.25 | 0.023 |
| PCB milk | -0.359 | 0.096 | -3.76 | 0.013 |
| Stage of lactation | 0.021 | 0.003 | 7.62 | 0.001 |
| Female tot body fat | -0.404 | 0.113 | -3.56 | 0.016 |

### Milk

The "maternal total body fat" variable was not included in this analysis because of its close relationship with the level of lipids in the milk ($r = -0.64$).

Dynamics of vitamin A in milk were investigated using vitamin A and PCB concentrations in maternal serum, the stage of lactation and the percentage of lipids in the milk (Table 2.3c). PCBs in milk were not incorporated in the regression because we observed that first, they were not significant and then, they obscured the effects of the other explanatory variables. The regression was highly significant and effective ($p < 0.001$, $R^2 = 0.823$). Vitamin A in milk was positively correlated with vitamin A in maternal serum ($p = 0.010$) and the stage of lactation ($p = 0.008$). Neither serum PCBs nor milk lipid percentage were correlated with vitamin A in milk.

### Pup serum

Vitamin A concentrations in pup serum were investigated according to PCB levels in pup serum, vitamin A and PCBs in milk, the stage of lactation and the female total body fat (Table 2.3d). The regression was significant and effective ($p < 0.001$, $R^2 = 0.995$). Vitamin A in pup serum was positively correlated with vitamin A in milk ($p = 0.023$) and the stage of lactation ($p = 0.001$). A negative correlation was established with PCBs in pup serum ($p = 0.058$): when PCBs in pup serum increased by 1%, vitamin A in pup serum decreased by 0.4%. A negative impact of PCBs in milk was also observed ($p = 0.013$): a rise of 1% in milk PCBs reduced vitamin A levels in pup serum by 0.3%. Vitamin A in pup serum tended to increase when maternal body fat decreased ($p = 0.016$).

## 2.4. Discussion

This work offers a unique opportunity to investigate the dynamics of vitamin A and PCBs in grey seals more thoroughly as, for the first

time, data on vitamin A in inner and outer blubber were included in such a longitudinal study. Moreover, the statistical model used here allowed us to consider the integrated dynamics of transfer of vitamin A from the mother to the pup from one body compartment to the next. In addition, the use of panel data analysis enabled exploration of relationships between vitamin A and PCBs in the same compartment but also between compartments.

### 2.4.1. Dynamics of vitamin A and PCBs throughout lactation

#### Maternal blubber

At early lactation, concentrations of vitamin A and PCBs were higher in the outer layer compared to the inner layer whereas these differences reversed or disappeared at late lactation. Differences between blubber layers may be explained by lipid stratification. Several studies have shown the heterogeneity of fatty acid composition in the blubber of marine mammals (Best *et al.*, 2003, Grahl-Nielsen *et al.*, 2003 and Wheatley *et al.*, 2007). Recently, vertical fatty acid profiles in the blubber column were investigated for the first time in a phocid seal (ringed seal, *Pusa hispida*), revealing 3 chemically distinct layers in terms of fatty acid composition (Strandberg *et al.*, 2008). It was suggested that the vertical distribution of fat-soluble vitamins and contaminants could exhibit stratification patterns similar to the vertical fatty acid profiles (Strandberg *et al.*, 2008). This would imply a simple relationship between some lipid components and the concentrations of vitamin A and PCBs. However, variations in the expression of the vitamin A storage system (cellular retinol binding protein I or CRBPI) through blubber layers may also explain the differences of vitamin A concentrations between inner and outer blubber and the lack of a similar relationship for PCBs.

Levels of vitamin A and PCBs underwent an increase at late lactation in the inner layer while they remained constant in the outer layer. These blubber layers are characterised by different physiological functions. The outer layer is relatively stable in terms of chemical

composition and mainly ensures insulation and buoyancy while the inner layer is more involved in the storage and mobilisation of lipids. The inner layer is the most metabolically active and likely reflects recent lipid mobilisation and deposition. Thus, it will be the first to be mobilised during a fast, as happens during grey seal lactation (Aguilar and Borrell, 1990, Koopman *et al.*, 1996, Hooker *et al.*, 2001, Best *et al.*, 2003 and Strandberg *et al.*, 2008). The increase in vitamin A and PCB concentrations in the inner layer may result from the fact that both types of compounds are less easily mobilised from blubber than triglycerides, as a result of their physico-chemical properties and are thus retained in the inner layer, which becomes thinner as lactation progresses (Debier *et al.*, 2002 and Debier *et al.*, 2004). The mobilisation of fatty acids from the adipose tissue has been shown to be a selective process that depends on the chain length and degree of unsaturation, with short and unsaturated fatty acids being mobilised first (Raclot *et al.*, 2001 and Raclot, 2003). If the same mechanism occurs in lactating seals, one can suggest that the mobilisation of apolar molecules such as PCBs remains slow at the beginning of the fast, when more hydrophilic fatty acids are mobilised from the adipose tissue (Debier *et al.*, 2006). PCBs would follow the mobilisation of less hydrophilic fatty acids, which are liberated later. The same hypothesis may be applied for vitamin A. However, a recent study investigating the mobilisation of blubber fatty acids in lactating Weddell seals (Wheatley *et al.*, 2008) showed that, even if there is evidence for a selective mobilisation of fatty acids during lactation, some of them did not conform to the molecular structure of those reported in Raclot (2003).

Specific mechanisms of storage and mobilisation of vitamin A are involved through the synthesis of CRBPI and RBP (the main serum carrier for retinol). Potential variations in the synthesis of those proteins over the course of lactation may also be at the origin of the observed phenomenon.

### Maternal serum

Vitamin A levels in maternal serum were quite stable during the first half of lactation and then underwent a slight increase at the end of lactation. Other studies of lactating seals also mentioned an increase between early and late lactation in maternal serum (Debier *et al.*, 2002 and Schweigert *et al.*, 2002). This slight increase at late lactation could be explained by the mobilisation of less hydrophilic triglycerides at the end of lactation as mentioned above. Other possible explanations are that the retention capacity of inner blubber for vitamin A is reached at late lactation, or that a greater amount of RBP is being synthesised in this layer at the end of the nursing period. In each case, the result will be a greater transfer of vitamin A in the bloodstream (Debier *et al.*, 2002, Debier *et al.*, 2004 and Debier *et al.*, 2005a). The dynamics of circulating PCBs were fairly similar to those of vitamin A and could also result from the pattern of mobilisation observed in the adipose tissue.

### Milk

As already observed in seal milk (Debier *et al.*, 2002, Debier *et al.*, 2003, Debier *et al.*, 2004 and Schweigert *et al.*, 2002), vitamin A and PCB levels increased at the end of lactation and did not follow the same pattern as milk lipids, which increased earlier. Such a curious parallelism between vitamin A and PCBs had already been observed in grey seal milk and the authors suggested a common mechanism of mobilisation from maternal blubber (Debier *et al.*, 2004). The present study brings additional data that go in the sense of this hypothesis.

### Pup serum

Reassuringly, the increase in vitamin A and PCBs observed in milk was coupled with an increase of both types of compounds in pup serum. Thus measurements of these compounds in pup sera offer a good indication of maternal milk levels. At late lactation, mean levels of both types of compounds were higher in pup sera than in their

mothers' sera. The dynamics observed here corroborate previous studies and could be explained by the high levels of vitamin A and PCBs found in milk and the important daily milk intake at the end of lactation (Pomeroy *et al.*, 1996, Debier *et al.*, 2002 and Debier *et al.*, 2003).

### 2.4.2. Relationships between compartments and effects of PCBs

The statistical model used in the present study highlighted several correlations between vitamin A and PCBs in the same compartment but also between compartments. However, because of the small size of sample ($N = 17$) and of the uneven number of samplings for some animals, certain explanatory variables, which may have explained the dynamics observed between compartments, were not significant.

#### Maternal blubber

Vitamin A levels in inner blubber were negatively correlated with the total body fat of the female. This result is in agreement with the hypothesis suggesting a sequestration and a redistribution of vitamin A in the reduced inner layer, as discussed in the previous section.

#### Maternal serum

The small number of observations for the regression in maternal serum allows us to consider the “vitamin A inner blubber” variable as significant. Therefore, a positive correlation between vitamin A in maternal serum and vitamin A contained in the blubber can be highlighted.

#### Milk

The positive correlation found between vitamin A in maternal serum and vitamin A in milk indicates that the vitamin A level found in

milk depends on the supply to the mammary gland. Vitamin A also showed a positive correlation with time.

### Pup serum

Pups depend on the vitamin A ingested via milk. It should be noted that the concentration of vitamin A in pup serum increased with time, suggesting that its storage in tissues such as liver and blubber does not compensate for its dietary supply. Two interesting relationships between vitamin A and PCBs were revealed. Levels of vitamin A in pup serum were affected negatively by the PCBs present in their serum and in milk. Negative relationships between PCBs and vitamin A have already been reported in serum (Rolland, 2000, Simms *et al.*, 2000, Simms and Ross, 2000, Jenssen *et al.*, 2003 and Debier *et al.*, 2005b), but this is the first study to display a negative impact of PCBs in milk on circulating vitamin A levels in pups.

## 2.5. Conclusions

Grey seal mothers fast throughout their short lactation period and during that time, large amounts of essential nutrients, such as vitamin A, are transferred into the milk to satisfy the demand of the newborn. At the same time, fat-soluble pollutants such as PCBs are also transferred to pups. We observed that in most of the body compartments studied, concentrations of vitamin A and PCBs increased at late lactation. The hypothesis of a similar mobilisation from maternal blubber could be investigated thanks to the incorporation of vitamin A blubber data. Moreover, the statistical model used here highlighted relationships between vitamin A in pup serum and PCBs in milk and pup serum. A general scheme of the dynamics of vitamin A and PCBs and the relationships found between both types of compounds is represented in Figure 2.1.

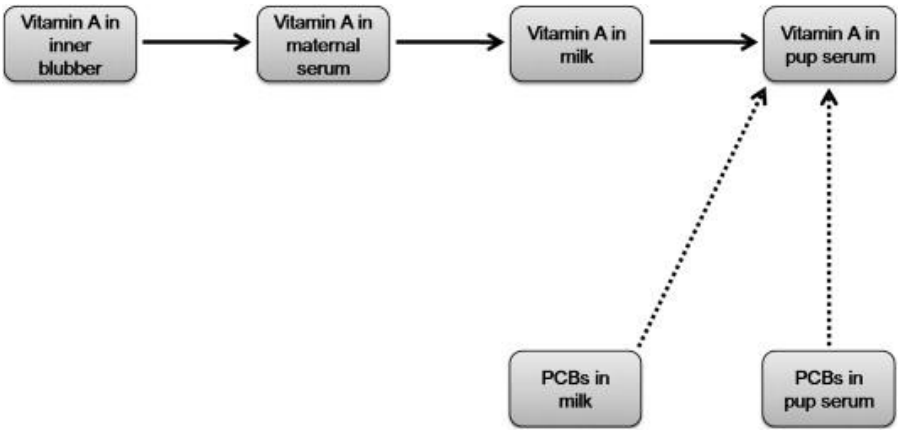


**Figure 2.1.** Schematic representation of the dynamics of vitamin A from maternal blubber to pup serum and impacts of PCBs on the vitamin A status in pup serum. Solid arrows show the positive correlations between the different compartments. Dotted arrows represent the negative relationships of PCBs in milk and in pup serum on vitamin A in pup serum.

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# Chapter 3

# Selective transfer of persistent organic pollutants and their metabolites in grey seals during lactation

## Foreword

In the previous chapter we have confirmed that PCBs were highly transferred from the mother to its pup during lactation in grey seal. We decided thus to investigate thoroughly the lactational transfer of additional POPs, such as the more recent PBDEs for which information in this field is still scarce. Additionally, although studies on humans showed that the transfer of HO-PCBs via the milk was limited[2,3], no data about metabolites are available for marine mammals. Therefore, we attempted to examine the lactational transfer of HO-PCBs as well as HO-PBDEs and the naturally-produced MeO-PBDEs in our grey seals. Changes in concentrations and profiles of PBDEs, PCBs, OCPs, their metabolites and MeO-PBDEs were examined at early and late lactation in maternal blubber (except for HO-metabolites), serum, milk and pup serum. This study has been

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published in *Environment International*. Some changes have been introduced in the original published version. These changes do not modify the conclusions of the published version. Therefore, this chapter is a redraft after the published version.

# Selective transfer of persistent organic pollutants and their metabolites in grey seals during lactation

*Redrafted after*

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Authors' contributions

The work presented here was carried out in collaboration between all authors. MVB and CD defined the research theme. SH and PP participated in the sample collection. MVB, LW and AC carried out the laboratory experiments. PP, AC and CD contributed to reagents/materials /analysis tools. MVB and CB performed the statistical analyses. MVB analysed the data. MVB, LW, AC and CD were engaged in active discussions. MVB wrote the paper. SH, KD, JFR and PP provided advice on the manuscript. CD supervised the work. All authors have contributed to, seen and approved the manuscript.

# Abstract

Twenty grey seal (*Halichoerus grypus*) mother–pup pairs from the colony of the Isle of May (Scotland) were sampled at early and late lactation in order to study the transfer of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and their metabolites (HO-PCBs and HO-PBDEs) as well as organochlorine pesticides (OCPs), such as DDT and metabolites (DDXs) and hexachlorobenzene (HCB). The transfer of the naturally produced MeO-PBDEs was also investigated. Generally, concentrations (on a lipid weight basis) of the sum of PCBs, PBDEs and DDXs tended to be higher in all tissues at late lactation (for maternal outer blubber ΣPCBs = 3860 ± 2091 ng/g, ΣPBDEs = 120 ± 74 ng/g and ΣDDXs = 559 ± 207 ng/g; for maternal inner blubber ΣPCBs = 4229 ± 3274 ng/g, ΣPBDEs = 148 ± 118 ng/g and ΣDDXs = 704 ± 353 ng/g; for maternal serum ΣPCBs = 1271 ± 796 ng/g, ΣPBDEs = 27 ± 16 ng/g and ΣDDXs = 242 ± 125 ng/g; for milk ΣPCBs = 1190 ± 747 ng/g, ΣPBDEs = 55 ± 36 ng/g and ΣDDXs = 357 ± 160 ng/g; for pup serum ΣPCBs = 1451 ± 901 ng/g, ΣPBDEs = 48 ± 31 ng/g and ΣDDXs = 395 ± 201 ng/g). In all tissues, ΣMeO-PBDEs were found at very low levels or even undetected and their concentrations appeared to increase at late lactation only in maternal inner blubber (2.7 ± 1.3 to 5.3 ± 2.9 ng/g for early and late lactation, respectively) and milk (0.6 ± 0.3 to 1.1 ± 0.5 ng/g for early and late lactation, respectively). The transfer from inner blubber to maternal serum was selective and strongly depended on the log $K_{ow}$ value of the compounds, with less lipophilic compounds being more efficiently released. Only a limited amount of HO-PCBs was transferred during lactation as 4-HO-CB-107 was the only metabolite detected in milk (29 to 40 pg/g lw). On the contrary, most of HO-PCB metabolites found in maternal serum were also detected in pup serum. These findings suggest not only a transplacental transfer of HO-PCBs from mothers to pups but also the possibility of endogenous biotransformation in suckling pups or accumulation of undetectable low amounts from milk.

**Keywords:** Grey seal, organochlorines, maternal transfer, blubber, milk, serum

## 3.1. Introduction

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs) are well known anthropogenic and lipophilic compounds that bioaccumulate throughout the trophic chain. PCB and PBDE metabolites such as hydroxylated PCBs (HO-PCBs) and hydroxylated PBDEs (HO-PBDEs) may have comparable or even higher toxic effects than their parent compounds in mammals, especially on thyroid and vitamin A metabolism (Meerts *et al.*, 2000; Meerts *et al.*, 2002). Although differences among species exist, it has been suggested that the formation of HO-PCBs results from the metabolic biotransformation of PCBs by cytochrome P450 enzymes (CYPs) in order to make them more water soluble and thus easier to eliminate. However, due to their high affinity for blood proteins (Letcher *et al.*, 2000), specific metabolites were found to be retained in human (Dirtu *et al.*, 2010) and marine mammal blood (Weijs *et al.*, 2009c; Gabrielsen *et al.*, 2011). By contrast, HO-PBDEs can be either biotransformation products of synthetic PBDEs mediated by CYPs or produced by marine organisms such as sponges or algae (Vetter *et al.*, 2002; Malmvärn *et al.*, 2005; Wiseman *et al.*, 2011). They can also result from the demethylation of other naturally-produced PBDE analogs, the methoxylated polybrominated diphenyl ethers (MeO-PBDEs), as demonstrated in microsomal fractions of several species of vertebrates (Wan *et al.*, 2009; Wiseman *et al.*, 2011). MeO-PBDEs have already been detected in wildlife and marine mammals at concentrations sometimes greater than PBDEs (Teuten *et al.*, 2005; Wan *et al.*, 2009; Weijs *et al.*, 2009a; Weijs *et al.*, 2010).

Because seals are top predators, have a long life span and are characterised by high lipid content (blubber), large concentrations of POPs are found in their tissues, especially in the blubber (Sørmo *et al.*, 2003; Weijs *et al.*, 2009b). When food intake is irregular or nonexistent during breeding, lactation and moulting in most species of seals, important amounts of POPs are mobilised from blubber into the bloodstream, exposing targeted organs to increased concentrations of toxicants (Debier *et al.*, 2006; Vanden Berghe *et al.*, 2010).

During lactation, seals produce lipid-rich milk containing high levels of POPs (Debier *et al.*, 2003b; Sørmo *et al.*, 2003; Vanden Berghe *et al.*, 2010; Frouin *et al.*, 2012). Suckling newborns are thus exposed to high amounts of toxic chemicals, while being in a critical developmental period of their life. Previous studies have shown that pup or juvenile seals exposed to POPs, even to low concentrations, had reduced immune competence and impairment of thyroid hormone and vitamin A homeostasis (Simms *et al.*, 2000; Hall *et al.*, 2003; Mos *et al.*, 2007; Sørmo, 2009). The lactational transfer of PCBs and OCPs in seals is more important at late lactation (Debier *et al.*, 2003b). In addition, it appears to be selective, with a preferential transfer of mostly less lipophilic compounds from mother to pup, as shown for grey seals (*Halichoerus grypus*) (Pomeroy *et al.*, 1996; Debier *et al.*, 2003a,; Sørmo *et al.*, 2003). The lactational transfer of PBDEs in marine mammals has been much less investigated. Studies on grey seals and hooded seals (*Cystophora cristata*) also report a selective transfer of PBDEs during lactation (Wolkers *et al.*, 2006; Ikonomou and Addison, 2008). However, those studies used a limited number of mother–pup pairs (5 to 6) and sampled only once during the lactation period. Therefore, the temporal trend in the transfer could not be established. To the best of our knowledge, only one recent study examined the changes in PBDE concentrations occurring in tissues of mothers and pups harp seal (*Phoca groenlandica*) sampled twice during the lactation (Frouin *et al.*, 2012). However, as for the previous studies, only a limited number of individuals could be sampled (n = 6). In addition, the authors discussed their results for total PBDEs rather than for each single PBDE congener.

The presence and activities of CYPs in several species of seals (Nyman *et al.*, 2001; Wolkers *et al.*, 2002) suggest that they are theoretically able to produce HO-PCBs and HO-PBDEs. Indeed, HO-PCBs were detected in significant amounts in adult and pup seals (Løken *et al.*, 2008; Gabrielsen *et al.*, 2011). On the contrary, no or only low levels of HO-PBDEs were found in serum or plasma of harbour (*Phoca vitulina*) and ringed (*Phoca hispida*) seals (Routti *et al.*, 2009; Weijs *et al.*, 2009c), suggesting that formation or retention of these molecules is low in these animals. Studies on humans and rodents reported that HO-PCBs were transferred to offspring mainly

through the placenta during gestation and to a lesser extent during lactation (Meerts *et al.*, 2002; Guvenius *et al.*, 2003; Fangström *et al.*, 2005). However, information concerning the maternal transfer of HO-PCBs and HO-PBDEs in marine mammals is lacking. Gabrielsen *et al.* (2011) found positive correlations between serum HO-PCB levels and the age and body mass of hooded seal pups. The authors suggested thus a transfer of HO-PCBs via the milk or an endogenous biotransformation of PCBs in pups. Nevertheless, that study did not investigate changes occurring during lactation and the presence of HO-PCBs in milk.

The present study investigated the lactational transfer of several POPs in grey seals. Grey seal females fast during a short lactation period (16 to 21 days), while producing milk rich in lipids (up to 60%) (Iverson *et al.*, 1993; Pomeroy *et al.*, 1996). During that time, females can lose between 25 and 50% of their initial body mass (Iverson *et al.*, 1993; Pomeroy *et al.*, 1999). In contrast, due to the important daily ingestion of milk, suckling pups rapidly gain mass (between 0.8 and 2.8 kg/day) (Iverson *et al.*, 1993; Pomeroy *et al.*, 1999). Maternal blubber, maternal serum, milk and pup serum from 20 mother–pup pairs were collected at early and late lactation in order to examine the changes in levels and profiles of PCBs, PBDEs, their related metabolites (HO-PCBs and HO-PBDEs), OCPs and MeO-PBDEs between early and late lactation. To the best of our knowledge, this is the first study to investigate the levels and profiles of PBDEs, HO-PCBs, HO-PBDEs and MeO-PBDEs in tissues of grey seal mother–pup pairs sampled at different times of the lactation period.

## 3.2. Material and methods

### 3.2.1. Seal sampling

Twenty grey seal mother–pup pairs from the Isle of May (IOM), Scotland, were studied during the breeding season in November–December 2008. On their arrival on the Isle, females were monitored and dates of birth were recorded by daily observations of the breeding

areas. Animals were sampled at early (days 2–5) and at late (days 14–17) lactation in order to collect maternal blubber, serum and milk as well as pup serum samples. Pup blubber samples could not be collected due to permit limitation.

**Table 3.1.** Morphometric data of the mother-pup pairs of grey seals (*Halichoerus grypus*) sampled from the breeding population on the Isle of May (Scotland) during the 2008 breeding season.

| ID | Female mass (kg) | | Pup sex | Pup age (day) | | Pup mass (kg) | |
|---|---|---|---|---|---|---|---|
| | *Early lactation* | *Late lactation* | | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* |
| OJ | 179 | 133 | M | 3 | 16 | 18 | 39 |
| OH | 151 | 102 | M | 4 | 17 | 24 | 50 |
| 4H | 139 | 104 | F | 4 | 15 | 17 | 34 |
| 4U | 131 | 90 | F | 2 | 16 | 15 | 33 |
| 5B | 168 | 130 | M | 2 | 15 | 17 | 37 |
| 5H | 203 | 149 | F | 2 | 17 | 16 | 40 |
| 6L | 232 | 182 | M | 3 | 17 | 17 | 35 |
| 7J | 134 | 104 | M | 3 | 16 | 15 | 32 |
| 7V | 187 | 129 | M | 2 | 16 | 18 | 48 |
| M10 | 154 | 114 | F | 3 | 15 | 19 | 43 |
| M11 | 125 | 100 | M | 4 | 15 | 20 | 35 |
| M20 | 187 | 151 | F | 4 | 16 | 17 | 36 |
| M24 | 159 | 128 | F | 2 | 14 | 16 | 26 |
| M25 | 158 | 127 | F | 3 | 15 | 18 | 37 |
| M28 | 146 | 102 | M | 3 | 16 | 17 | 43 |
| M29 | 163 | 114 | F | 3 | 16 | 18 | 47 |
| M33 | 181 | 128 | M | 5 | 18 | 21 | 49 |
| M44 | 186 | 145 | F | 6 | 15 | 20 | 47 |
| M46 | 127 | 103 | M | ? | ? + 10 | 12 | 24 |
| M6 | 160 | 127 | F | 2 | 15 | 15 | 33 |

? = Not determined

However, Frouin *et al.* (2011) reported a strong relationship between ΣPBDE levels in blubber and serum of harbour, harp and grey seal pups. The investigators concluded that serum was a reliable indicator for ΣPBDE contaminations in seal pups. In addition, another study from the same authors (Frouin *et al.*, 2012) recently measured several

POPs in harp seal pup blubber and serum and the concentrations (expressed per unit of lipid weight) reported in both tissues fell within the same ranges. Data concerning pup age as well as body weights of mothers and pups at each capture are presented in Table 3.1. Sampling techniques are described elsewhere (Debier *et al.*, 2002 and 2003b). Animals were handled and weighed under UK Home Office licence as described in Pomeroy *et al.* (1996). All the samples were stored at − 20 °C until analyses.

### 3.2.2. Analysis

In all samples, 35 PCB congeners (IUPAC numbers: CB-18, -28, -44, -47, -49, -52, -87, -95, -99, -101, -105, -110, -118, -128, -138, -146, -149, -151, -153, -156, -158, -170, -171, -172, -174, -177, -180, -183, -187, -194, -195, -199, -205, -206 and -209), 6 PBDEs (IUPAC numbers: BDE-28, -47, -99, -100, -153 and -154), 6 DDXs (*o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDT), HCB and 2 MeO-PBDEs (2'-MeO-BDE-68 and 6-MeO-BDE-47) were targeted. Additionally, twenty HO-PCB congeners were investigated in serum and milk: 3-HO-CB (118, 138, 153, 180), 4-HO-CB (109, 120, 127, 130, 146, 162, 163, 172, 177, 187, 193, 198, 199, 202, 208) and 4-diHO-CB202. Three HO-PBDEs were targeted in all serum and milk samples as well (6-HO-BDE-47, 5-HO-BDE-47 and 4-HO-BDE-49).

The method used for the extraction and clean-up of blubber samples is described in details in Covaci *et al.* (2008). In brief, approximately 150 mg of each end of the blubber biopsy (outer and inner parts) was homogenised with anhydrous $Na_2SO_4$ and internal standards (CB 143 and BDE 77) were added. The samples were extracted by hot Soxhlet with hexane/acetone (3/1; v/v) and the remaining extract was cleaned up on acid silica. After elution of the analytes with 20 ml hexane and 15 ml dichloromethane (DCM), the cleaned extract was evaporated and dissolved in 150 µl iso-octane.

Serum samples were analysed as already described in Weijs *et al.* (2009c) with small adaptations in order to include the analysis of MeO-PBDEs. Briefly, serum (about 1.5 ml) was spiked with internal

standards (CB-143 and BDE-77 for compounds eluting in the 1st fraction, e.g. neutrals; 4′-HO-CB-159 and 3-MeO-BDE-28 for compounds eluting in the 2nd fraction, e.g. phenolics and MeO-PBDEs), diluted with Milli-Q water, mixed with formic acid and sonicated for 20 min. The samples were extracted on solid-phase-extraction (SPE) cartridges (Oasis HLB, 3 ml/200 mg) and eluted with MeOH/DCM (1/1; v/v). The extract was evaporated to dryness and reconstituted in 500 µl hexane before being fractionated on silica SPE cartridges (3 ml/500 mg). The 1st fraction containing neutral compounds (PCBs, PBDEs and OCPs) was eluted with 6 ml hexane, evaporated to dryness and resolubilised in 100 µl iso-octane. The 2nd fraction containing the phenolic compounds (HO-PCBs and HO-PBDEs) and MeO-PBDEs, was eluted with 8 ml DCM, evaporated to dryness and derivatised for 30 min with trimethylsilyldiazomethane to form methyl-derivatives. After solvent evaporation, the dry residue was resolubilised in 75 µl iso-octane.

The same extraction method as for blubber was applied to milk samples with minor adaptations. Approximately 400 mg of milk was mixed with anhydrous $Na_2SO_4$ and spiked with internal standards CB-143, BDE-77 and 4′-HO-CB-159. After extraction by hot Soxhlet and clean-up, the extract was eluted with a 50 ml hexane/DCM mixture (1/1; v/v), evaporated and dissolved in 150 µl iso-octane. After analysis of the neutral compounds, the extract was further fractionated on silica SPE cartridges, as described above for serum samples. The 2$^{nd}$ fraction was derivatised, evaporated and resolubilised in 50 µl iso-octane.

Lipid determination for blubber and milk was performed gravimetrically on an aliquot of the extract directly after the Soxhlet extraction. For serum, the different lipid classes (total cholesterol (CHOL), phospholipids (PPL), triacylglycerols (TG) and nonesterified fatty acids (NEFA)) were determined using enzymatic kits from Diasys (Diasys Diagnostics System, Holzheim, Germany) and Wako (Wako Chemicals USA Inc., Richmond, VA). Serum total lipid concentrations were calculated by summing the individual lipid classes. The concentrations of the different lipid classes were

calculated on the basis of standard equivalents, as recommended by the respective kits.

Quality assurance and quality control were performed through the analysis of procedural blanks and a standard reference material from the National Institute of Standards and Technology (NIST) (SRM 1945, OCPs, PCBs, and PBDEs in whale blubber). For the SRM 1945, the relative standard deviations (RSD) were < 10% for most analytes. Additionally, the method performance was assessed through successful participation to interlaboratory studies organised by NIST. Procedural blanks were consistent (RSD < 20%) and therefore, the mean value of each analyte in the procedural blanks was used for subtraction. Method quantification limits (LOQs) for individual OCPs, PCB and PBDE congeners were based on procedural blanks (3 times the standard deviation) and the amount of sample taken for analysis.

For OCPs, PCBs and PBDEs in serum, QC was assessed through regular participation to interlaboratory comparison exercises organised by AMAP (POPs in serum). Obtained values were deviating no more than 20% from the consensus values. For HO-PCBs and MeO-PBDEs, the mean recovery of internal standards 4′-HO-CB 159 and 3-MeO-BDE28 in serum was 96 ± 2% and 94 ± 5%, respectively. Recoveries of individual HO-PCBs and MeO-PBDEs assessed through spiking experiments at 25 and 125 pg/ml ranged between 90 and 93% with good precision (RSD < 5%).

All compounds were measured with an Agilent 6890 gas chromatograph coupled with a 5973 mass spectrometer system (GC–MS). For the analysis of PBDEs and phenolics, the MS operated in electron capture negative ionisation (ECNI) mode equipped with a 30 m × 0.25 mm × 0.25 µm DB-5 capillary column. For the PCB, HCB and DDX analysis, the MS was operated in electron ionisation (EI) mode and was equipped with a 25 m × 0.22 mm × 0.25 µm HT-8 capillary column. The MS was used in the SIM mode with 2 ions monitored for each PCB homologue group.

### 3.2.3. Partitioning between tissues

The ratios between maternal blubber and serum as well as between maternal serum and milk were calculated for all POPs, using lipid adjusted measurements. To do so, levels detected in maternal serum were divided by levels detected in maternal inner blubber (partitioning between blubber and serum) and levels found in milk were divided by levels found in maternal serum (partitioning between maternal serum and milk). Values > 1 indicate a higher preference of the compounds for maternal serum or milk compared to maternal inner blubber or maternal serum, respectively. These ratios were calculated only for late lactation as this is the stage of lactation with the highest POP transfer (Debier *et al.*, 2003b; Sørmo *et al.*, 2003; Vanden Berghe *et al.*, 2010).

### 3.2.4. Statistical analyses

Statistical analyses were conducted using SPSS 17 for Windows. Contaminant levels were LN-transformed to normalise the data and to reduce inter-individual variability. For concentrations below the limit of quantification (LOQ), a value of LOQ*detection frequency (f) for each tissue was used. Linear mixed models (LMMs) were used to test differences in contaminant concentrations and profiles between both lactation periods within the same tissue. The individual was considered as a random explanatory variable, while the lactation period (early and late) was determined as a fixed variable. When possible, the transfer of contaminants between tissues (maternal blubber → maternal serum → milk → pup serum) was investigated by adding the concentrations found in the "previous" tissue in the pre-cited model (e.g., PCB levels found in maternal inner blubber were used as a fixed variable in order to explain PCB levels in maternal serum). The level of statistical significance was set at $p \leq 0.05$ for all analyses.

## 3.3. Results

### 3.3.1. Lipids

The lipid percentage of maternal inner and outer layers decreased significantly between early and late lactation (Table 3.2). In maternal serum samples, the PPL fraction was the most important for both periods, representing about 60% of the total lipid content (Table 3.3).

The total lipid content in maternal serum decreased significantly between early and late lactation (Table 3.2). On the other hand, the lipid percentage in milk (hexane/acetone-extractable fat) significantly increased at late lactation (Table 3.2). Similar to maternal serum, the dominant lipid class in pup serum was PPL, representing about 45% of the total lipid content (Table 3.3). The total lipid content in pup serum increased significantly between early and late lactation (Table 3.2). Changes in PCB, PBDE, OCP and MeO-PBDE levels between early and late lactation in all tissues were discussed on a lipid weight basis. However, since HO-PCBs and HO-PBDEs have also a high affinity for other serum constituents than lipids, such as blood proteins, changes in hydroxylated metabolite levels between both lactation periods in serum were examined on a wet weight basis.

**Table 3.3.** Mean ± standard deviation of the lipid fractions (%) measured in maternal and pup serum of 20 grey seal mother-pup pairs.

| Lipid fraction (%) | Maternal serum | | Pup serum | |
|---|---|---|---|---|
| | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* |
| TG | 0.01 ± 0.00 | < 0.01 | 0.25 ± 0.12 | 0.38 ± 0.13 |
| CHOL | 0.24 ± 0.04 | 0.18 ± 0.05 | 0.21 ± 0.03 | 0.29 ± 0.04 |
| NEFA | 0.04 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| PLL | 0.54 ± 0.07 | 0.36 ± 0.09 | 0.44 ± 0.07 | 0.57 ± 0.07 |
| **Σ lipids** | **0.82 ± 0.11** | **0.58 ± 0.13** | **0.93 ± 0.18** | **1.27 ± 0.21** |

TG = Triacylglycerols; CHOL= Total cholesterol; NEFA = Non Esterified Fatty Acids; PPL = Phospholipids

**Table 3.2.** Mean ± standard deviation (expressed per unit of lipid weight) of levels of PCBs, PBDEs, OCPs and MeO-PBDEs measured in maternal blubber and serum, milk and pup serum of 20 grey seal mother-pup pairs.

| | Outer blubber (ng/g)* | | Inner blubber (ng/g)* | | Maternal serum (ng/g)* | | Milk (ng/g)* | | Pup serum (ng/g)* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* |
| Lipid content (%) | 86.7 ± 3.8 | 77.5 ± 8.8£ | 84.6 ± 5.0 | 66.9 ± 12.9£ | 0.8 ± 0.1 | 0.6 ± 0.1£ | 44.9 ± 6.0 | 57.5 ± 5.2§ | 0.9 ± 0.2 | 1.3 ± 0.2 |
| Σ tri-CBs | 1 ± 0 | 7 ± 12 | 1 ± 0 | 4 ± 7 | N.D | N.D | N.D | N.D | 2 ± 1 | 5 ± 1 |
| Σ tetra-CBs | 17 ± 6 | 21 ± 11 § | 15 ± 4 | 27 ± 16§ | 14 ± 5 | 19 ± 6 | 17 ± 5 | 20 ± 7 | 23 ± 9 | 34 ± 13§ |
| Σ penta-CBs | 146 ± 61 | 179 ± 76§ | 116 ± 40 | 260 ± 146§ | 65 ± 2 | 103 ± 45§ | 83 ± 27 | 118 ± 52 | 115 ± 49 | 182 ± 84§ |
| Σ hexa-CBs | 1431 ± 895 | 1913 ± 1010§ | 850 ± 435 | 2292 ± 1709§ | 350 ± 136 | 770 ± 476 | 416 ± 151 | 726 ± 469 | 585 ± 269 | 865 ± 551§ |
| Σ hepta-CBs | 928 ± 705 | 1301 ± 802§ | 446 ± 360 | 1285 ± 1092§ | 114 ± 51 | 329 ± 235§ | 134 ± 53 | 280 ± 206 | 199 ± 102 | 309 ± 227§ |
| Σ octa-CBs | 230 ± 198 | 326 ± 221§ | 87 ± 94 | 265 ± 258§ | 12 ± 10 | 43 ± 42 | 34 ± 14 | 41 ± 32 | 29 ± 18 | 47 ± 40§ |
| Σ nona/deca-CBs | 79 ± 73 | 114 ± 82§ | 28 ± 38 | 97 ± 106§ | 2 ± 2 | 8 ± 12 | 4 ± 2 | 6 ± 6 | 5 ± 5 | 9 ± 9§ |
| **Σ PCBs** | **2832 ± 1870** | **3860 ± 2091§** | **1543 ± 922** | **4229 ± 3274§** | **556 ± 210** | **1271 ± 796** | **688 ± 234** | **1190 ± 747** | **958 ± 431** | **1451 ± 901§** |
| BDE 28 | 0.4 ± 0.2 | 0.5 ± 0.2§ | 0.4 ± 0.2 | 0.7 ± 0.4§ | N.D | N.D | 0.2 ± 0.1 | 0.3 ± 0.2§ | 0.3 ± 0.2 | 0.3 ± 0.2 |
| BDE 47 | 52.5 ± 30.2 | 64.4 ± 37.8§ | 40.5 ± 21.5 | 87.3 ± 60.7§ | 10.8 ± 5.5 | 18.8 ± 11.2 | 23.8 ± 12.6 | 42.7 ± 27.1 | 27.7 ± 13.8 | 38.2 ± 23.8§ |
| BDE 99 | 5.8 ± 3.3 | 8.5 ± 6.6§ | 4.7 ± 4.6 | 12.8 ± 14.0§ | 0.7 ± 0.4 | 1.9 ± 1.7 | 1.3 ± 0.9 | 3.0 ± 3.1 | 3.3 ± 8.3 | 2.2 ± 2.1 |
| BDE 100 | 12.8 ± 10.2 | 16.9 ± 11.2§ | 7.9 ± 6.1 | 21.7 ±19.9§ | 1.1 ± 0.6 | 3.2 ± 2.2§ | 2.5 ± 1.2 | 5.6 ± 4.2 | 2.8 ± 1.4 | 4.1 ± 2.9 |
| BDE 153 | 12.2 ± 11.2 | 17.6 ± 12.6§ | 5.2 ± 7.0 | 13.0 ± 13.5§ | 0.4 ± 0.3 | 1.3 ± 1.0§ | 0.8 ± 0.5 | 1.7 ± 1.5 | 1.4 ± 0.6 | 1.8 ± 1.2 |
| BDE 154 | 8.7 ± 11.5 | 11.8 ± 12.8§ | 4.5 ± 5.2 | 11.6 ± 13.2§ | 0.3 ± 0.2 | 1.3 ± 1.0§ | 0.7 ± 0.5 | 1.6 ± 1.7 | 1.0 ± 0.9 | 1.3 ± 1.7 |
| **Σ PBDEs** | **93.0 ± 62.5** | **120.3 ± 74.2§** | **63.6 ± 44.8** | **148.1± 117.7§** | **13.4 ± 7.0** | **26.5 ± 16.0** | **29.4 ± 15.1** | **54.9 ± 36.3** | **36.4 ± 16.3** | **48.0 ± 30.5** |
| *p,p'*-DDD | 5 ± 3 | 6 ± 4 | 6 ± 3 | 10 ± 7§ | 2 ± 2 | 3 ± 3§ | 7 ± 3 | 9 ± 3§ | < 1 | 1 ± 2 |
| *p,p'*-DDE | 413 ± 184 | 509 ± 190§ | 294 ± 96 | 637 ± 316§ | 147 ± 50 | 218 ± 111§ | 203 ± 70 | 313 ± 138§ | 259 ± 113 | 373 ± 186§ |
| *p,p'*-DDT | 34 ± 24 | 44 ± 25§ | 24 ± 15 | 57 ± 42§ | 14 ± 5 | 20 ± 14 | 21 ± 8 | 35 ± 21§ | 17 ± 9 | 21 ± 14 |
| **Σ DDXs** | **452 ± 201** | **559 ± 207§** | **323 ± 106** | **704 ± 353§** | **162 ± 55** | **242 ± 125** | **230 ± 78** | **357 ± 160§** | **276 ± 120** | **395 ± 201§** |
| **HCB** | **7 ± 8** | **9 ±11§** | **7 ± 8** | **11 ± 13** | **7 ± 8** | **7 ± 10** | **11 ± 10** | **12 ±13** | **16 ± 23** | **17 ± 20** |
| 6-MeO-BDE47 | 2.3± 0.0 | 2.4 ± 1.2 | 2.2± 1.1 | 4.2 ± 2.3§ | 0.4± 0.2 | 0.6 ± 0.3 | 0.5± 0.2 | 0.9 ± 0.5§ | N.D | N.D |
| 2'-MeO-BDE68 | 0.6 ± 0.3 | 0.6 ± 0.3 | 0.6 ± 0.3 | 1.2 ± 0.7§ | N.D | N.D | 0.1 ± 0.0 | 0.2 ± 0.1§ | N.D | N.D |
| **Σ MeO-PBDEs** | **2.9 ± 1.1** | **3.1 ± 1.4** | **2.7 ± 1.3** | **5.3 ± 2.9§** | **0.4± 0.2** | **0.6 ± 0.3** | **0.6 ± 0.3** | **1.1 ± 0.5§** | **N.D** | **N.D** |

For a defined tissue, values within a row followed by £ are significantly lower at late lactation ($p \leq 0.05$). For a defined tissue, values within a row followed by § are significantly higher at late lactation ($p \leq 0.05$). N.D = Not detected; N.M = Not measured.

*Statistics were performed on LN transformed values

### 3.3.2. PCBs

PCB concentrations were the highest of all POPs measured in the investigated tissues (Table 3.2). In all tissues and for both periods, CB-153 was the predominant PCB congener followed by CB-138 or CB-180. Together, these three PCB congeners accounted for more than 55% of the total PCBs. CB-99, -101, -118, -128, -146, -149, -170, -183 and -187 were also systematically found in all samples but their contribution varied with the tissue and the period. The detection frequency of the different congeners in a given tissue can be found in Table 3.4a.

The statistical treatments were performed on congener groups according to their degree of chlorination, namely tri-CBs (CB-18 and -28), tetra-CBs (CB-44, -47, -49 and -52), penta-CBs (CB-87, -95, -99, -101, -105, -110 and -118), hexa-CBs (CB-128, -132, -146, -149, -151, -153 and -156), hepta-CBs (CB-170, -171, -172, -174, -177, -180, -183 and -187), octa-CBs (CB-194, -195, -199 and -205) and nona/deca-CBs (CB-206 and -209). Because tri-CBs were only detected at very low concentrations in a limited number of samples in all investigated tissues statistical analyses were not carried out for this group.

#### Variation in concentrations

Significantly higher concentrations were found at late lactation in maternal outer and inner blubber for ΣPCBs and this observation was true for all PCB groups ($p$-values between < 0.001 and 0.009) (Table 3.2). In maternal serum, we observed an increase in all PCB groups and thus ΣPCBs at late lactation (Table 3.2). However, this increase appeared to be significant only for the penta- and hepta-CBs ($p_{5\text{-}Cl} < 0.011$ and $p_{7\text{-}Cl} = 0.019$) (Table 3.2). In milk, although concentrations of ΣPCBs and all PCB groups tended to be higher at late lactation, no significant change could be found (Table 3.2).

**Table 3.4a.** Detection frequency (f, in %) and limit of quantification (LOQ, on a lipid weight basis) for each PCB congener in the different investigated tissues.

| Congener | f MB | LOQ MB (ng/g) | f MS | LOQ MS (ng/g) | f M | LOQ M (ng/g) | f PS | LOQ PS (ng/g) |
|---|---|---|---|---|---|---|---|---|
| PCB 18 | 10 | 10.0 | 0 | 5.0 | 0 | 5.0 | 0 | 5.0 |
| PCB 28 | 1 | 10.0 | 0 | 5.0 | 0 | 5.0 | 40 | 5.0 |
| PCB 44 | 0 | 10.0 | 0 | 4.0 | 8 | 5.0 | 8 | 4.0 |
| PCB 47 | 17 | 10.0 | 47 | 4.0 | 65 | 5.0 | 85 | 4.0 |
| PCB 49 | 1 | 10.0 | 5 | 4.0 | 20 | 5.0 | 53 | 4.0 |
| PCB 52 | 86 | 10.0 | 93 | 4.0 | 98 | 5.0 | 100 | 4.0 |
| PCB 87 | 27 | 5.0 | 2 | 2.0 | 93 | 2.5 | 53 | 2.0 |
| PCB 95 | 83 | 5.0 | 37 | 2.0 | 98 | 2.5 | 88 | 2.0 |
| PCB 99 | 100 | 5.0 | 100 | 2.0 | 100 | 2.5 | 100 | 2.0 |
| PCB 101 | 100 | 5.0 | 100 | 2.0 | 100 | 2.5 | 100 | 2.0 |
| PCB 105 | 100 | 2.5 | 79 | 2.0 | 98 | 1.5 | 93 | 2.0 |
| PCB 110 | 39 | 5.0 | 81 | 2.0 | 95 | 2.5 | 73 | 2.0 |
| PCB 118 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 128 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 95 | 2.0 |
| PCB 132 | 98 | 2.5 | 49 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 138 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 146 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 149 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 151 | 98 | 2.5 | 9 | 2.0 | 100 | 1.5 | 78 | 2.0 |
| PCB 153 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 156 | 100 | 2.5 | 91 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 170 | 100 | 2.5 | 100 | 2.0 | 98 | 1.5 | 100 | 2.0 |
| PCB 171 | 100 | 2.5 | 51 | 2.0 | 95 | 1.5 | 88 | 2.0 |
| PCB 172 | 100 | 2.5 | 33 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 174 | 96 | 2.5 | 72 | 2.0 | 100 | 1.5 | 98 | 2.0 |
| PCB 177 | 100 | 2.5 | 81 | 2.0 | 98 | 1.5 | 90 | 2.0 |
| PCB 180 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 183 | 100 | 2.5 | 100 | 2.0 | 95 | 1.5 | 88 | 2.0 |
| PCB 187 | 100 | 2.5 | 100 | 2.0 | 100 | 1.5 | 100 | 2.0 |
| PCB 194 | 100 | 2.5 | 53 | 2.0 | 93 | 1.5 | 43 | 2.0 |
| PCB 195 | 100 | 2.5 | 30 | 2.0 | 95 | 1.5 | 98 | 2.0 |
| PCB 199 | 100 | 2.5 | 23 | 2.0 | 78 | 1.5 | 83 | 2.0 |
| PCB 205 | 83 | 2.5 | 21 | 2.0 | 70 | 1.5 | 55 | 2.0 |
| PCB 206 | 99 | 2.5 | 26 | 2.0 | 43 | 1.5 | 40 | 2.0 |
| PCB 209 | 95 | 2.5 | 9 | 2.0 | 9 | 1.5 | 9 | 2.0 |

MB = Maternal Blubber; MS = Maternal Serum; M = Milk; PS = Pup Serum

In serum of suckling pups, all PCB groups as well as ΣPCBs increased significantly between early and late lactation ($p$-values between < 0.001 and 0.004) (Table 3.2). In addition to some period effects, our LMMs indicated significant and positive relationships between ΣPCBs in maternal outer blubber and maternal inner blubber, ΣPCBs in maternal inner blubber and maternal serum and ΣPCBs in maternal serum and milk ($p$-values between < 0.001 and 0.028).

### Variations in profiles

The percentages of tetra- to hexa-CBs in maternal outer blubber decreased significantly at late lactation ($p$-values between 0.009 and 0.011), while they increased significantly for hepta- to nona/deca-CBs ($p$-values between 0.012 and 0.015) (Figure 3.1a and Table 3.5). In inner blubber, the proportions of tetra- and penta-CBs declined significantly at late lactation ($p_{4\text{-Cl}} < 0.001$ and $p_{5\text{-Cl}} = 0.002$), while the ratios of hepta-CB increased significantly ($p = 0.019$) (Figure 3.1a and Table 3.5). The percentages of hexa-, octa- and nona/deca-CBs remained stable. In maternal serum, the percentages of tetra- to hexa-CBs decreased significantly between early and late lactation ($p \leq 0.001$ for all), while the proportions of hepta- and octa-CBs increased significantly ($p_{7\text{-Cl}} < 0.001$ and $p_{8\text{-Cl}} = 0.002$). No significant change was observed for nona/deca-CBs (Figure 3.1a and Table 3.5). In milk, the proportions of tetra-, penta-, octa- and nona/deca-CBs decreased significantly at late lactation ($p$-values between < 0.001 and 0.013), while the ratios of hepta-CBs increased significantly ($p = 0.047$) and the percentages of hexa-CBs remained stable (Figure 3.1a and Table 3.5). In pup serum, no significant changes between early and late lactation were observed in the proportions of all PCB groups (Figure 3.1a and Table 3.5).

**Table 3.5.** Mean ± standard deviation of the proportions* (%) of PCBs, PBDEs, DDXs, HO-PCBs and MeO-PBDEs in maternal blubber and serum, milk and pup serum of 20 grey seal mother-pup pairs.

| | Outer blubber | | Inner blubber | | Maternal serum | | Milk | | Pup serum | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* |
| tri-CBs | 0.1 ± 0.0 | 0.2 ± 0.3 | 0.1 ± 0.2 | 0.1 ± 0.3 | N.D | N.D | N.D | N.D | 0.3 ± 0.1 | 0.4 ± 0.2 |
| tetra-CBs | 0.8 ± 0.5 | 0.6 ± 0.3£ | 1.1 ± 0.5 | 0.8 ± 0.3£ | 2.7 ± 1.0 | 1.9 ± 1.0£ | 2.6 ± 0.9 | 2.0 ± 0.6£ | 2.6 ± 0.7 | 2.5 ± 0.8 |
| penta-CBs | 6.3 ± 2.5 | 4.9 ± 1.2£ | 8.5 ± 2.6 | 7.1 ± 1.8£ | 12.2 ± 2.1 | 9.1 ± 2.2£ | 12.4 ± 2.0 | 11.0 ± 2.5£ | 12.3 ± 2.6 | 12.7 ± 3.5 |
| hexa-CBs | 52.6 ± 6.5 | 50.1 ± 5.5£ | 56.8 ± 4.8 | 55.7 ± 4.5 | 62.8 ± 2.1 | 60.9 ± 3.0£ | 60.2 ± 3.3 | 60.9 ± 5.2 | 60.6 ± 3.0 | 58.9 ± 3.4 |
| hepta-CBs | 30.8 ± 5.1 | 33.1 ± 3.5§ | 27.0 ± 4.6 | 28.9 ± 3.5§ | 20.1 ± 2.9 | 24.8 ± 3.5§ | 19.2 ± 3.0 | 22.3 ± 6.5§ | 20.7 ± 3.6 | 21.4 ± 4.1 |
| octa-CBs | 7.1 ± 3.1 | 8.2 ± 2.5§ | 4.9 ± 2.0 | 5.5 ± 1.9 | 1.9 ± 1.0 | 2.9 ± 1.4§ | 4.9 ± 1.4 | 3.3 ± 1.0£ | 3.1 ± 1.3 | 3.4 ± 1.5 |
| nona/deca-CBs | 2.4 ± 1.5 | 2.8 ± 1.4§ | 1.5 ± 1.0 | 1.9 ± 1.1 | 0.3 ± 0.3 | 0.5 ± 0.4 | 0.6 ± 0.3 | 0.4 ± 0.2£ | 0.5 ± 0.3 | 0.7 ± 0.5 |
| BDE 28 | 0.5 ± 0.2 | 0.4 ± 0.1 | 0.8 ± 0.3 | 0.6 ± 0.2£ | N.D | N.D | 0.9 ± 0.2 | 0.7 ± 0.2£ | 0.9 ± 0.5 | 0.7 ± 0.3£ |
| BDE 47 | 59.5 ± 8.7 | 54.8 ± 6.9£ | 66.5 ± 8.2 | 62.4 ± 7.1£ | 79.9 ± 5.3 | 71.6 ± 7.9£ | 80.7 ± 2.7 | 78.8 ± 5.1 | 76.5 ± 13.2 | 78.3 ± 7.3 |
| BDE 99 | 6.5 ± 1.9 | 6.8 ± 1.9 | 6.8 ± 2.5 | 8.4 ± 2.8§ | 5.6 ± 2.1 | 6.9 ± 2.8§ | 4.3 ± 1.5 | 5.2 ± 2.9 | 7.4 ± 15.1 | 4.5 ± 2.4 |
| BDE 100 | 13.3 ± 2.0 | 14.0 ± 1.5§ | 12.3 ± 1.7 | 13.9 ± 2.1§ | 8.5 ± 1.7 | 11.7 ± 2.9§ | 8.8 ± 1.1 | 9.8 ± 2.2§ | 8.1 ± 2.1 | 9.0 ± 2.6 |
| BDE 153 | 12.4 ± 4.9 | 15.1 ± 4.3§ | 7.3 ± 5.3 | 7.8 ± 2.7 | 3.3 ± 1.5 | 4.8 ± 2.2§ | 2.8 ± 0.9 | 2.8 ± 1.1 | 4.5 ± 1.7 | 4.5 ± 2.6 |
| BDE 154 | 7.8 ± 3.9 | 8.8 ± 3.8§ | 6.3 ± 2.6 | 6.8 ± 2.7 | 2.7 ± 1.4 | 5.0 ± 3.0§ | 2.5 ± 0.9 | 2.7 ± 1.4 | 2.7 ± 2.4 | 3.0 ± 2.4 |
| *p,p'*-DDD | 1.2 ± 0.5 | 1.1 ± 0.5 | 1.9 ± 2 | 1.5 ± 1 | 1.0 ± 0.7 | 1.4 ± 1.2 | 3.0 ± 0.8 | 2.6 ± 0.8£ | 0.2 ± 0.1 | 0.2 ± 0.2 |
| *p,p'*-DDE | 91.4 ± 3.3 | 91.0 ± 3.4 | 90.9 ± 82 | 90.7 ±4 | 90.7 ± 1.9 | 90.6 ± 1.7 | 87.9 ± 1.8 | 87.8 ± 1.6 | 93.3 ± 1.6 | 94.1 ± 1.0§ |
| *p,p'*-DDT | 7.4 ± 3.2 | 7.9 ± 3.1§ | 7.2 ± 12 | 7.8 ± 3§ | 8.4 ± 1.8 | 8.0 ± 1.8 | 9.1 ± 1.3 | 9.6 ± 1.7 | 6.3 ± 1.6 | 5.3 ± 0.9£ |
| 3-HO-CB138 | N.M | N.M | N.M | N.M | 1.2 ± 1.1 | 1.7 ± 1.1 | N.D | N.D | 2.5 ± 0.9 | 2.0 ± 0.5£ |
| 4-HO-CB107 | N.M | N.M | N.M | N.M | 72.2 ± 6.9 | 74.0 ± 5.3 | N.A | N.A | 65.8 ± 6.5 | 77.3 ± 3.3§ |
| 4-HO-CB120 | N.M | N.M | N.M | N.M | 3.4 ± 1.1 | 3.4 ± 1.0 | N.D | N.D | 3.3 ± 1.2 | 3.3 ± 0.6 |
| 4-HO-CB146 | N.M | N.M | N.M | N.M | 2.4 ± 1.2 | 2.4 ± 1.8 | N.D | N.D | 2.0 ± 0.7 | 1.5 ± 0.7£ |
| 4-HO-CB162 | N.M | N.M | N.M | N.M | 16.6 ± 3.7 | 14.5 ± 3.1£ | N.D | N.D | 26.0 ± 5.2 | 15.6 ± 3.2£ |
| 4-HO-CB187 | N.M | N.M | N.M | N.M | 1.6 ± 1.7 | 1.4 ± 1.0 | N.D | N.D | 0.5 ± 0.3 | 0.3 ± 0.4 |
| 4-HO-CB202 | N.M | N.M | N.M | N.M | 2.8 ± 1.9 | 2.6 ± 1.4 | N.D | N.D | N.D | N.D |
| 6-MeO-BDE47 | 78.9 ± 5.7 | 79.0 ± 4.4 | 78.8 ± 5.4 | 78.3 ± 4.0 | N.A | N.A | 81.2 ± 5.8 | 81.3 ± 6.8 | N.D | N.D |
| 2'-MeO-BDE68 | 21.1 ± 5.7 | 21.0 ± 4.4 | 21.2 ± 5.4 | 21.7 ± 4.0 | N.D | N.D | 18.8 ± 5.8 | 18.7 ± 6.8 | N.D | N.D |

For a defined tissue, values within a row followed by £ are significantly lower at late lactation ($p \leq 0.05$). For a defined tissue, values within a row followed by § are significantly higher at late lactation ($p \leq 0.05$). N.D = Not detected; N.M = Not measured; N.A = Not applicable.

*Proportion = compound/Σ compound type (e.g., proportion of BDE 47 = BDE 47 levels/ ΣPBDEs).

### 3.3.3. PBDEs

BDE-47 was the most dominant congener in all samples representing between 50% and 80% of the total PBDEs depending on the tissue. Other important congeners were BDE-99, BDE-100 or BDE-153 depending on the period and the tissue considered. The detection frequency of various congeners in each tissue is summarised in Table 3.4b.

**Table 3.4b.** Detection frequency (f, in %) and limit of quantification (LOQ, on a lipid weight basis) for each PBDE congener in the different investigated tissues.

| Congener | f MB | LOQ MB (ng/g) | f MS | LOQ MS (ng/g) | f M | LOQ M (ng/g) | f PS | LOQ PS (ng/g) |
|---|---|---|---|---|---|---|---|---|
| BDE 28 | 99 | 0.2 | 0 | 0.5 | 100 | 0.1 | 33 | 0.5 |
| BDE 47 | 100 | 0.2 | 100 | 0.5 | 100 | 0.1 | 100 | 0.5 |
| BDE 99 | 100 | 0.2 | 65 | 0.5 | 100 | 0.1 | 80 | 0.5 |
| BDE 100 | 100 | 0.2 | 93 | 0.5 | 100 | 0.1 | 100 | 0.5 |
| BDE 153 | 100 | 0.2 | 42 | 0.5 | 100 | 0.1 | 98 | 0.5 |
| BDE 154 | 100 | 0.2 | 40 | 0.5 | 100 | 0.1 | 63 | 0.5 |

MB = Maternal Blubber; MS = Maternal Serum; M = Milk; PS = Pup Serum

#### Variation in concentrations

In maternal outer and inner blubber, ΣPBDEs as well as all PBDE congeners increased significantly at late lactation (*p*-values between < 0.001 and 0.005) (Table 3.2). In maternal serum, although levels of ΣPBDEs and individual PBDE congeners tended to be higher at late lactation, significant differences were found only for BDE-100, BDE-153 and BDE-154 (*p*-values between < 0.001 and 0.011). In milk, concentrations of ΣPBDEs and individual PBDE congeners tended also to be higher at late lactation but no significant change was observed (Table 3.2). Similarly, no significant change between early and late lactation was found in pup serum (Table 3.2).

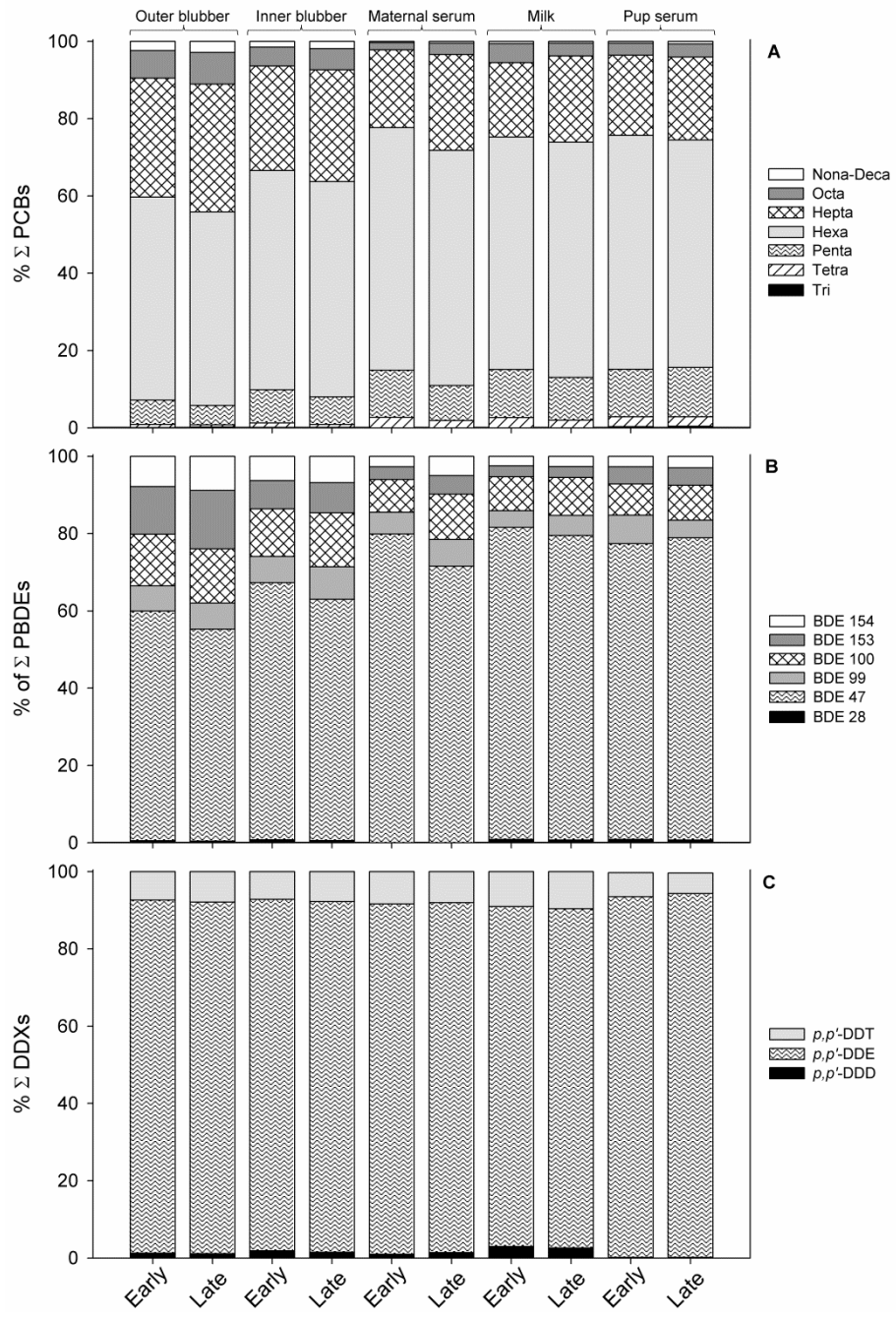


**Figure 3.1.** Proportions of the different groups of PCBs (A), congeners of PBDEs (B) and DDXs (C) in maternal blubber, maternal serum, milk and pup serum of the 20 grey seals mother-pup pairs.

The LMMs highlighted significant and positive relationships between ΣPBDEs in maternal outer blubber and maternal inner blubber, ΣPBDEs in maternal inner blubber and maternal serum and ΣPBDEs in maternal serum and milk ($p$-values ≤ 0.001 for all).

### Variation in profiles

The proportions of BDE-47 decreased significantly at late lactation in maternal outer blubber ($p = 0.014$), while the percentages of BDE-100, BDE-153 and BDE-154 increased significantly ($p$-values between 0.018 and 0.023) and those of BDE-28 and BDE-99 remained the same (Figure 3.1b and Table 3.5). In maternal inner blubber, the percentages of BDE-28 and BDE-47 decreased significantly at late lactation ($p_{BDE\text{-}28} = 0.003$ and $p_{BDE\text{-}47} = 0.032$), while proportions of BDE-99 and BDE-100 increased significantly ($p_{BDE\text{-}99} = 0.013$ and $p_{BDE\text{-}100} = 0.001$) and those of BDE-153 and BDE-154 remained unchanged (Figure 3.1b and Table 3.5). In maternal serum, the percentages of BDE-47 were significantly lower at late lactation ($p < 0.001$), while those of other congeners increased significantly ($p$-values between 0.001 and 0.007) (Figure 3.1b and Table 3.5). In milk, proportions of BDE-28 decreased significantly ($p = 0.004$), while those of BDE-100 increased significantly ($p = 0.019$) and those of other congeners remained unchanged between early and late lactation (Figure 3.1b and Table 3.5). In pup serum, the percentages of BDE-28 dropped significantly at late lactation, while proportions of other congeners remained stable ($p = 0.043$) (Figure 3.1b and Table 3.5).

## 3.3.4. HO-PCBs and HO-PBDEs

Because of the larger affinity of hydroxylated metabolites for blood proteins compared to blubber lipids (Letcher *et al.*, 2000), HO-PCBs and HO-PBDEs were not targeted in maternal blubber. Their presence was, however, investigated in milk in order to elucidate a possible lactational transfer pathway to pups. No HO-PBDEs were found in either milk or maternal and pup serum samples.

**Table 3.6.** Mean ± standard deviation of levels of HO-PCBs measured in maternal serum (in pg/ml), milk (in ng/g lipid weight) and pup serum (in pg/ml) of 20 grey seal mother-pup pairs.

| HO-PCBs | Maternal serum (pg/ml)* | | Milk (ng/g lipid weight)* | | Pup serum (pg/ml)* | |
|---|---|---|---|---|---|---|
| | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* | *Early lactation* | *Late lactation* |
| 3-HO-CB138 | 6 ± 5 | 11 ± 10§ | N.D | N.D | 10 ± 6 | 21 ± 10§ |
| 4-HO-CB107 | 408 ± 210 | 486 ± 250§ | 0.03 ± 0.01 | 0.04 ± 0.02§ | 335 ± 286 | 827 ± 355§ |
| 4-HO-CB120 | 19 ± 10 | 22 ± 12 | N.D | N.D | 14 ± 10 | 35 ± 15§ |
| 4-HO-CB146 | 12 ± 7 | 16 ± 9 | N.D | N.D | 9 ± 7 | 18 ± 11§ |
| 4-HO-CB162 | 93 ± 51 | 94 ± 53 | N.D | N.D | 130 ± 116 | 173 ± 107§ |
| 4-HO-CB187 | 7 ± 6 | 9 ± 6 | N.D | N.D | 3 ± 4 | 3 ± 5 |
| 4-HO-CB202 | 14 ± 8 | 16 ± 10 | N.D | N.D | N.D | N.D |
| **Σ HO-PCBs** | **558 ± 271** | **654 ± 329§** | **0.03 ± 0.01** | **0.04 ± 0.02§** | **501 ± 421** | **1077 ± 484§** |

*Statistics were performed on LN transformed values;
For a defined tissue, values within a row followed by § are significantly higher at late lactation ($p \leq 0.05$);
N.D = Not detected

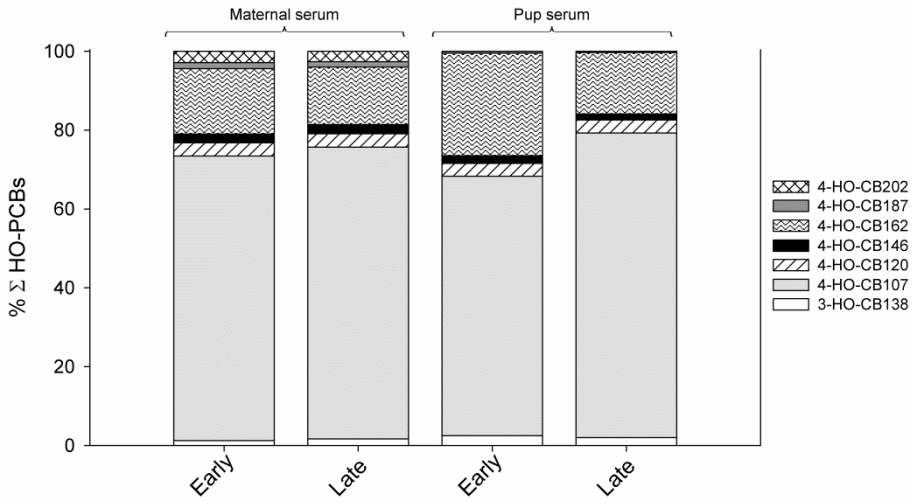


**Figure 3.2.** Proportions of the different metabolites of HO-PCBs in maternal and pup serum of the 20 grey seals mother-pup pairs.

Among the 20 HO-PCBs targeted in maternal serum, milk and pup serum, 4-HO-CB-107 was the most abundant metabolite, representing between 66 and 100% of the total HO-PCB concentrations. In particular, 4-HO-CB-107 was the only HO metabolite found in milk at very low levels (Table 3.6). Details on the detection frequency of each metabolite in various tissues can be found in Table 3.4c.

### Variation in concentrations

ΣHO-PCBs in maternal serum increased significantly at late lactation ($p = 0.002$) (Table 3.6).

Among all the detected metabolites, only levels of 3-HO-CB-138 and 4-HO-CB-107 appeared to increase significantly ($p_{3HOCB138} = 0.032$ and $p_{4HOCB107} < 0.001$). In milk, concentration of 4-HO-CB-107 increased significantly at late lactation ($p = 0.022$) (Table 3.6). In pup serum, ΣHO-PCBs increased significantly at late lactation and this increase was observed for all the detected metabolites ($p$-values $\leq 0.001$ for all), except for 4-HO-CB-187 (Table 3.6). No relationships between a defined tissue and the "previous" one could be observed.

### Variation in profiles

In maternal serum, only the proportions of 4-HO-CB-162 decreased significantly at late lactation ($p = 0.002$) (Figure 3.2 and Table 3.6). In pup serum, a significant increase in the ratios of 4-HO-CB-107 was observed at late lactation ($p < 0.001$), while the percentages of 3-HO-CB-138, 4-HO-CB-146 and 4-HO-CB-162 decreased significantly ($p$-values between $< 0.001$ and 0.038) (Figure 3.2 and Table 3.6). Proportions of 4-HO-CB-120 and 4-HO-CB-187 remained unchanged.

**Table 3.4c.** Detection frequency (f, in %) and limit of quantification (LOQ, on a lipid weight basis) for each HO-PCB metabolite in the different investigated tissues.

| Congener | f MB | f MS | LOQ MS (ng/g) | f M | LOQ M (pg/g) | f PS | LOQ PS (ng/g) |
|---|---|---|---|---|---|---|---|
| 3-HO-CB118 | N.M | 0 | 2.0 | 0 | 20.0 | 0 | 2.0 |
| 3-HO-CB138 | N.M | 26 | 1.5 | 0 | 20.0 | 53 | 1.5 |
| 3-HO-CB153 | N.M | 0 | 1.5 | 0 | 20.0 | 0 | 1.5 |
| 3-HO-CB180 | N.M | 2 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-diHO-CB202 | N.M | 2 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB107 | N.M | 100 | 2.0 | 90 | 20.0 | 100 | 2.0 |
| 4-HO-CB120 | N.M | 51 | 2.0 | 0 | 20.0 | 53 | 2.0 |
| 4-HO-CB127 | N.M | 0 | 1.5 | 0 | 20.0 | 3 | 1.5 |
| 4-HO-CB130 | N.M | 2 | 1.5 | 0 | 20.0 | 0 | 1.5 |
| 4-HO-CB146 | N.M | 44 | 1.5 | 0 | 20.0 | 40 | 1.5 |
| 4-HO-CB162 | N.M | 100 | 1.0 | 0 | 20.0 | 100 | 1.0 |
| 4-HO-CB163 | N.M | 2 | 1.5 | 0 | 20.0 | 5 | 1.5 |
| 4-HO-CB172 | N.M | 2 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB177 | N.M | 0 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB187 | N.M | 35 | 1.0 | 0 | 20.0 | 13 | 1.0 |
| 4-HO-CB193 | N.M | 5 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB198 | N.M | 0 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB199 | N.M | 0 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB202 | N.M | 63 | 1.0 | 0 | 20.0 | 0 | 1.0 |
| 4-HO-CB208 | N.M | 2 | 1.0 | 0 | 20.0 | 0 | 1.0 |

MB = Maternal Blubber; MS = Maternal Serum; M = Milk; PS = Pup Serum; N.M = Not measured

### 3.3.5. Pesticides

For DDXs, the most prevalent metabolite was *p,p'*-DDE which contributed for up to 94% of the total DDXs. *p,p'*-DDT represented more than 5% of the total DDXs. The detection frequency of each compound in a given tissue is presented in Table 3.4d.

**Table 3.4d.** Detection frequency (f, in %) and limit of quantification (LOQ, on a lipid weight basis) for each DDX metabolite and HCB in the different investigated tissues.

| Congener | f MB | LOQ MB (ng/g) | f MS | LOQ MS (ng/g) | f M | LOQ M (ng/g) | f PS | LOQ PS (ng/g) |
|---|---|---|---|---|---|---|---|---|
| HCB | 51 | 4.0 | 40 | 2.0 | 100 | 2.0 | 95 | 2.0 |
| op-DDD | 0 | 10.0 | 0 | 5.0 | 0 | 5.0 | 10 | 5.0 |
| op-DDE | 0 | 10.0 | 0 | 5.0 | 0 | 5.0 | 0 | 5.0 |
| op-DDT | 0 | 10.0 | 0 | 5.0 | 0 | 5.0 | 0 | 5.0 |
| pp-DDD | 40 | 10.0 | 16 | 5.0 | 85 | 5.0 | 8 | 5.0 |
| pp-DDE | 100 | 10.0 | 100 | 5.0 | 100 | 5.0 | 100 | 5.0 |
| pp-DDT | 96 | 10.0 | 91 | 5.0 | 100 | 5.0 | 98 | 5.0 |

MB = Maternal Blubber; MS = Maternal Serum; M = Milk; PS = Pup Serum

#### Variation in concentrations

ΣDDXs increased significantly at late lactation in maternal outer blubber ($p < 0.001$) and this increase was also observed for *p,p'*-DDE and *p,p'*-DDT levels (*p*-values < 0.001 for both) (Table 3.2). Similarly, HCB levels were also higher at late lactation ($p = 0.018$) (Table 3.2). In inner blubber, concentrations of the 3 detected DDX metabolites and thus ΣDDXs increased significantly at late lactation (*p*-values between < 0.001 and 0.002), while HCB levels remained unchanged (Table 3.2). In maternal serum, no difference between early and late lactation was observed for ΣDDXs but *p,p'*-DDE and *p,p'*-DDD increased significantly at late lactation ($p_{DDE} = 0.020$ and $p_{DDD} = 0.016$) (Table 3.2). HCB levels remained stable (Table 3.2). In milk, ΣDDXs increased significantly at late lactation ($p = 0.001$) and this increase was seen for the 3 DDX compounds (*p*-values between

0.001 and 0.013) (Table 3.2). By contrast, HCB concentrations remained stable (Table 3.2). In pup serum, a significant increase was observed for ΣDDXs as well as for *p,p'*-DDE ($p_{DDE} = 0.002$ and $p_{\Sigma DDXs} = 0.003$) (Table 3.2). HCB levels remained stable (Table 3.2). Our LMMs indicated significant and positive relationships between HCB levels in maternal outer blubber and maternal inner blubber, HCB levels in maternal inner blubber and maternal serum, HCB levels in maternal serum and milk and HCB levels in milk and in pup serum (*p*-values between < 0.001 and 0.010). Regarding ΣDDXs, positive relationships were found only between levels in maternal outer blubber and maternal inner blubber ($p < 0.001$).

### Variation in DDX profiles

In maternal outer and inner blubber, only the percentages of *p,p'*-DDT increased significantly at late lactation ($p = 0.033$ and $p = 0.036$ for outer and inner blubber, respectively) (Figure 3.1c and Table 3.5). In maternal serum, the percentages of the different isomers remained stable between early and late lactation (Figure 3.1c and Table 3.5). A significant decrease in *p,p'*-DDD proportions was observed at late lactation in milk ($p = 0.024$), while the ratios of *p,p'*-DDE and *p,p'*-DDT remained stable (Figure 3.1c and Table 3.5). Finally, in pup serum, proportions of *p,p'*-DDE increased significantly at late lactation ($p = 0.012$), while those of *p,p'*-DDT decreased significantly ($p = 0.002$) (Figure 3.1c and Table 3.5).

## 3.3.6. MeO-PBDEs

Two naturally-produced MeO-PBDEs, 2'-MeO-BDE-68 and 6-MeO-BDE-47, were detected in very low concentrations in maternal blubber and milk with a predominance of 6-MeO-BDE-47 (up to 90% of total MeO-PBDEs) (Table 3.2). In maternal serum, only 6-MeO-BDE-47 could be detected in 44% of the samples. None of the 2 congeners were found in pup serum.

### Variation in concentrations

ΣMeO-PBDEs increased significantly in maternal inner blubber ($p < 0.001$) but not in outer blubber. This increase was observed for both congeners ($p$-values < 0.001 for both) (Table 3.2). In maternal serum, concentrations of 6-MeO-BDE-47 did not change between early and late lactation (Table 3.2). In milk, ΣMeO-PBDEs increased significantly at late lactation and this increase was noticed for both congeners ($p$-values between 0.001 and 0.050) (Table 3.2). The only significant relationship found in our LMMs was the positive one between ΣMeO-PBDEs in maternal outer blubber and ΣMeO-PBDEs in maternal inner blubber ($p < 0.001$).

### Variation in profiles

The percentages of both congeners remained constant between early and late lactation in each tissue (Table 3.5).

## 3.3.7. Transfer ratios

Decreasing ratios from maternal inner blubber to maternal serum with the number of chlorine and bromine atoms were observed for the different PCB homologue groups and PBDE congeners (Figure 3.3a). In addition, a significant inverse relationship was observed between transfer ratios of PCBs, PBDEs and OCPs and their respective log $K_{ow}$ values ($R^2 = 0.810$; $p < 0.001$) (Figure 3.3a). However, since the standard deviations of OCPs were quite important and since variations in profiles were particularly apparent for PCBs and PBDEs, we have thus performed our statistics on PCBs and PBDEs only. Figure 3.4 shows significant inverse relationships between transfer ratios for both type of compounds from maternal inner blubber to maternal serum and their respective log $K_{ow}$ values ($R^2 = 0.877$; $p = 0.006$ and $R^2 = 0.806$; $p = 0.039$ for PCBs and PBDEs, respectively). By contrast, no clear relationship could be observed between POP transfer ratios from maternal serum to milk and their respective log $K_{ow}$ values ($R^2 = 0.126$; $p = 0.194$) (Figure 3.3b).

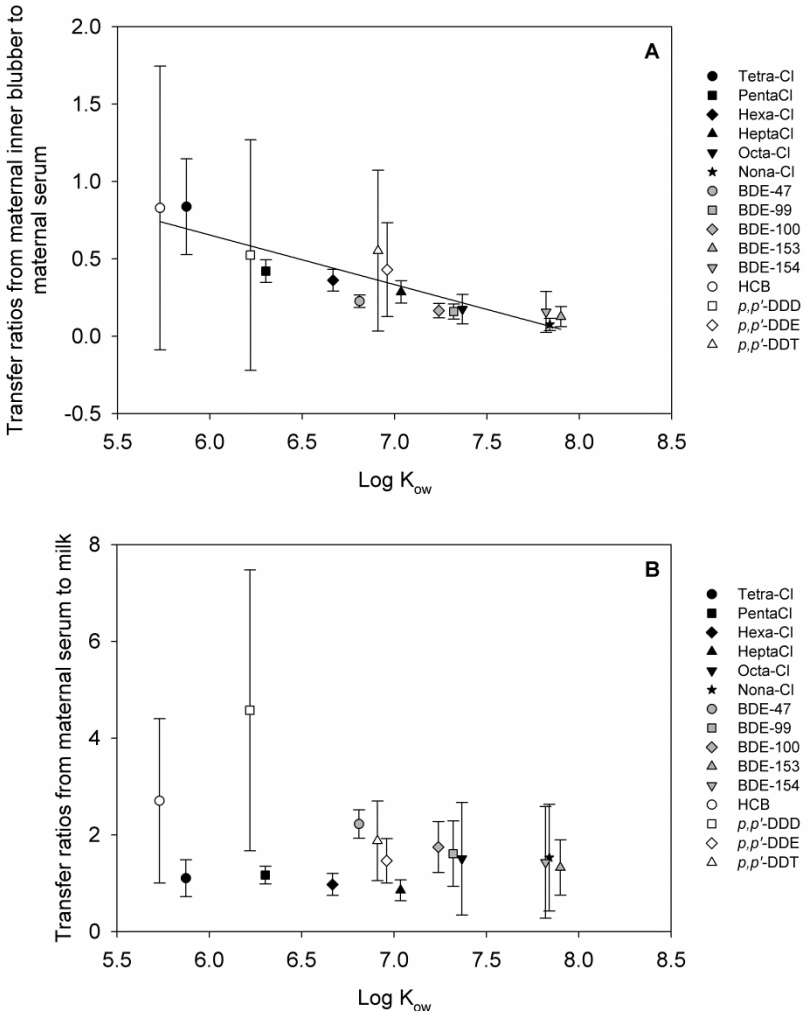


**Figure 3.3.** Relationship between transfer ratios from maternal inner blubber to maternal serum of PCBs, PBDEs and OCPs and their respective Log $K_{ow}$ (A) and relationship between transfer ratios from maternal serum to milk of PCBs, PBDEs and OCPs and their respective Log $K_{ow}$ (B). Log $K_{ow}$ values were compiled from Hansen *et al.*, 1999 for PCBs, Braekevelt *et al.*, 2003 for PBDEs, De Bruijn *et al.*, 1989 for pesticides and Yu *et al.*, 2008 for MeO-PBDEs. Log $K_{ow}$ for the different PCB groups was calculated as the mean of Log $K_{ow}$ of each PCB congener present in the corresponding group (e.g. Log $K_{ow}$ penta-Cl corresponds to the mean of log $K_{ow}$ of CB-87, -95, -99, -101, -105, -110 and -118).

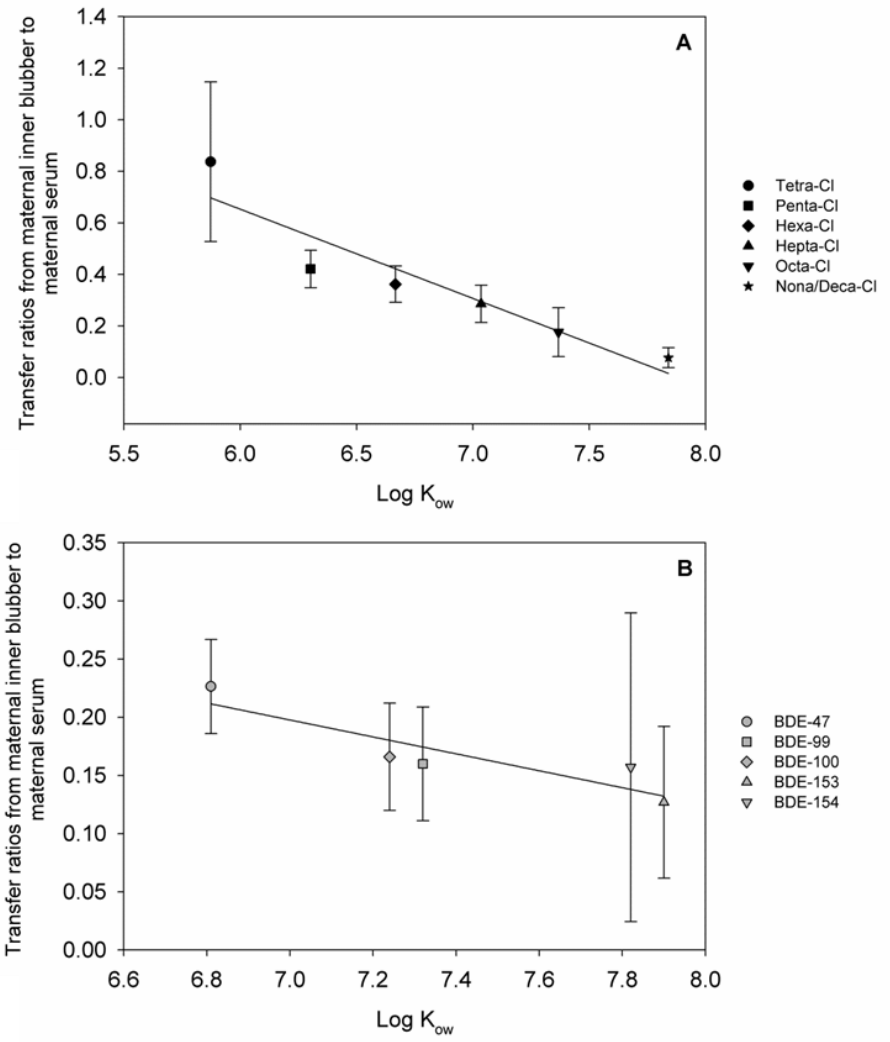


**Figure 3.4.** Relationship between transfer ratios from maternal inner blubber to maternal serum of PCBs (A) and PBDEs (B) and their respective Log $K_{ow}$.

## 3.4. Discussion

### 3.4.1. General levels and profiles

The PCB profiles were dominated by hexa- and hepta-CBs, mainly CB-153, CB-138 and CB-180. Not surprisingly, BDE-47 represented the major PBDE congener and *p,p'*-DDE the dominant DDX metabolite. Similar profiles are usually reported in seal species around the world (Espeland *et al.*, 1997; Sørmo *et al.*, 2003 ; Wolkers *et al.*, 2006; Miranda Filho *et al.*, 2009). ΣPCBs found in maternal blubber in the present study were comparable to those reported for the same grey seal colony sampled 10 and 2 years earlier, respectively (Debier *et al.*, 2003b; Vanden Berghe *et al.*, 2010). Such a similar degree of contamination indicates that, although they have been banned for several decades, these contaminants are still present in significant amounts in the northern UK environment and bioaccumulate in organisms. Law *et al.* (2010) observed an increase of PCBs in the blubber of Scottish harbour porpoises (*Phocoena phocoena*) between 1995 and 2005 and suggested an atmospheric transport from more contaminated adjacent UK regions. ΣPBDEs in maternal blubber were similar to those reported for lactating Canadian grey seal females from Sable Island sampled in 1995 (Ikonomou and Addison, 2008), but 2 to 3 times higher than those found in lactating Canadian harp seal females from Magdalen Islands sampled in 2008 (Frouin *et al.*, 2012). ΣDDXs found in maternal blubber of the present work were much lower than concentrations measured in blubber of lactating grey seal from Canada sampled in 1995 (Sørmo *et al.*, 2003), but similar than those detected in the lactating harp seal females from Magdalen Islands (Frouin *et al.*, 2012). Finally, HCB levels found in the studies of Sørmo *et al.* (2003) and Frouin *et al.* (2012) were 2 to 3 times higher than those measured in the present study.

4-HO-CB-107 followed by 4-HO-CB-162 was the prevalent metabolite in maternal and pup sera, which agrees with profiles reported for seals in other studies (Løken *et al.*, 2008; Weijs *et al.*, 2009c; Gabrielsen *et al.*, 2011). ΣHO-PCBs measured in the present study in

maternal and pup sera were lower than those found in the blood of hooded seal mother–pup pairs (Gabrielsen *et al.*, 2011) and in other non-lactating phocid seals (Løken *et al.*, 2008; Weijs *et al.*, 2009c). The only data existing about HO-PCBs in milk concern human breast milk. Similar to our grey seal milk, Guvenius *et al.* (2003) found a predominance of 4-HO-CB-107 in mothers' milk from Sweden, while 4-HO-CB-187 was the prevalent metabolite in human milk from Canada and the Faroe Islands (Newsome and Davies, 1996; Fangström *et al.*, 2005). Levels detected in grey seal milk were generally comparable or higher than those detected in human milk (Newsome and Davies, 1996; Fangström *et al.*, 2005), except for the Faroese population which is exposed to important levels of POPs due to their consumption of pilot whale meat and blubber (Fangström *et al.*, 2005).

None of the investigated HO-PBDEs could be detected in either serum or milk. This result corroborates previous findings that reported no or only low concentrations of circulating HO-PBDEs in harbour and ringed seals (Routti *et al.*, 2009 and Weijs *et al.*, 2009c). The authors suggested that biotransformation of PBDEs or retention of HO-PBDEs was low in those species. Additionally, it has been suggested that the primary source of HO-PBDEs may originate from the demethylation of naturally-produced MeO-PBDEs (Wan *et al.*, 2009). In the present study, MeO-PBDEs were absent or found at very low concentrations in all tissues. 6-MeO-BDE47 was the predominant congener. This is in agreement with previous studies reporting that 6-MeO-BDE47 was the main congener found in marine mammals from the Northern hemisphere (Covaci *et al.*, 2010 and Weijs *et al.*, 2009a). Levels of MeO-PBDEs in grey seals of the present study were relatively low compared to those found in blubber of harbour seal females from the North Sea (Weijs *et al.*, 2009a).

### 3.4.2. Maternal transfer of PCBs, PBDEs and OCPs

#### Variation in concentrations

The blubber of marine mammals is composed primarily of fatty acids stored as triacylglycerols (Wheatley *et al.*, 2008). Earlier studies reported that fatty acid mobilisation during fasting in seals occurred principally from the inner blubber layer, while the outer part remained more stable (Strandberg *et al.*, 2008). However, in the present study, we observed a slight but significant decrease in lipid content in the outer layer as well. Wheatley *et al.* (2007) observed changes in the fatty acid composition of outer blubber between early and late lactation in lactating Weddell seals (*Leptonychotes weddellii*). Together, these findings indicate that outer blubber may also be involved, to some extent, in the lipid dynamics during lactation in seals. The decrease in lipid content in maternal blubber layers was associated with an increase of ΣPCBs, ΣPBDEs and ΣDDXs on a lipid weight basis in both layers. The increase was, however, more profound in the inner layer than in the outer layer.

Similar findings were found for PCBs in lactating and fasting seals (Debier *et al.*, 2003b; Debier *et al.*, 2006; Vanden Berghe *et al.*, 2010) and the authors suggested a less efficient mobilisation of PCBs from blubber than triacylglycerols, resulting in increasing concentrations in the reduced blubber layer. It is also possible that POPs are mobilised from maternal blubber into the bloodstream together with the lipids but due to their higher affinity for the remaining blubber triacylglycerols, some POPs may be reabsorbed by the blubber, while the rest may end up into the milk. In both cases, the result is an accumulation of POPs in blubber over the course of lactation, especially the inner part (levels more than 2 times higher at late lactation compared to early lactation). In addition, a mobilisation of POPs from other tissues such as the liver, muscles or kidneys may also occur during lactation, as observed for lactating rodents (Vodicnik and Lech, 1980; Poul, 1991; Lee *et al.*, 2009). Once into the blood circulation, one part of these compounds may be absorbed by

the blubber because of the larger amount of fat present in this tissue and their higher affinity for triacylglycerols, as mentioned previously.

For most POPs, our LMMs showed a significant effect of total concentrations found in the "previous" compartment of transfer on total concentrations encountered in the "following" compartment of transfer (outer blubber → inner blubber → serum → milk — see Section 2.2.4). These findings suggest that the contamination of milk depends in great part on the mobilisation of POPs from maternal blubber, transport into the bloodstream and uptake by the mammary gland. Although not always statistically significant, total concentrations of most POPs tended to be also higher in maternal serum and milk at late lactation. When results were expressed per unit of wet weight, concentrations of PCBs, PBDEs and DDXs were significantly higher at late lactation as compared to early lactation in milk (results not shown). Similar dynamics have already been observed in maternal serum and milk for PCBs in lactating grey seals from the same colony (Debier *et al.*, 2003b; Vanden Berghe *et al.*, 2010). The authors suggested that the greatest part of the PCB mobilisation from maternal blubber may occur at late lactation, resulting in increasing PCB levels in maternal serum and then in milk at that same period. In addition, as suggested earlier, POPs from other tissues than blubber may also be released into the blood, contributing to the increase of concentrations observed in maternal serum and milk.

The important daily ingestion of contaminated milk, with higher POP concentrations at late lactation, leads to the accumulation of POPs in the suckling pups, as shown by the higher POP concentrations in their serum at late lactation, despite the important body dilution due to a rapid accumulation of weight.

### Variation in profiles

Profiles also changed over the course of lactation, which was especially apparent for PCBs and PBDEs. In blubber, the proportions of lower chlorinated PCBs (tetra- to hexa-CBs) and the lower

brominated BDE-47 tended to decrease at late lactation, while the percentages of higher halogenated congeners remained stable or increased. It thus appears that the mobilisation of POPs from maternal blubber is a selective phenomenon. Indeed, the transfer ratios of PCBs and PBDEs from maternal inner blubber to maternal serum decreased with the number of chlorine and bromine atoms and a significant inverse relationship was observed between the transfer ratios of PCBs, PBDEs and OCPs and their respective log $K_{ow}$ values. These results indicate that compounds with a high log $K_{ow}$ and thus with a high lipophilicity are less easily transferred into the bloodstream, which is characterised by more polar lipids than blubber. HCB, which has the lowest log $K_{ow}$ value, has a comparable transfer ratio to tetra-CBs and both compounds appear thus to be more efficiently transferred into blood. Such a selective transfer from blubber into blood has already been reported for PCBs and OCPs in seals (Debier *et al.*, 2003a; Sørmo *et al.*, 2003), but the present study is, to our knowledge, the first to highlight a similar pattern for PBDEs.

In maternal serum, profiles also changed towards lower proportions of tetra- to hepta-CBs and BDE-47 and higher proportions of octa- to deca-CBs and BDE-99 to BDE-154 at late lactation. Several hypotheses may explain these findings. A higher mobilisation of highly halogenated compounds from maternal blubber may start at late lactation, as already suggested by Debier *et al.* (2003a). Such dynamics may be linked, at least in part, to a selective mobilisation of fatty acids according to their physico-chemical properties, as detailed in Debier *et al.* (2006) and Vanden Berghe *et al.* (2010). In addition, a biological barrier towards some POPs may exist at the mammary gland, resulting in the accumulation of higher halogenated PCBs and PBDEs in maternal serum at late lactation as suggested in Debier *et al.* (2003a) and Sørmo *et al.* (2003). The percentages of higher halogenated compounds increased at late lactation in maternal serum, while they decreased or remained stable in milk. These opposite trends between both tissues for higher halogenated congeners support the hypothesis of the existence of a physiological barrier at the mammary gland for those compounds. However, contrary to what was observed for the transfer from maternal inner

blubber to maternal serum, no clear relationship was found between POP transfer ratios from maternal serum to milk and their degree of lipophilicity (log $K_{ow}$). In addition, highly halogenated compounds had transfer ratios from maternal serum to milk > 1, suggesting an efficient transfer of those compounds from maternal serum to the milk at the end of the lactating period. LaKind *et al.* (2009) reported similar findings for lactating women and concluded that the hypothesis that persistent lipophilic chemicals, on a lipid-adjusted basis, have consistent concentrations across matrices was likely too simplistic. Indeed, it is important to keep in mind that such physiological matrices are not static and that milk is constantly renewed, a phenomenon that is not taken into account by simple transfer ratios.

In pup serum, the profiles for most investigated POPs remained relatively stable between early and late lactation.

### 3.4.3. Maternal transfer of HO-PCBs

Total HO-PCB concentrations increased significantly at late lactation in maternal serum. This result suggests an increasing biotransformation of PCBs over the course of lactation. All potential precursors of the HO-PCBs encountered in lactating females (see Table 3.6) were indeed present in their serum in significant amounts (Table 3.6). However, this may also be explained by a mobilisation from other tissues such as blubber and liver, which are known to contain HO-PCBs (Gebbink *et al.*, 2008), as suggested for the parent compounds. Although rodent studies reported generally a down-regulation of CYP expression during lactation (Borlakoglu *et al.*, 1993; He *et al.*, 2005), it is important to keep in mind that in our case, grey seal females fast throughout the entire lactation period. This particular case may also modulate the expression and activity of CYPs. Such an observation has already been described in Arctic charr (*Salvelinus alpinus*), a fish species that undertakes prolonged fasting periods (Vijayan *et al.*, 2006). CYP1A protein expression was significantly enhanced in the liver of fasting fish that were experimentally treated by PCBs, indicating that an extended fasting may increase the impact of PCBs

on the liver metabolism in charr. As grey seal females also undertake an important fasting during lactation, a similar phenomenon may occur in these animals.

**Table 3.7.** HO-PCB metabolites and their potential precursors in maternal and pup serum grey seals.

| HO-metabolites | Potential precursors |
|---|---|
| 3-HO-CB138 | CB1-38 |
| 4-HO-CB107 | CB-118, CB-105 |
| 4-HO-CB120 | CB-118 |
| 4-HO-CB146 | CB-138, CB-153, CB-146 |
| 4-HO-CB187 | CB-183, CB-187 |
| 4-HO-CB202* | CB-199 |

*Not detected in pup serum

Contrary to their parent compounds, hydroxylated metabolites are preferentially retained in the blood due to their affinity for plasma proteins (Letcher *et al.*, 2000). This is probably why only 4-HO-CB107 was detected in milk at very low concentrations compared to those found in maternal and pup sera. Studies on human milk also indicated that only a low amount of HO-PCBs is transferred through lactation (Guvenius *et al.*, 2003; Fangström *et al.*, 2005). Interestingly, in addition to 4-HO-CB-107 which was found in all pup serum samples, most of the metabolites detected in maternal serum were also found in pup serum. Similar findings were observed by Gabrielsen *et al.* (2011) in hooded seal mother–pup pairs. The presence of HO-PCBs in pup serum may have different origins. HO-PCBs were transferred to the foetus during gestation as reported for humans and rodents (Meerts *et al.*, 2002; Guvenius *et al.*, 2003). Nevertheless, we also observed that concentrations of ΣHO-PCBs as well as most detected HO-metabolites increased significantly at late lactation in pup serum, most probably resulting from two possibilities. First, since low hepatic CYP activities have been demonstrated in suckling harp seal and hooded seal pups sampled once during lactation (Wolkers *et al.*, 2002 and 2009), it is possible that pups accumulate these HO-PCBs via endogenous biotransforma-

tion of PCBs. Indeed, all possible precursors of the HO-PCBs encountered in pup serum in the present study (see Table 3.7) were present in significant amounts (Table 3.6). It has been shown that CYP activities in humans are reduced during the neonatal period but increase with advancing age (Milsap and Jusko, 1994). Phase I reactions (e.g., hydroxylation) develop quickly and reach adult capacities at the age of 6 months (Milsap and Jusko, 1994). Increasing CYP activities may thus explain the increasing HO-PCB levels observed in the serum of our grey seal pups. This hypothesis is supported by the findings of Gabrielsen *et al.* (2011) who reported positive correlations between HO-PCB levels and the age of hooded seal pups. The second explanation for the increasing HO-PCB levels in pup serum may result from a transfer through the milk. Indeed, although only 4-HO-CB-107 could be detected in milk, the other metabolites found in pup serum may be present in trace amounts in milk, allowing pups to bioaccumulate these metabolites in their blood through lactation. However, the LMMs did not show any effect of the concentrations of HO-PCBs in maternal serum on the concentrations of HO-PCBs in milk. Similarly, there was no effect of HO-PCBs in milk on HO-PCBs in pup serum, which suggests that the transfer of HO-PCBs through the milk is probably not the major route.

### 3.4.4. Maternal transfer of the naturally-produced MeO-PBDEs

This is the first study reporting on the maternal transfer of MeO-PBDEs in marine mammals during lactation. Levels of MeO-PBDEs increased at late lactation in maternal inner blubber and milk. MeO-PBDEs are highly lipophilic compounds (log $K_{ow}$ = 6.91 and 7.17 for 2′-MeO-BDE-68 and 6-MeO-BDE-47, respectively) (Yu *et al.*, 2008) and tend to accumulate preferentially in blubber. Indeed, only limited amounts of MeO-PBDEs seemed to be transferred through milk as reflected by the very low levels found in maternal serum and milk compared to maternal blubber. This may result either from a higher retention of those compounds by the blubber or from a mobilisation combined to a direct reabsorption by this tissue, as explained in Section 2.3.2. The LMMs did not show any effect of the concentrations

of MeO-PBDEs in maternal inner blubber on the concentrations of MeO-PBDEs in maternal serum. Similarly, there was no effect of the concentrations of MeO-PBDEs in maternal serum on the concentrations of MeO-PBDEs in milk, supporting that the transfer of MeO-PBDEs from maternal blubber to milk was limited. Interestingly, none of the 2 MeO-PBDEs could be found in pup serum. Contrary to their mothers that were exposed to MeO-PBDEs via their diet prior to lactation, pups are exposed to relatively low levels of MeO-PBDEs through milk. It is possible that these metabolites are present in pup serum but at concentrations below the limit of quantification. Another hypothesis could be that pups are not efficient at absorbing these molecules. However, Covaci *et al.* (2010) detected significant levels of MeO-PBDEs in harbour seal pup liver and blubber, indicating an efficient absorption and storage of these compounds by seal pups. Due to their high lipophilic properties, MeO-PBDEs may be rapidly deposed in pup blubber following intestinal absorption, as suggested for adult females, leading to low or undetectable levels in pup serum.

## 3.5. Conclusions

This study advances the knowledge on the transfer of POPs during lactation in grey seals, especially concerning PBDEs, HO-PCBs and MeO-PBDEs. For the first time, we showed that PBDEs were selectively transferred from maternal blubber into serum of lactating grey seals. We have also observed a very low lactational transfer of HO-PCBs in grey seals, as previously reported for humans and laboratory animals. In addition, HO-PCB metabolites were found to accumulate in pups, suggesting, among other things, that the biotransformation rate of PCBs increased with the advancing age of the suckling pup. Finally, only low levels of MeO-PBDEs were found in maternal tissues and none of the congeners could be detected in pup serum, suggesting that the presence of organisms producing these compounds is low in the UK marine environment.

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Chapter 4

# Effects of polychlorobiphenyls, polybromodiphenylethers, organochlorine pesticides and their metabolites on vitamin A status in lactating grey seals

## Foreword

In the 1st chapter we reported that total PCB concentrations found in milk and pup serum tended to decrease circulating vitamin A levels in pups. In the 2nd chapter, we demonstrated that, in addition to PCBs, other POPs such as PBDEs and DDXs were found at important concentrations in maternal blubber and serum. Additionally, we showed that significant concentrations of HO-PCBs were present in maternal serum. We found thus relevant to investigate the interactions between vitamin A status and those other POPs in lactating grey seal females. The present chapter is devoted to study the potential relationships between vitamin A and the POPs measured in Chapter 3 in maternal blubber and serum. Potential correlations between vitamin A and MeO-PBDEs were also examined. This study should bring totally new information on the interactions between POPs and vitamin A status in lactating marine mammals.

Results of this chapter have been accepted in *Environmental Research* (in Press). Some changes have been introduced in the final accepted version. These changes do not modify the conclusions of the accepted version. Therefore, this chapter is a redraft after the accepted version.

# Effects of polychlorobiphenyls, polybromodiphenylethers, organochlorine pesticides and their metabolites on vitamin A status in lactating grey seals

*Redrafted after*

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Authors' contributions

The work presented here was carried out in collaboration between all authors. MVB and CD defined the research theme. SH and PP participated in the sample collection. MVB, LW and AC carried out the laboratory experiments. PP, AC and CD contributed to reagents/materials/analysis tools. MVB and CB performed the statistical analyses. MVB analysed the data. MVB, LW, AC and CD were engaged in active discussions. MVB wrote the paper. SH, KD, SP, JFR and PP provided advice on the manuscript. CD supervised the work. All authors have contributed to, seen and approved the manuscript.

# Abstract

Polychlorobiphenyls (PCBs), polybromodiphenylethers (PBDEs) and organochlorine pesticides (OCPs), such as dichlorodiphenyl-trichloroethane (DDT) and hexachlorobenzene (HCB), are considered as endocrine disruptors in laboratory and wild animals. This study investigated whether these compounds and their hydroxylated metabolites (HO-PCBs and HO-PBDEs) may affect the homeostasis of vitamin A, a dietary hormone, in the blubber and serum of twenty lactating grey seals sampled at early and late lactation on the Isle of May, Scotland. The effect of naturally produced compounds such as the methoxylated (MeO)-PBDEs was also examined. Vitamin A levels in inner blubber (37 ± 9 μg/g wet weight (ww) and 92 ± 32 μg/g ww at early and late lactation, respectively) and serum (408 ± 143 ng/ml and 390 ± 98 ng/ml at early and late lactation, respectively) appeared to be positively related to ΣPCBs, ΣPBDEs and several individual PCB and PBDE congeners in inner blubber and serum. These findings may suggest enhanced mobilisation of hepatic retinoid stores and redistribution in the blubber, a storage site for vitamin A in marine mammals. We have also reported that serum concentrations of ΣHO-PCBs and 4-OH-CB107 tended to increase with circulating vitamin A levels. Although the direction of the relationships may sometimes differ from those reported in the literature, our results are in agreement with previous findings highlighting a disruption of vitamin A homeostasis in the blubber and bloodstream following exposure to environmental pollutants. The fact that vitamin A and PCBs appeared to share common mechanisms of mobilisation and transfer during lactation in grey seals (Debier *et al.*, 2004; Vanden Berghe *et al.*, 2010) may also play a role in the different relationships observed between vitamin A and lipophilic pollutants.

**Keywords:** grey seal females, lactation, POPs, metabolites, vitamin A

## 4.1. Introduction

Persistent organic pollutants (POPs), such as polychlorobiphenyls (PCBs), polybromodiphenylethers (PBDEs) and organochlorine pesticides (OCPs), biomagnify in adipose tissue through the marine food web due to their persistence and lipophilicity (Law *et al.*, 2003; Muir *et al.*, 2003; Borja *et al.*, 2005; Wolkers *et al.*, 2006). Top predators, such as marine mammals, are therefore particularly sensitive to the accumulation of these pollutants that have been associated with reproduction impairment, endocrine disruption and/or immunotoxicity in several species (Sørmo *et al.*, 2009; Letcher *et al.*, 2010; Routti *et al.*, 2010a). Various types of biomarkers for environmental contaminant exposure or effect have been proposed in order to provide information on the overall health of marine mammals. Vitamin A (or retinol) represents one of these biomarkers (Simms and Ross, 2000). Although vitamin A is a non-specific biomarker of effect and thus does not point to an individual cause of the effect, it may reflect the overall impact due to a complex mixed exposure. Vitamin A is essential for vision, reproduction, immune functions, growth and development (Blomhoff, 1994; Debier and Larondelle, 2005) and a deficiency of this micronutrient induces important health consequences (West and Darnton-Hill, 2001).

The relationships between environmental pollutants and vitamin A homeostasis have been well documented in humans, laboratory animals and wildlife species (Brouwer *et al.*, 1998; Lans *et al.*, 1993; Hallgren *et al.*, 2001; Ellis-Hutchings *et al.*, 2006; Novák *et al.*, 2008). Most of these studies focussed on the impact of PCBs, and to a lesser extent of DDTs, HCB and PBDEs on vitamin A metabolism. Studies on laboratory and wild animals generally report depleted hepatic and adipose tissue retinoid stores as well as increased levels in the kidneys (Zile, 1992; Håkansson *et al.*, 1994; Hallgren *et al.*, 2001; Nyman *et al.*, 2003; Ellis-Hutchings *et al.*, 2006; Mos *et al.*, 2007; Novák *et al.*, 2008). In plasma, both an increase and a decrease of vitamin A levels have been observed, probably depending on the species, the physiological state and the concentrations at which the animals were exposed (Zile, 1992; Nyman *et al.*, 2003; Novák *et al.*, 2008; Cesh *et al.*, 2010; Delport *et al.*, 2011).

The mechanisms involved in the disruption of vitamin A homeostasis by environmental pollutants are still not fully understood and a lot remains to be elucidated. Several environmental contaminants are known to disrupt the activity of enzymes involved in the storage, mobilisation and/or catabolism of vitamin A, interact with proteins of transport or alter the regulation of vitamin A nuclear receptors (Figure 4.1). In the liver, contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), PCBs and pesticides were able to modulate the regulation of some cytochrome P450 (CYP) enzymes that are also involved in the metabolism and the catabolism of retinoic acid (Figure 4.1, points 1 and 2) (Boyer *et al.*, 2000, Leiva-Presa *et al.*, 2006; Novák *et al.*,2008). The activity of enzymes that esterify retinol and hydrolyse retinyl esters such as retinyl ester hydrolase (REH), acyl-CoA:retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT) were also shown to be modulated by PCBs and TCDD in fish and laboratory animals (Figure 4.1, points 3, 4 and 8) (Chen *et al.*, 1992; Nilsson *et al.*, 1996; Ndayibagira and Spear, 1999; Nilsson *et al.*, 2000). In addition, some hydroxylated PCB metabolites (HO-PCBs) can interact with transthyretin (TTR), as reported *in vivo* and *in vitro* in humans and rats. TTR is a protein involved in the blood transport of thyroxine ($T_4$) and the retinol binding protein (RBP)–retinol complex. The binding of HO-PCBs to TTR instead of $T_4$ lowers its affinity for the RBP-retinol complex, resulting in a greater urinary excretion of the RBP-retinol complex and thus a loss of vitamin A (Figure 4.1, point 5) (Brouwer *et al.*, 1998; Lans *et al.*, 1993). Similar interactions between some hydroxylated PBDE metabolites (HO-PBDEs) and human TTR have been found *in vitro* (Meerts *et al.*, 2000), suggesting that these metabolites may also be able to disrupt vitamin A homeostasis (Figure 4.1, point 5). Finally, PCBs and TCDD may affect vitamin A metabolism at the nuclear level as reported in human keratinocytes and seal blubber (Figure 4.1, points 6 and 7) (Lorick *et al.*, 1998; Mos *et al.*, 2007).

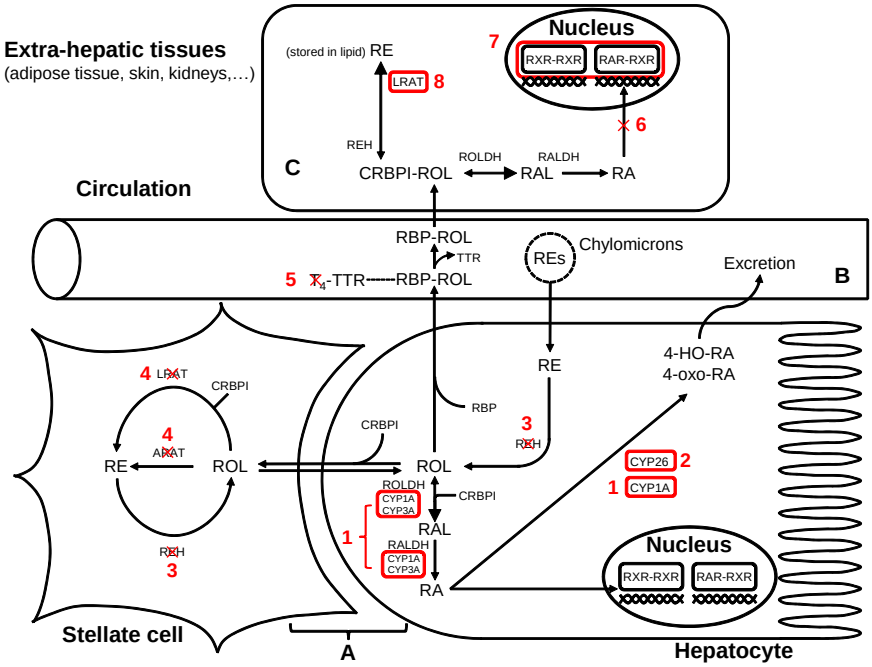


**Figure 4.1.** Possible mechanisms of action of environmental pollutants on vitamin A physiology in vertebrates. (**A**) *Impact on the metabolism of vitamin A in the liver (stellate cells and hepatocytes)*: (**1**) Induction of CYPs such as CYP1A (following activation of AhR) and CYP3A (following activation of PXR) involved in the metabolism of vitamin A and catabolism of RA (TCDD, PCBs, pesticides); (**2**) Downregulation of CYP26, the major enzyme of RA catabolism (*p,p'*-DDE); (**3**) Decrease of REH activity (CB-77 and CB-126); (**4**) Decreased LRAT and ARAT activity in stellate cells (TCDD). (**B**) *Interactions with vitamin A transport proteins in blood*: (**5**) Disruption of the TTR-RBP complex (HO-PCBs, HO-PBDEs). (**C**) *Impact on the metabolism of vitamin A in extra-hepatic tissues:* (**6**) Inhibition of atRA-RAR binding in human keratinocytes (TCDD); (**7**) Increase of the expression of RARα in seal blubber (PCBs); (**8**) Increased LRAT activities in rat kidneys (TCDD). AhR, aryl hydrocarbon receptor; ARAT, acyl-CoA:retinol acyltransferase; atRA, *all-trans* retinoic acid; CRBPI, Cellular retinol-binding protein type I: CYPs, cytochromes P450; LRAT, lecithin:retinol acyltransferase; PCBs, polychlorobiphenyls; PXR, pregnane X receptor; RA, retinoic acid; RAL, retinaldehyde; RALDH, retinal dehydrogenases; RAR, retinoic acid receptor; RBP, retinol binding protein; RE, retinyl ester; REH, retinyl ester hydrolase; ROL, retinol; ROLDH, retinol dehydrogenases; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; $T_4$, thyroxine; TTR, transthyretin. Figure adapted from Ghyselinck *et al.*, 1999, Simms *et al.*, 2000 and Novák *et al.*, 2008.

Contrary to HO-PCBs, HO-PBDEs may result either from the biotransformation of synthetic PBDEs or be produced by marine sponges and algae (Wiseman *et al.*, 2011). In addition, recent studies have shown that the demethylation of methoxylated polybrominated diphenyl ethers (MeO-PBDEs), compounds also naturally produced by marine organisms such as sponges and algae, could lead to the formation of HO-PBDEs, as demonstrated *in vitro* in microsomal fractions of several species of vertebrates (Wan *et al.*, 2009; Wiseman *et al.*, 2011). The presence of MeO-PBDEs in marine organisms may therefore be of particular concern. MeO-PBDEs can biomagnify in the food chain and have been measured in several marine mammals species, such as True's beaked whale (Mesoplodon mirus), beluga whale (*Delphinapterus leucas*), harbour porpoise (*Phocoena phocoena*) and harbour seal (*Phoca vitulina*) (Wolkers *et al.*, 2004; Teuten *et al.*, 2005; Weijs *et al.*, 2009). In addition, the concentrations of MeO-PBDEs and especially tetrabrominated-MeO-PBDEs, have exceeded those of the anthropogenic PBDEs in several Australian marine mammals (Vetter *et al.*, 2002).

While several studies investigated the effects of PCBs and DDTs on vitamin A metabolism in marine mammals (Nyman *et al.*, 2003; Debier *et al.*, 2005; Tornero *et al.*, 2006; Mos *et al.*, 2007; Routti *et al.*, 2010b; Vanden Berghe *et al.*, 2010), to our knowledge, none focussed on the relationships between vitamin A levels and PBDE contamination. However, PCBs and PBDEs share similar chemical properties and comparable adverse effects have been reported in several marine mammal species (Hall *et al.*, 2003; Beineke *et al.*, 2005; Peden-Adams *et al.*, 2005). Furthermore, an impact of PBDEs on the status of vitamin A has been reported in laboratory animals, as well as in birds (Hallgren *et al.*, 2001; Fernie *et al.*, 2005; Ellis-Hutchings *et al.*, 2006; Cesh *et al.*, 2010). One can therefore assume that PBDEs may also affect vitamin A status in marine mammals.

The present study aimed to investigate the potential relationships between environmental pollutants (PBDEs, PCBs, their HO-metabolites and OCPs) and the levels of vitamin A in the blubber and serum of lactating grey seals (*Halichoerus grypus*). The relationships between the naturally produced MeO-PBDEs and vitamin A concen-

trations in maternal blubber and serum were also examined. Grey seals are characterised by having a short (from 16 to 21 days) and intense lactation period (Pomeroy *et al.*, 1996). During that time, females produce milk extremely rich in lipids (up to 60%), while fasting (Iverson *et al.*, 1993; Pomeroy *et al.*, 1996). Because females do not eat throughout the lactation, milk lipids derive only from their own body stores. The mobilisation of such huge amounts of lipids from blubber results in the release of lipophilic pollutants into the bloodstream of the lactating females (Debier *et al.*, 2003, Sørmo *et al.*, 2003; Vanden Berghe *et al.*, 2010), exposing tissues to the potential harmful effects of these com- pounds. To our knowledge, this is the first study to examine the impact of PBDEs, HO-PCBs, HO-PBDEs and MeO-PBDEs on the vitamin A status in a free-ranging marine mammal species during the lactation period.

## 4.2. Material and methods

### 4.2.1. Seal sampling

Lactating grey seals from the Isle of May (IOM), Scotland, were studied during the breeding season in November–December 2008. Twenty females were captured and sampled at early (days 2–5) and mid-late (days 14–17) lactation in order to collect blubber and blood serum samples. Sampling techniques are described elsewhere (Debier *et al.*, 2002a and 2003; Pomeroy *et al.*, 1996). Briefly, blood samples were obtained from the extradural vein and blubber biopsies were taken from the lateral pelvic area of each female with a 6-mm biopsy punch. To protect vitamin A against UV light, samples collected from each female were directly stored in an individual light–tight container. Samples were then transported to the field laboratory where blood samples were centrifuged and serum was immediately transferred into light–tight Eppendorf tubes. Blubber samples for POP analyses were stored in aluminium foil while blubber samples for vitamin A analyses were placed into light–tight Eppendorf tubes. In the field laboratory, all the samples were frozen before being

shipped on dry ice to Belgium, where they were stored at -80°C and -20°C, for vitamin A and POP analyses, respectively.

### 4.2.2. Vitamin A analysis

Vitamin A in serum and blubber was extracted and analysed as described elsewhere (Debier *et al.*, 2002b; Vanden Berghe *et al.*, 2010). Briefly, three successive extractions with hexane were carried out on each serum sample. The extracts were pooled, evaporated and the residue was reconstituted with methanol. For blubber, approximately 150 mg of each end of the biopsy (outer and inner parts) were saponified with ethanolic KOH under nitrogen. Each sample was then extracted twice with diethyl ether. The extracts were pooled and washed at least 4 times with MilliQ water before being filtered and evaporated. The residue was dissolved in methanol. Vitamin A concentrations were quantified with an external standard solution of retinol (for serum) or retinyl acetate (for blubber) in ethanol that underwent the same extraction procedure as the samples. Vitamin A was determined by high-performance liquid chromatography (HPLC) – DAD detector operated at 325 nm and equipped with a Supelcosil LC18 column (25 cm x 4.5 mm x 5 µm). Vitamin A was eluted isocratically with a 100% methanol mobile phase at 1.5 mL $min^{-1}$. The concentrations of the standard solutions were determined spectrophotometrically ($E^{1\%1cm}$ = 1835 and 1524 at 325 nm for retinol and retinyl acetate, respectively). The limit of detection (LOD) was defined as 3 times the noise of the background line (0.01 and 0.02 ppm for serum and blubber, respectively) and the limit of quantitation (LOQ) as 10 times the noise of the background line (0.03 and 0.06 ppm for serum and blubber, respectively).

### 4.2.3. Lipid and contaminant analysis

Details on the lipid determination as well as the blubber and serum sample preparation, extraction, clean-up, quality assurance and chromatography analyses are described elsewhere (Vanden Berghe *et al.*, 2012). In all samples, 35 PCB congeners (IUPAC numbers: CB-18,

-28, -44, -47, -49, -52, -87, -95, -99, -101, -105, -110, -118, -128, -138, -146, -149, -151, -153, -156, -158, -170, -171, -172, -174, -177, -180, -183, -187, -194, -195, -199, -205, -206 and -209), 6 PBDEs (IUPAC numbers: BDE-28, -47, -99, -100, -153 and -154), 6 DDXs (*o,p'*-DDD, *o,p'*-DDT, *o,p'*-DDE, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDT), HCB and 2 MeO-PBDEs (2'-MeO-BDE 68 and 6-MeO-BDE 47) were measured. Additionally, twenty HO-PCBs were investigated in serum: 3HO-CB (118, 138, 153, and 180), 4HO-CB (107, 120, 127, 130, 146, 162, 163, 172, 177, 187, 193, 198, 199, 202, and 208) and 4-di-HO-CB202. The following 3 HO-PBDEs were targeted in all serum samples: 6-HO-BDE-47, 5-HO-BDE-47 and 4-HO-BDE-49.

### 4.2.4. Statistical analyses

Statistical analyses were conducted using SPSS 17 for Windows. Vitamin A and all contaminant levels were ln-transformed to normalise the data. Linear mixed models (LMM) were used to test the impact of pollutants on vitamin A concentrations. The individual was considered as a random explanatory variable, whereas the period of lactation, contaminant levels (on a lipid-weight basis for blubber and on a wet weight basis for serum), as well as the concentration of vitamin A in the "previous" tissue (e.g. vitamin A levels in inner blubber in order to explain vitamin A levels in serum) were included in the model as fixed variables. Some of the studied contaminants were highly inter-correlated in all investigated tissues (Table 4.1) and thus, could not be included together into the same statistical model in order to explain vitamin A variations. Therefore, we performed a different vitamin A model for each individual compound (e.g. CB-153, BDE-47 and *p,p'*-DDE), as well as for the sum of each class of contaminants (ΣPCBs, ΣPBDEs, ΣDDXs and ΣMeO-PBDEs). Only tables summarising the results for total contaminant concentrations are shown in Tables 4.3 to 4.5. The level of statistical significance was set at $p \leq 0.05$.

**Table 4.1.** Pearson correlation coefficients among contaminant groups in the different compartments.

| a) Outer blubber | | | | |
|---|---|---|---|---|
| | PBDEs | DDXs | MeO-PBDEs | HCB |
| PCBs | 0.843*** | 0.875*** | -0.385* | 0.032 |
| PBDEs | | 0.785*** | -0.289 | -0.066 |
| DDXs | | | -0.325* | 0.212 |
| MeO-PBDEs | | | | -0.085 |

| b) Inner blubber | | | | |
|---|---|---|---|---|
| | PBDEs | DDXs | MeO-PBDEs | HCB |
| PCBs | 0.905*** | 0.889*** | 0.146 | -0.098 |
| PBDEs | | 0.854*** | 0.242 | -0.188 |
| DDXs | | | 0.097 | 0.088 |
| MeO-PBDEs | | | | -0.247 |

| c) Serum | | | | | |
|---|---|---|---|---|---|
| | PBDEs | DDXs | HO-PCBs | MeO-PBDEs | HCB |
| PCBs | 0.879*** | 0.027 | 0.706*** | -0.042 | -0.109 |
| PBDEs | | 0.222 | 0.650*** | 0.089 | -0.174 |
| DDXs | | | -0.084 | 0.296 | 0.204 |
| HO-PCBs | | | | 0.181 | -0.199 |
| MeO-PBDEs | | | | | -0.226 |

All correlations are made on ln-transformed values. The significance of correlation is presented as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

## 4.3. Results

Details concerning POP levels and profiles, as well as lipid contents in maternal blubber and serum can be found elsewhere (Vanden Berghe *et al.*, 2012).
Mean concentrations of total vitamin A in blubber and serum are reported in Table 4.2.

**Table 4.2.** Mean concentrations ± standard deviation of the total vitamin A concentrations in blubber and serum of 20 lactating grey seals.

| Tissues | Vitamin A concentrations | |
|---|---|---|
| | *Early lactation* | *Late lactation* |
| Outer blubber (µg/g ww) | 55 ± 20 | 61 ± 18 |
| Inner blubber (µg/g ww) | 37 ± 9 | 92 ± 32 |
| Serum (ng/ml) | 408 ± 143 | 390 ± 98 |

### 4.3.1. Blubber

There was no relationship between vitamin A and any of the investigated contaminants in outer blubber (Table 4.3). By contrast, vitamin A levels were positively related to ΣPCBs, ΣPBDEs, ΣDDXs and ΣMeO-PBDEs, but not to HCB, in inner blubber (Figure 4 and Table 4.4). Concerning individual congeners, we observed that vitamin A was positively correlated ($p$-values between < 0.001 and 0.035) to all PCB congeners in inner blubber, except CB-18, -28, -47, -49 and -110. Vitamin A in inner blubber was also positively correlated to all PBDE congeners measured in inner blubber ($p$-values between < 0.001 and 0.007), except BDE-28. Concerning DDXs, the correlation was positive only for *p,p'*-DDE ($p$ = 0.004). Finally, among MeO-PBDEs, a positive relationship was found only with 2'-MeO-BDE-68 in inner blubber ($p$ = 0.023).

### 4.3.2. Serum

In serum, vitamin A concentrations were positively related to ΣPCBs, ΣPBDEs and ΣHO-PCBs (Figure 3 and Table 4.5). Significant relationships were found with several individual PCB congeners (CB-99, -101, -105, -118, -128, -138, -146, -149, -174, -177 and -187) (*p*-values between 0.001 and 0.047). Among individual PBDE congeners and HO-PCB metabolites, positive relationships were observed between vitamin A levels and BDE-47 ($p = 0.015$) and 4-HO-CB-107 ($p = 0.030$). No correlation with either MeO-PBDEs or OCPs could be found. Relationships between vitamin A and HO-PBDEs in serum could not be assessed because none of the investigated PBDE metabolites were detected in the present samples.

**Table 4.3.** Results of linear mixed models performed to test the relationships between total vitamin A concentrations and the different contaminant levels in the outer blubber of lactating grey seals. The period of lactation was included as a confounding variable.

| Explanatory variables | Estimate | STD error | t | P value |
|---|---|---|---|---|
| *Model 1 with ΣPCBs* | | | | |
| Period | -0.201 | 0.093 | -2,156 | 0.041 |
| Σ PCBs outer blubber | -0.150 | 0.106 | -1.416 | 0.167 |
| *Model 2 with ΣPBDEs* | | | | |
| Period | -0.160 | 0.088 | -1.822 | 0.083 |
| Σ PBDEs outer blubber | -0.151 | 0.147 | -1.026 | 0.317 |
| *Model 3 with ΣDDXs* | | | | |
| Period | -0.209 | 0.091 | -2.291 | 0.031 |
| Σ DDXs outer blubber | -0.277 | 0.154 | -1.804 | 0.084 |
| *Model 4 with HCB* | | | | |
| Period | -0.151 | 0.087 | -1.741 | 0.097 |
| HCB outer blubber | -0.050 | 0.067 | -0.755 | 0.459 |
| *Model 5 with ΣMeO-PBDEs* | | | | |
| Period | -0.134 | 0.085 | -1.575 | 0.132 |
| Σ MeO-PBDEs outer blubber | 0.200 | 0.150 | 1.331 | 0.196 |

All LMM are made on ln-transformed values.

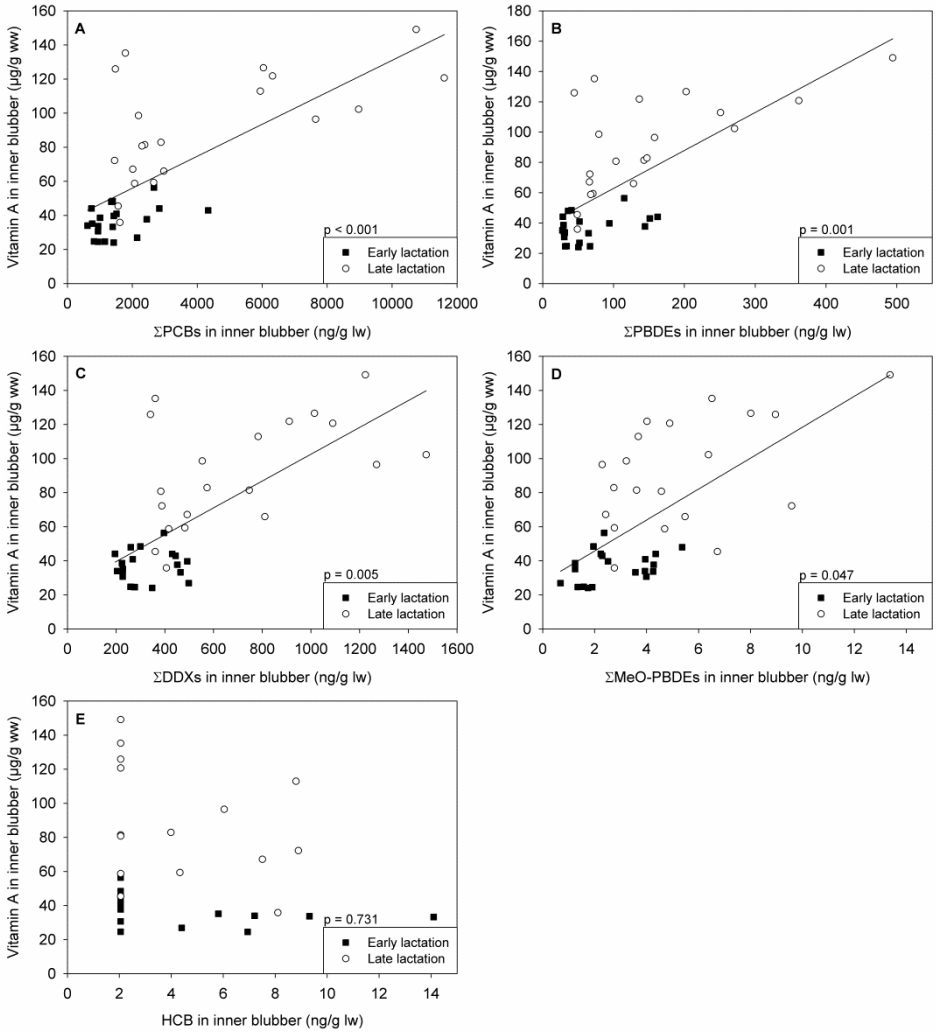


**Figure 4.2.** Relationships between vitamin A levels in inner blubber and total PCBs (A), PBDEs (B), DDXs (C), MeO-PBDEs (D) and HCB (E) concentrations measured in inner blubber of lactating grey seals. The *p*-values represent the level of significance of the linear mixed models which combined early and late lactation stages.

**Table 4.4.** Results of linear mixed models performed to test the relationships between total vitamin A concentrations and the different contaminant levels in the inner blubber of lactating grey seals. The period of lactation and vitamin A in outer blubber were included as confounding variables.

| Explanatory variables | Estimate | STD error | t | P value |
|---|---|---|---|---|
| *Model 1 with ΣPCBs* | | | | |
| Period | -0.603 | 0.100 | -6.049 | 0.000 |
| Vit A outer blubber | 0.371 | 0.114 | 3.251 | 0.002 |
| Σ PCBs inner blubber | 0.259 | 0.066 | 3.915 | 0.000 |
| *Model 2 with ΣPBDEs* | | | | |
| Period | -0.654 | 0.097 | -6.745 | 0.000 |
| Vit A outer blubber | 0.357 | 0.118 | 3.034 | 0.004 |
| Σ PBDEs inner blubber | 0.229 | 0.064 | 3.561 | 0.001 |
| *Model 3 with ΣDDXs* | | | | |
| Period | -0.596 | 0.116 | -5.144 | 0.000 |
| Vit A outer blubber | 0.427 | 0.122 | 3.508 | 0.001 |
| Σ DDXs inner blubber | 0.323 | 0.106 | 3.046 | 0.005 |
| *Model 4 with HCB* | | | | |
| Period | -0.839 | 0.094 | -8.931 | 0.000 |
| Vit A outer blubber | 0.373 | 0.142 | 2.635 | 0.013 |
| HCB inner blubber | -0.019 | 0.050 | -0.373 | 0.713 |
| *Model 5 with ΣMeO-PBDEs* | | | | |
| Period | -0.708 | 0.104 | -6.796 | 0.000 |
| Vit A outer blubber | 0.310 | 0.136 | 2.279 | 0.029 |
| Σ MeO-PBDEs inner blubber | 0.200 | 0.095 | 2.102 | 0.047 |

All LMM are made on ln-transformed values.

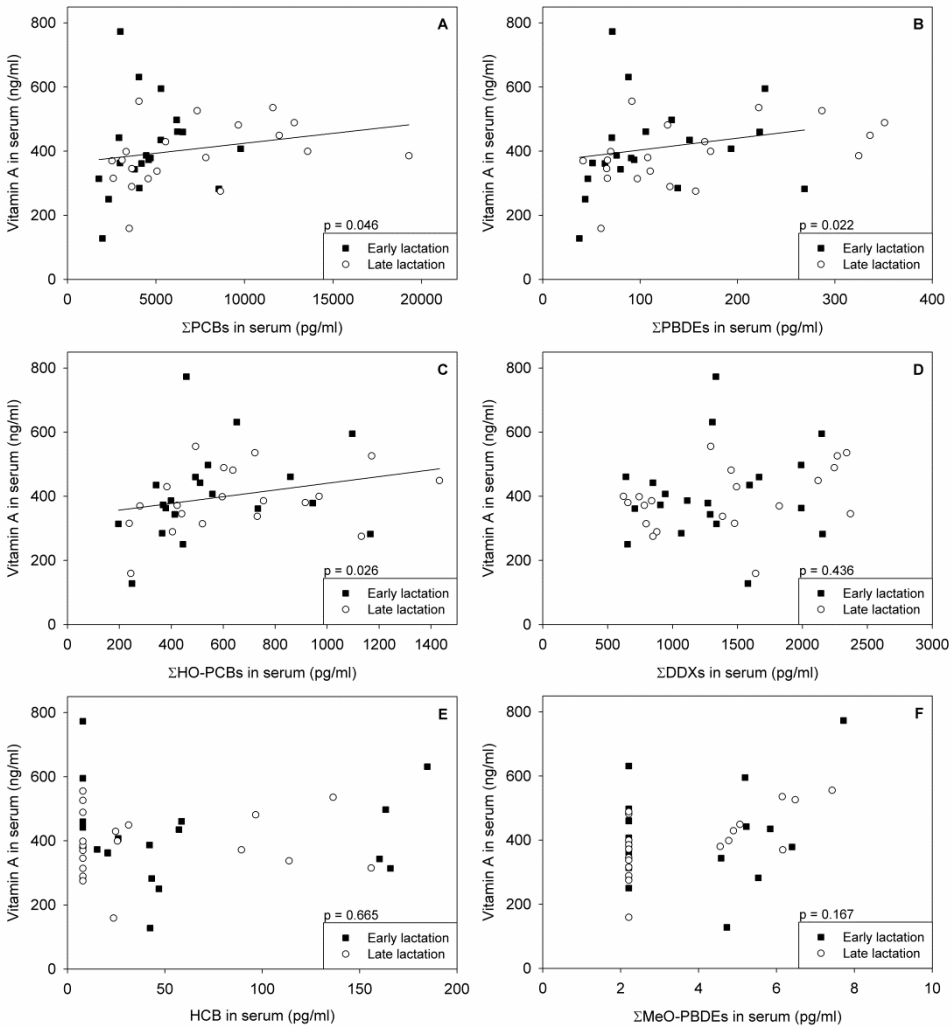


**Figure 4.3**. Relationships between vitamin A levels in serum and total PCBs (A), PBDEs (B), HO-PCBs (C), DDXs (D), HCB (E) and MeO-PBDEs (F) concentrations measured in serum of lactating grey seals. The *p*-values represent the level of significance of the linear mixed models which combined early and late lactation stages.

**Table 4.5.** Results of linear mixed models performed to test the relationships between total vitamin A concentrations and the different contaminant levels in the serum of lactating grey seals. The period of lactation and vitamin A in inner blubber were included as confounding variables.

| Explanatory variables | Estimate | STD error | t | P value |
|---|---|---|---|---|
| *Model 1 with Σ PCBs* | | | | |
| Period | 0.198 | 0.164 | 1.207 | 0.236 |
| Vit A inner blubber | 0.100 | 0.167 | 0.600 | 0.553 |
| Σ PCBs serum | 0.218 | 0.104 | 2.083 | 0.046 |
| *Model 2 with Σ PBDEs* | | | | |
| Period | 0.177 | 0.163 | 1.090 | 0.283 |
| Vit A inner blubber | 0.100 | 0.161 | 0.618 | 0.541 |
| Σ PBDEs serum | 0.228 | 0.092 | 2.466 | 0.022 |
| *Model 3 with Σ DDXs* | | | | |
| Period | 0.201 | 0.172 | 1.164 | 0.253 |
| Vit A inner blubber | 0.193 | 0.168 | 1.148 | 0.258 |
| Σ DDXs serum | 0.109 | 0.138 | 0.790 | 0.436 |
| *Model 4 with HCB* | | | | |
| Period | 0.222 | 0.173 | 1.288 | 0.206 |
| Vit A inner blubber | 0.209 | 0.167 | 1.250 | 0.219 |
| HCB serum | -0.023 | 0.053 | -0.438 | 0.665 |
| *Model 5 with 6-MeO-BDE47* | | | | |
| Period | 0.187 | 0.170 | 1.099 | 0.280 |
| Vit A inner blubber | 0.180 | 0.164 | 1.094 | 0.281 |
| 6-MeO-BDE47 | 0.165 | 0.117 | 1.412 | 0.167 |
| *Model 6 with Σ HO-PCBs* | | | | |
| Period | 0.202 | 0.161 | 1.250 | 0.220 |
| Vit A inner blubber | 0.153 | 0.157 | 0.972 | 0.338 |
| Σ HO-PCBs serum | 0.277 | 0.115 | 2.400 | 0.026 |

All LMM are made on ln-transformed values.

## 4.4. Discussion

We examined the relationships between vitamin A levels and the concentrations of PCBs, PBDEs, their metabolites and OCPs in the blubber and serum of lactating grey seals. Correlations with naturally produced MeO-PBDEs were also investigated. The introduction of important factors, such as the period of lactation, in our LMM allowed the exclusion of the effects of time and thus, body mass and adipose lipid stores loss associated with lactation. Although several studies have already examined the relationships between vitamin A and PCBs or OCPs in seals (Nyman *et al.*, 2003; Jenssen *et al.*, 2003; Debier *et al.*, 2005; Mos *et al.*, 2007; Vanden Berghe *et al.*, 2010), this is the first study to explore the relationships between vitamin A and PBDEs, HO-PCBs and MeO-PBDEs in lactating seals.

### 4.4.1. Relationships between PCBs, HO-PCBs and vitamin A

Significant positive associations were found between vitamin A concentrations and ΣPCBs, as well as with most individual PCB congeners in inner blubber, except those which were found in less than 50% of the blubber samples, e.g. CB-18, -28, -47, -49 and -110 (Vanden Berghe *et al.*, 2012). This indicates that, regardless of the period of lactation, females with higher PCB levels in their inner blubber also tended to have higher vitamin A concentrations in that same layer. In addition, circulating vitamin A concentrations were positively related to ΣPCBs, as well as to some tetra-CBs (CB-99, -101, -105 and -118), penta-CBs (CB-128, -138, -146 and -149) and hexa-CBs (CB-174, -177 and -187) measured in the serum. Vitamin A was also positively associated to serum levels of ΣHO-PCBs and 4-OH-CB107, the major HO metabolite detected in serum (Vanden Berghe *et al.*, 2012).

The relationships between PCBs and vitamin A status have been investigated in laboratory animals, as well as in wildlife. Several molecular mechanisms have been suggested and are summarised in Figure 4.1. Increases in the circulating vitamin A after exposure to

PCBs have already been reported in rats and were linked to a reduction of hepatic retinoid stores (Bank *et al.*, 1989; Zile, 1992), suggesting an enhanced mobilisation of hepatic vitamin A in PCB exposed animals. A reduction of hepatic retinoid stores following PCB exposure has also been observed in fish (Boyer *et al.*, 2000), birds (Bishop *et al.*, 1999; Murk *et al.*, 1994) and aquatic mammals (Murk *et al.*, 1998; Nyman *et al.*, 2003). Although several *in vivo* and *in vitro* studies showed that the activity of different liver enzymes involved in the retinoid metabolism were modified following PCB exposure (Figure 4.1), the mechanisms by which an increased mobilisation of vitamin A from the liver may occur are still unknown. One explanation may lie in the decreased LRAT and ARAT activities, two enzymes involved in the conversion of retinol into retinyl esters in hepatic stellate cells (D'Ambrosio *et al.*, 2011), and thus in the hepatic storage of vitamin A. Indeed, it has been shown that the treatment of rats with TCDD induced a decreased activity of LRAT and ARAT (Nilsson *et al.*, 1996). As coplanar PCBs are agonists of the AhR in a similar way to TCDD and the impact of TCDD on vitamin A metabolism occurs entirely via AhR (Novák *et al.*, 2008), coplanar PCBs, such as CB-105 and -118, may also be able to induce comparable enzyme inhibition. An additional contributory mechanism could be an increased release of retinol bound to RBP into the blood circulation (Zile, 1992). However, to the best of our knowledge, such a mechanism has not been described in detail.

Although liver vitamin A concentrations were not measured in the present study, it is possible that the positive relationships between PCBs and vitamin A observed here are due to an enhanced mobilisation of vitamin A from hepatic stores. The surplus released from the liver into the bloodstream could be either taken up by the kidneys, which play an important role in vitamin A metabolism (Käkëla *et al.*, 1997) or stored in the blubber, which is an important storage site for vitamin A in marine mammals (Schweigert *et al.*, 1987; Käkëla *et al.*, 1997; Vanden Berghe *et al.*, 2010). Nevertheless, in a similar study performed on Scottish grey seals, including the same seal population, Vanden Berghe *et al.* (2010) did not observe any relationship between PCBs and vitamin A levels in serum of lactating females, although comparable vitamin A and PCBs

concentrations were measured in both studies. In that previous study, some individuals could not be sampled at each period. The number of samples was thus sometimes unequal for some animals and tissues, which may have lowered the statistical power to detect some effects. Additionally, our current analysis used a more rigorous LMM approach, whereas panel data regressions were performed in Vanden Berghe *et al.* (2010). The potential effects of PCBs on vitamin A in inner blubber could not be assessed in the previous study because there were too few matched samples for that tissue (11 out of 17).

Positive correlations between PCBs in blubber and circulating vitamin A were observed in weaned and fasted harbour seal pups (Simms *et al.*, 2000). Similarly, Tornero *et al.* (2006) reported a positive correlation between retinoids and PCBs in the blubber of male common dolphins (*Delphinus delphis*). Individuals had, however, 10-fold higher PCB levels than in the present study. Both studies suggested an enhanced release of vitamin A from liver retinoid stores because of PCB contamination, contributing to increasing concentrations of vitamin A in blubber and serum. By contrast, Mos *et al.* (2007) found negative associations between PCBs and vitamin A levels in blubber of harbour seal pups exposed to similar PCB concentrations to the present study. Although Nyman *et al.* (2003) observed positive correlations between PCBs in liver and circulating vitamin A, they reported negative relationships between liver and blubber vitamin A contents and hepatic PCBs levels in grey seals. These studies suggested an increased mobilisation of vitamin A stores from the liver, but also from the blubber, leading to the depletion of vitamin A levels in these tissues and an increase of vitamin A in blood.

Most studies on seals report negative relationships between vitamin A and ΣPCB concentrations in the circulation (Jenssen *et al.*, 2003; Debier *et al.*, 2005; Mos *et al.*, 2007). Although these studies did not investigate the presence of circulating HO-PCBs, they suggest that the decrease in retinol concentrations was likely due to a disruption of the TTR-RBP-retinol complex by specific HO-PCBs. This can result in a greater excretion of circulating vitamin A by glomerular filtration, as observed in humans and laboratory animals (Brouwer *et*

*al.*, 1998; Lans *et al.*, 1993) (Figure 4.1). Several recent studies support this hypothesis. In a recent *in vitro* study using [$^{125}$I]–$T_4$ and [$^{14}$C]-4-OH-CB107, Gutleb *et al.* (2010) have shown that 4-OH-CB107 completely saturated the binding sites on TTR for $T_4$ in wild living polar bears (*Ursus maritimus*). Nonetheless, the concentrations of 4-HO-CB107 detected in those polar bears were more than 25 times higher than levels detected in the seals of the present study (Vanden Berghe *et al.*, 2012). Gabrielsen *et al.* (2011) reported negative relationships between several HO-PCBs and thyroid hormones in *in vivo* studies with hooded seals (*Cystophora cristata*). Similarly, although not demonstrated in wild mammals yet, Cesh *et al.* (2010) found negative correlations between HO-PCBs and retinol in plasma of nestling bald eagle (*Haliaeetus leucocephalus*). These observations favour a competitive binding of HO-PCBs to TTR in wild animals.

The positive correlations between HO-PCBs and vitamin A observed in the present study do not mean that HO-PCBs did not exert an impact on the blood transport of vitamin A, through the binding to TTR. Indeed, other molecular mechanisms occurring at different levels (e.g. enzymes, nuclear receptors) in liver and/or blubber may also take place and be at the origin of the positive relationships observed. Moreover, contrary to the present study, the previously cited works were carried out on non-lactating animals and, except for the study of Simms *et al.* (2000), on non-fasting animals. Lactation is known to affect circulatory and tissue vitamin A concentrations in female seals, reflecting the fasting-associated mobilisation of vitamin A from blubber stores (Debier *et al.*, 2002 and 2012). Although the stage of lactation was used in our statistical models to control for temporal variation in lipid loss associated with lactation and fasting, lactation represents a physiological state associated with important metabolic adaptations. Therefore, other mechanisms may be at the origin of the positive relationships observed in our grey seal mothers. The fact that mobilisation and transfer of PCBs and vitamin A during lactation in grey seals appear to share common biochemical pathways may also contribute to the relationships observed in the present study (Debier *et al.*, 2004; Vanden Berghe *et al.*, 2010).

### 4.4.2. Relationships between PBDEs, MeO-PBDEs and vitamin A

Vitamin A concentrations in inner blubber were positively correlated with ΣPBDEs, as well as with BDE-47, -99, -100, -153 and -154. Similarly, positive relationships were observed in serum between vitamin A concentrations and ΣPBDEs, as well as BDE-47, the prevalent PBDE congener found in the serum samples (Vanden Berghe *et al.*, 2012). The positive relationships between vitamin A and PBDE levels in serum and blubber may result, in part, from an enhanced mobilisation of vitamin A from the liver, as suggested earlier for PCBs.

PBDEs have been shown to decrease hepatic retinoid levels in rats (Hallgren *et al.*, 2001; Ellis-Hutchings *et al.*, 2006), which may be explained in part by an enhanced vitamin A metabolism in the liver. For example, technical PBDE mixtures have been shown to induce both phase I and phase II xenobiotic metabolising enzymes in *in vitro* and *in vivo* experiments (Siddiqi *et al.*, 2003) and may thus have adverse effects comparable to TCDD and dioxin-like PCBs on hepatic vitamin A homeostasis. However, Sanders *et al.* (2005) reported only a weak up-regulation of CYP1A1 (a biomarker for activation of the AhR) by DE-71, a PBDE technical mixture, and BDE-47, -99 and -100 in rats exposed to high concentrations. On the contrary, PBDEs appeared to be more able to up-regulate CYP2B and CYP3A, both biomarkers for activation of Constitutive Androstane and Pregnane X Receptors (CAR and PXR, respectively). Sanders *et al.* (2005) suggested thus that PBDEs more likely mediate their toxicity through AhR-independent mechanisms. It has been shown recently that CYP3A was involved in the hepatic metabolism of retinoic acid (Ross and Zolfaghari, 2011), suggesting that PBDEs may affect vitamin A metabolism, in part, through this pathway.
Mechanisms by which PBDEs may increase mobilisation of hepatic vitamin A have never been investigated. Few studies have analysed the effects of PBDEs on vitamin A status and most of them have been performed on laboratory animals or birds and often used non-environmentally relevant concentrations. Hallgren *et al.* (2001)

showed that rats exposed to 18 and 36 mg/kg of Bromkal 70-5 DE, a technical mixture containing tetra- and penta-PBDEs (40% of BDE-47), had reduced hepatic vitamin A levels compared to controls. Although PBDE concentrations found in our grey seals are about 200 times lower, a comparable decrease following PBDE contamination may have occurred, leading to higher circulating vitamin A concentrations. However, although Ellis-Hutchings *et al.* (2006) also observed a significant decrease in vitamin A liver content in DE-71 exposed rats during gestation and lactation, no change in circulating retinol could be detected, suggesting that the mobilisation of vitamin A from the liver was not enhanced. Similarly, no significant correlation between ΣPBDEs and retinol in blood plasma were found in bald eagles with levels of contamination 10 times higher than the grey seals of the present study (Cesh *et al.*, 2010). On the other hand, American kestrels (*Falco sparverius*) exposed to environmentally relevant PBDE congener concentrations had lower plasma retinol levels when exposed to BDE-99 and -100, while reduced hepatic retinol and retinyl palmitate concentrations were observed when kestrels were exposed to BDE-47 (Fernie *et al.*, 2005). The direction of the relationships between PBDEs and vitamin A appeared thus to be highly variable according to the species, the physiological status of the animal, but also the congeners/mixtures and the level of exposure.

Positive correlations were observed between vitamin A, ΣMeO-PBDE and 2-MeO-BDE-68 levels in inner blubber. Since this is the first study to investigate the relationships between vitamin A and naturally produced MeO-PBDEs in any living organism, comparison with the literature was not possible. Moreover, the toxicity of MeO-PBDEs is still unclear. Some *in vitro* studies on human cell lines observed alterations of enzyme activity such as CYP17 and aromatase (Cantón *et al.*, 2005; Cantón *et al.*, 2006; Song *et al.*, 2008). However, one major concern is likely the demethylation of MeO-PBDEs, which produces OH-PBDEs, a process that has been observed in some fish species (Wan *et al.*, 2009). A competitive binding of HO-PBDEs on human TTR has been demonstrated *in vitro* (Meerts *et al.*, 2000) and negative relationships between ΣHO-PBDEs and circulating retinol in bald eagles were observed (Cesh *et al.*, 2010). Therefore, the presence of MeO-PBDEs in organisms may have toxicological

implications for vitamin A homeostasis. Nevertheless, HO-PBDEs were not detected in grey seals serum of the present study, although the limit of quantification was rather low (5 pg/ml). Other studies have also reported no or low concentrations of circulating HO-PBDEs in seals (Routti *et al.*, 2009; Weijs *et al.*, 2009) and the authors suggested either low biotransformation or low retention of those compounds. PBDEs were present at low concentrations in blubber and serum in our lactating grey seals (Vanden Berghe *et al.*, 2012), so it is possible that HO-PBDEs are formed at only low rates. In addition, MeO-PBDEs which may also be a source of HO-PBDEs (demethylation) were also detected at low concentrations in blubber and were absent in serum (Vanden Berghe *et al.*, 2012). The low concentrations or absence of two potential sources of HO-PBDEs may explain the absence or undetectable levels of HO-PBDEs in serum.

### 4.4.3. Relationships between OCPs and vitamin A

Vitamin A concentrations in inner blubber were positively correlated with ΣDDXs and with *p,p'*-DDE in particular, the dominant DDX metabolite (Vanden Berghe *et al.*, 2012). In serum, no effect of DDXs on vitamin A was observed. Similarly, no association between vitamin A concentrations and low levels of HCB (Vanden Berghe *et al.*, 2012) was found in any investigated tissues. In contrast to our results, Nyman *et al.* (2003) reported negative relationships between vitamin A and ΣDDX content in the liver but also in the blubber of grey seals. This decrease was coupled to an increase in circulating vitamin A. The authors suggested an exacerbated mobilisation of vitamin A from the liver and blubber, as already discussed for PCBs. On the other hand, vitamin A negatively correlated with ΣDDX concentrations in serum of juvenile sea lions (Debier *et al.*, 2005). Little is known about mechanisms of action of DDXs on vitamin A status. Delport *et al.* (2011) showed that long-term DDT uptake reduced RBP concentrations in human blood.

## 4.5. Conclusions

We observed positive relationships between vitamin A and PCBs, PBDEs and DDXs in inner blubber and between vitamin A and PCBs, PBDEs and HO-PCBs in serum of lactating grey seal females. These results suggest that vitamin A may be mobilised from the liver and redistributed into the blubber. Even though some of the relationships observed in the present study differed from previously published studies, our data corroborate previous findings showing that environmental contaminants may disrupt vitamin A homoeostasis in wild seals. Although the lactation was taken into account, this particular state very likely plays a role in the relationships between vitamin A and pollutants. Among others, the fact that vitamin A and PCBs seem to be mobilised and transferred through similar pathways in lactating grey seals may contribute to the relationships observed. Finally, it is essential to remember that, in environmental studies, animals are exposed to a wide and complex mixture of compounds that interact with each other and that may interfere with the metabolism of vitamin A as mixtures or single compounds. Therefore, the relevance of vitamin A as a biomarker of POP exposure in wildlife will greatly depend on the ability to better understand and account for the various confounding factors. Besides, the alteration of vitamin A status resulting from the exposure to environmental contaminants can affect the immune response with potential consequences on the resistance to infection at large (Mora *et al.*, 2008). Moreover, direct effects of retinol on morbillivirus infections have recently been partially elucidated (Trottier *et al.*, 2009). These impacts of vitamin A on marine mammal resistance to viral infections certainly deserve more attention. Additionally, it would be worth investigating the potential impacts of POPs and their metabolites in suckling pups. Indeed, in one of our previous works (Vanden Berghe *et al.*, 2010), we showed that circulating vitamin A in suckling grey seal pups was adversely affected by PCBs found in milk and pup serum. These negative impacts on vitamin A status during a period of rapid growth and development could likely affect the health of young grey seals.

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# Chapter 5

# Effect of PCBs, PBDEs and vitamin A in C57BL/6 murine splenocytes and the 11B7501 lymphoma B cell line from harbour seal - Preliminary results

## Foreword

We have shown in previous chapters that vitamin A homeostasis in grey seals may be disrupted by POPs. Vitamin A is an important regulator of the immune system. Therefore, an alteration of the vitamin A status resulting from POP exposure may affect the immune system and therefore possibly increase the risk of infections[4]. The influence of vitamin A on the immune system of marine mammals and its possible interactions with the immunotoxic effects of POPs deserves further investigation. In this last experimental part, we performed experiments on 2 *in vitro* models: C57BL/6 mouse splenocytes and a harbour seal lymphoma B cell line. We attempted to assess the effect of several PCB and PBDE congeners as well as vitamin A on the apoptosis, proliferation and cell cycle. These models

[4] Mora, J.R., Iwata, M., von Andrian, U.H., 2008. Vitamin effects on the immune system: vitamins A and D take centre stage. Nature Reviews Immunology 8, 685-698.

have been set up in order to help to better understand the effects of vitamin A and POPs on the immune system, especially the one of wild marine mammals. This chapter presents the preliminary results of our experiments.

# Effect of PCBs, PBDEs and vitamin A in C57BL/6 murine splenocytes and the 11B7501 lymphoma B cell line from harbour seal - Preliminary results

## 5.1. Introduction

Environmental pollutants such as polychlorobiphenyls (PCBs) and more recent polybromodiphenylethers (PBDEs) are persistent, widely distributed in the environment and biomagnify throughout the food chain. PCB-induced immunotoxicity has already been well documented in laboratory animals (Stack *et al.*, 1999, Smithwick *et al.*, 2003 and 2004; Mori *et al.*, 2006 and 2008), humans (Nakata *et al.*, 2002; Levin *et al.*, 2005a; Sandal *et al.*, 2008) and wildlife (Luebke *et al.*, 1997; Keller *et al.*, 2006; Ross and De Guise, 2006). On the other hand, some studies have also reported an absence of effect of PCBs on immune cell functions in different mammal species (Belpaeme *et al.*, 1996; Fernlöf *et al.*, 1998; Mori *et al.*, 2008). The immunotoxicity of PCBs seems to be linked to their substitution pattern. While immunological effects of co-planar congeners appear to be mediated through the aryl hydrocarbon receptor (AhR) (Silkworth and Antrim, 1985), other studies have demonstrated that non dioxin-like PCBs exerted their immunotoxicity via AhR-independent mechanisms (Stack *et al.*, 1999, Smithwick *et al.*, 2003 and 2004; Levin *et al.*, 2005a).

In contrast to PCBs, only a limited number of studies have reported the potential effects of PBDEs on the immune system. Adult mice treated subchronically with a high dose of DE-71, a commercial mixture of PBDEs, (1000 mg/kg body weight (bw)) had reduced antibody response to sheep red blood cells (SRBC) (Fowles *et al.*, 1994) and adult mice exposed daily to 18 mg BDE-47/kg bw had a reduced number of splenocytes (Thuvander and Darnerud, 1999). Leijs *et al.* (2009) reported a negative correlation between PBDE levels in serum of adolescents and the lymphocyte counts. In harbour seal (*Phoca vitulina*), phagocytosis was affected when cells were exposed *in vitro* to several PBDE congeners (Frouin *et al.*, 2010a). In addition, recent studies have suggested that a possible mechanism of PBDE immunotoxicity may be found in the reactive oxygen species (ROS) formation (Reistad et Mariussen, 2005; Yan *et al.*, 2011). On the other hand, in some other models, PBDEs had no effect on immunological parameters. For example, Fernölf *et al.* (1998)

observed no effect of several PBDE congeners on human blood lymphocyte proliferation. Similarly, skin response to phytohaemagglutinin (PHA) was not modulated in minks that fed diets supplemented with 1 to 100 ppm of DE-71 (Martin *et al.*, 2007) and natural killer (NK) cell activity in mice was not altered after exposure to up to 1000 mg DE-71/kg bw for 14 days (Fowles *et al.*, 1994). The differences observed among all those studies may be linked to the studied animal species, the investigated function and/or the PBDEs (in mixture or as individual congener) used during the exposure.

Vitamin A is an important regulator of the immune system in mammals. A deficiency in vitamin A generally alters several immune functions such as phagocytosis and NK cell activity and decreases the number as well as the activity of T lymphocytes (Friedman and Sklan, 1989; Stephensen, 2001). A supplementation of vitamin A can usually restore the normal immune cell functions (Stephensen, 2001). It is also well known that an optimal vitamin A intake is important to protect from infections. Therefore, vitamin A deficiency observed in developing countries is thought to be an important contributing factor of child death associated with infectious diseases (Blomhoff *et al.*, 2004). Vitamin A deficiency is associated with gastrointestinal and respiratory infections, while vitamin A supplementation tends to significantly reduce mortality in HIV-infected or malnourished children (Mora *et al.*, 2008). In addition, studies in several African countries reported that vitamin A deficiency was responsible, in part, for the massive measles deaths in children and that treatment with vitamin A can reduce the mortality associated with measles (Sommer, 2008). Consequently, the United Nation Children's fund (UNICEF) and the World Health Organisation (WHO) recommended vitamin A treatment for children suffering from acute measles (Sommer, 2008). Since PCBs and PBDEs have been demonstrated to potentially alter vitamin A status in both laboratory and wild animals (Zile, 1992; Hallgren *et al.*, 2001; Debier *et al.*, 2005; Ellis-Hutchings *et al.*, 2006; Novák *et al.*, 2008), the immunotoxic effects of these compounds may result either from direct impacts on immune cells or from indirect mechanisms such as an alteration of the vitamin A status.

At the top of the marine food web, marine mammals can be heavily exposed to environmental contaminants such as PCBs and PBDEs, as demonstrated by the relatively high concentrations found in their blubber (Weijs *et al.*, 2009; Vanden Berghe *et al.*, 2012). Numerous studies have already reported the immunotoxic effects of PCBs in several marine mammal species (Ross and De Guise, 2006; Mori *et al.*, 2008), while only one investigated the impact of PBDEs in harbour seals and showed that some immune parameters were affected after *in vitro* exposure to individual congeners (Frouin *et al.*, 2010a). The presence of such immunotoxic contaminants in the organism of marine mammals may facilitate the emergence of infectious diseases, as suggested to explain the fact that morbillivirus canine distemper virus (CDV) caused a high mortality rate in several marine mammal species in the 1990s (Ross, 2002). Additionally, since negative relationships between environmental contaminants and vitamin A status have already been observed in marine mammals (Jenssen *et al.*, 2003; Debier *et al.*, 2005; Mos *et al.*, 2007, Vanden Berghe *et al.* 2010) and since CDV is closely related to the human measles virus, a reduction of vitamin A levels in contaminated animals may also have facilitated the CDV deaths, as reported earlier for explaining measles deaths in African children.

In the present work, we used C57BL/6 mice and a harbour seal lymphoma B cell line (11B7501 B lymphoma cell line) as models to investigate 1) the potential immunotoxic effect of several PCB and PBDE congeners and 2) the impact of vitamin A on the immune system. These experiments were carried out in the perspective of determining the effects of vitamin A exposure on the potential immunosuppressive effect of PCBs and PBDEs. The C57BL/6 is the commonly mouse strain used for such immunological experiments and the 11B7501 B lymphoma cell line has been demonstrated to be an useful *in vitro* model to address immunotoxic effects on environmental contaminants on seal immune cells (Frouin *et al.*, 2010a and 2010b). Apoptosis, proliferation and cell cycle progression were investigated because they represent key functions in the homeostasis of the immune system. These 2 models were tested in order to bring new insights on the effects of vitamin A and POPs on the immune system, especially the one of wild marine mammals.

## 5.2. Material and methods

Experiments were realised in 2 phases in 2 different laboratories (Laboratory of Prof. Michel Fournier, Institut National de la Recherche Scientifique - Institut Armand-Frappier, Canada and laboratory of Prof. Pierre Gianello, Pôle de chirurgie expérimentale et transplantation, UCL, Belgium). Some of them were performed in both laboratories and since similar results were obtained, data were combined. Details of all the experiments can be found in Table 5.1.

### 5.2.1. Cultured cell line

The 11B7501 B lymphoma cell line (American Type Culture Collection, ATCC) was developed from the peripheral blood mononuclear cells derived from a male harbour seal with malignant leukemia lymphoma.

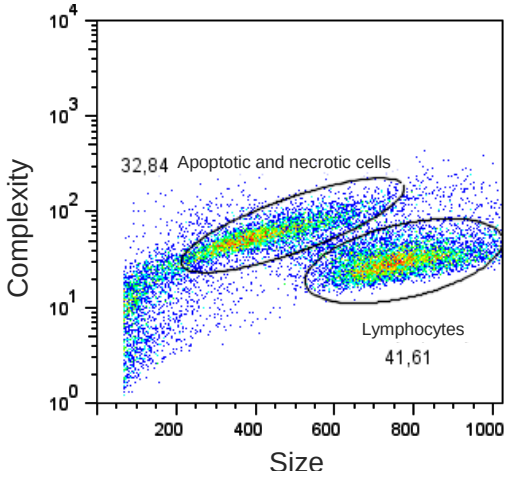


**Figure 5.1**. Scattergram of the flow cytometric profile of the 11B7501 lymphoma B cell line.

The cell line was maintained in suspension in complete RPMI (RPMI-1640 (Sigma-Aldrich, St-Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% Peniciline (100 U/ml) – Streptomycine (100 mg/ml) (Sigma-Aldrich) and 10 mM Hepes (Sigma-Aldrich)) at 37°C under 5% $CO_2$ humidified air. The cell viability was assessed by the trypan blue exclusion method before culturing the cells. Cells were adjusted to a concentration of $2.5x10^6$ cells/ml. A scattergram of the flow cytometric profile of this cell line is presented in Figure 5.1.

### 5.2.2. Murine splenocyte isolation

C57BL/6 female mice were obtained from Charles River Canada Inc (Qc, Canada) or from the Faculty of Medicine animal facilities of the Université catholique de Louvain (UCL, Belgium) and used between 6 and 11 weeks of age. The mice were killed by $CO_2$ inhalation and the spleen was aseptically removed and placed in RPMI medium at 4°C. The spleen was then triturated with the end of a 3-ml syringe and the suspension obtained was filtered through nylon wool into Pasteur pipettes. The crude cell suspension was allowed to sediment for 5 min and the supernatant containing the lymphocytes was collected and resuspended in complete RPMI. Viability of cells was determined by trypan blue dye exclusion before culturing the cells. Cells were adjusted to a concentration of $5x10^5$ cells/ml.

### 5.2.3. Chemical preparation and treatment

2,2',4,4'-tetrabromodiphenyl ether (BDE-47, purity 100%), 2,2',4,4',5-pentabromodiphenyl ether (BDE-99, purity 99.2%), 2,2',4,4',6-pentabromodiphenyl ether (BDE-100, purity 100%), 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE-153, purity 99.3%), 2,3',4,4',5-pentachlorobiphenyl (CB-118, purity 99.5%), 3,3',4,4',5-pentachlorobiphenyl (CB-126, purity 99.4%), 2,2',3,4,4',5-hexachlorobiphenyl (CB-138, purity 99.9%), 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153, purity 99.5%) and 2,2',3,4,4',5,5'-pentachlorobiphenyl (CB-180, purity 100%) were obtained from AccuStandard (New Haven, CT, USA). CB-118,

-138, -153, -180 and BDE-47, -99, -100, and -153 were chosen because they are all well representative of the PCB and PBDE contamination in the environment and in tissues of top predators such as marine mammals (Weijs *et al.*, 2009; Vanden Berghe *et al.*, 2012). CB-126 was selected because it is a coplanar congener that is known to be highly toxic. Stock solutions were prepared by dissolving each compound in dimethylsulfoxide (DMSO) (Sigma-Aldrich) or absolute ethanol (EtOH) (Sigma-Aldrich). In order to avoid the adsorption of PCBs and PBDEs onto the polypropylene round bottom 96-well plates during treatment, cells were cultured into borosilicate tubes. Working dilutions of PBDEs and PCBs were prepared in complete RPMI and contaminants were added to the culture medium at final concentrations of $10^{-8}$, $10^{-7}$ and $10^{-6}$ mol/l. The lowest concentration reflects the one detected in human serum and marine mammals tissues (Dirtu *et al.*, 2010; Weijs *et al.*, 2009). PCBs and PBDEs were tested as individual congeners as well as in PCB or PBDE mixtures. However, due to technical problems, all immune parameters and/or PCB, PBDE congeners and their mixtures could not be tested for all experiments. For example, some mixtures were tested only for apoptosis and cell cycle, while the proliferation could not be investigate for the harbor seal cell line. The PCB and PBDE congeners and their corresponding mixtures tested for each functions are summarised in Table 5.1. The PCB mixture was tested both as a non-additive mixture (mixture A) and additive mixture (mixture B) of the 5 PCB congeners, while the PBDE mixture was tested only as a non-additive mixture (mixture C) of the 4 PBDE congeners. The concentration of each PCB congener in mixture A ranged from $2x10^{-9}$ to $2x10^{-7}$ mol/l to obtain a final concentration of the mixure ranging from $10^{-8}$ to $10^{-6}$ mol/l. The concentration of each PCB congener in mixture B ranged from $10^{-8}$ to $10^{-6}$ mol/l to obtain a final concentration of the mixure ranging from $5x10^{-8}$ to $5x10^{-6}$ mol/l. The concentration of each PBDE congener in mixture C ranged from $2.5x10^{-9}$ to $2.5x10^{-7}$ mol/l to obtain a final concentration of the mixure ranging from $10^{-8}$ to $10^{-6}$ mol/l. For the cell cycle experiments with murine splenocytes, only CB-126, -138 and -153 could be tested. As a result, the corresponding PCB mixture included only those 3 PCB congeners (mixture D). Control experiments were performed with the same concentrations of DMSO or EtOH found in the most

concentrated PCBs or PBDEs working solutions (vehicle control) and did not exceed 0.2% (v:v). Those vehicle concentrations had no effect on function cells.

**Table 5.1.** Summary of the different immune parameters and treatments tested for the 2 investigated models (mouse and cell line).

| Immune parameters | Mouse | | | Cell line[c] | | |
|---|---|---|---|---|---|---|
| | *PCBs* | *PBDEs* | *Vit A* | *PCBs* | *PBDEs* | *Vit A* |
| **Apoptosis** | 118[a]<br>126[a]<br>138[a]<br>153[a]<br>180[a]<br>mixture A[a]<br>mixture B[b] | 47[a]<br>99[a]<br>100[a]<br>153[a]<br>mixture C[a] | ReOH[b]<br>ATRA[b] | 118<br>126<br>138<br>153<br>180<br>mixture A | 47<br>99<br>100<br>153<br>mixture C | ReOH<br>ATRA |
| **Proliferation** | 118[b]<br>126[b]<br>138[b]<br>153[b]<br>180[b] | 47[b]<br>99[b] | ReOH[b]<br>ATRA[b] | not tested | not tested | not tested |
| **Cell cycle** | 126[c]<br>138[c]<br>180[c]<br>mixture D[c] | 47[c]<br>99[c]<br>100[c]<br>153[c]<br>mixture C[c] | ReOH[b]<br>ATRA[b] | 118<br>126<br>138<br>153<br>180<br>mixture A | 47<br>99<br>100<br>153<br>mixture C | ReOH<br>ATRA |

Mixture A = CB-118+126+138+153+180 (non additive); Mixture B = CB-118+126+138+153+180 (additive); Mixture C = BDE-47+99+100+153 (non additive); Mixture D = CB-118+138+180 (non additive).

[a]Experiments performed in Canada and Belgium; [b]Experiments performed in Belgium only; [c]Experiments performed in Canada only.

Retinol (ReOH) and all-*trans* retinoic acid (ATRA) were purchased from Sigma-Aldrich. The retinoid compounds were dissolved in EtOH or DMSO, flushed with azote and stored in lightproof containers at -20°C. Vitamin A experiments were performed under yellow light. In

order to understand how physiological levels of vitamin A may influence the immune system, working dilutions of ReOH and ATRA were prepared in complete RPMI and added to the culture medium at final concentrations of $10^{-9}$ to $10^{-7}$ mol/l for ATRA and $10^{-7}$ to $10^{-5}$ mol/l for ReOH. In normal conditions, ReOH is found in the circulation at concentrations ranging from 1 to 3 µmol/l and ATRA is present in the nanomolar range (5–7 nmol/l) (Ross and Harrison, 2007). Control experiments were performed with the same concentrations of DMSO or EtOH found in the most concentrated working solutions and did not exceed 0.2% (v:v). Those vehicle concentrations had no effect on function cells.

### 5.2.4. Determination of PCB concentrations in cell culture medium

In order to be sure that cells were exposed to the right PCB concentrations, a liquid-liquid extraction was performed on the supernatant medium, as decribed in Bourez *et al.* (2012). Brifely, five ml of *n*-hexane were added to 400 µl of supernatant medium in an EPA vial. The samples were vortexed thouroughly for 5 min and were stored at 4°C until further analysis. Samples were then purified and analysed as described elsewhere (Debier *et al.*, 2003).

### 5.2.5. Viability

Viability determination was assessed in parallel of each test, except for proliferation. It was evaluated by flow cytometry using propidium iodide (PI, Sigma-Aldrich) (100 µg/mL) as described by Brousseau *et al.* (1999). Samples were analysed with a FACScalibur™ flow cytometer (Becton Dickinson) and the fluorescence of at least 5000 events was read in FL3. Data were expressed as a percentage of the vehicle control.

### 5.2.6. Apoptosis

Apoptotic cells were analysed by flow cytometry. For staining with the impermeant nuclear dye YO-PRO-1/propidium iodide (Vybrant Apoptosis Assay Kit #4, Molecular Probes Inc., Eugene, Oregon, USA), cells were collected and resuspended in 500 µl sterile cold phosphate buffered saline (PBS, Sigma-Aldrich) after 6h- (splenocytes) and 24h- (cell line) treatment with the different concentrations of PCBs, PBDEs or vitamin A. The cell line was incubated longer than splenocytes because it appeared to be more resistant. One µl of the YO-PRO-1 stock solution and 1 µl PI stock solution were added to the cell suspension and cells were incubated at 4°C for 30 min in the dark. Stained cells were analysed by flow cytometry using FL1 flurorescence for YO-PRO-1 and FL3 for PI. At least 5000 cells were counted for each sample. Dexamethasone (10 µmol/l) (Sigma-Aldrich) or stauroporine (5 µmol/l) (Sigma-Aldrich) was used as a positive control. In order to investigate the potential protective effect of vitamin A on apoptosis, cells were exposed to vitamin A in presence of dexamethasone. Vitamin A treatments in absence of dexamethasone were run in parallel as a "vitamin A" control. Results were expressed as percentage of the vehicle control.

### 5.2.7. Splenocyte proliferation

Lymphocyte proliferation was measured by incorporation of [$^3$H]-thymidine as described by Brousseau *et al.* (1999). Briefly, splenocytes were plated in triplicate with mitogens and the different concentrations of PCBs, PBDEs or vitamin A for 48h at 37°C and 5% $CO_2$ in 96-well round-bottom plates for vitamin A and in borosilicate tubes for contaminants. The optimal concentration used to stimulate T- and B lymphocytes was 2.5 µg/ml concanavalin A (Con A) (Sigma-Aldrich) and 50 µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich), respectively. These concentrations were determined previously with a standard curve (range of concentrations from 0 to 40 µg/ml and from 0 to 100 µg/ml for Con A and LPS, respectively). After 48h of incubation, 1µCi [$^3$H]-thymidine (MP Biomedicals Europe, Illkirch,

France) was added to each well. Regarding treatment with contaminants in borosilicate tubes, cells were distributed in 96-well round-bottom plates, prior to thymidine addition. Plates were incubated for a further 18h. The cells were harvested on fiberglass filter with a cell harvester and the amount of incorporated radioactivity was measured with a RackBeta or a Beckman ß-scintillation counter. Data were expressed as percentage of the vehicle control.

### 5.2.8. Cell cycle analysis

The different phases (G0, S and G2/M) of the cell cycle were analysed by flow cytometry after DNA staining with the Cell Cycle Staining Reagent containing 95.5 ml of 0.1% Triton X-100 (Sigma-Aldrich), 2 ml of DNAse-free RNAse (10 mg/ml) (Sigma-Aldrich) and 2.5 ml of PI (1mg/ml). Murine splenocytes were incubated with Con A or LPS and after 48h-treatment with vitamin A or contaminants, cells were centrifuged at 450 x g for 5 min, washed with PBS, resuspended in cold EtOH (70%) and kept at 4°C for at least 12h. Cells were then washed twice with PBS and stained with 500 µl Cell Cycle Staining Reagent. After incubation at 37°C in the dark for 15 min, samples were analysed by flow cytometry and the fluorescence of at least 5000 events was read in FL3. The protocol for the cell line was similar, except that no mitogen was added into the culture medium since cell lines have the ability to proliferate on their own. Results were expressed as the percentage of cells in each phase of the cell cycle.

### 5.2.9. Statistical analyses

All the results were expressed as mean ± standard deviation (SD). Data were analysed by a one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test to identify means with significant difference compared to the vehicle control. The calculations were performed using SPSS 9 for Windows and the level of significance was set at a $p$-value < 0.05.

# 5.3. Results and discussion

## 5.3.1. PCB concentrations in medium

Table 5.2 reports the PCB concentrations measured in the supernatant medium for the lowest ($10^{-8}$ mol/l) and the highest ($10^{-6}$ mol/l) concentrations after 6h and 48h of incubation in glass tubes. Although concentrations were not exactly the same than the theoretical ones, they fell into the right range.

**Table 5.2.** PCB concentrations found in the supernatant medium of the cultured cells after 6h and 48h incubation in glass tubes compared to the theoritical concentrations ($10^{-8}$ mol/l for the lowest and $10^{-6}$ mol/l for the highest).

| PCB congener | Concentrations measured after 6h incubation (mol/l) | | Concentrations measured after 48h incubation (mol/l) | |
|---|---|---|---|---|
| | Highest | Lowest | Highest | Lowest |
| 118 | $1.85x10^{-6}$ | $9.20x10^{-9}$ | $1.61x10^{-6}$ | $6.99x10^{-9}$ |
| 126 | $1.21x10^{-6}$ | $1.16x10^{-8}$ | $7.51x10^{-7}$ | $9.06x10^{-9}$ |
| 138 | $1.36x10^{-6}$ | $9.98x10^{-9}$ | $1.01x10^{-6}$ | $8.95x10^{-9}$ |
| 153 | $8.81x10^{-7}$ | $8.57x10^{-9}$ | $6.73x10^{-7}$ | $5.43x10^{-9}$ |
| 180 | $7.34x10^{-7}$ | $4.41x10^{-9}$ | $4.78x10^{-7}$ | $5.57x10^{-9}$ |

## 5.3.2. Effects of PCBs and PBDEs in murine splenocytes

### Apoptosis

Exposure to 5 µmol/l of staurosporine or 10 µmol/l of dexamethasone for 6h induced apoptosis in murine splenocytes and represents our positive control (Figures 5.2a and b). The different PCB and PBDE congeners tested as well as their non-additive mixture A had no impact on apoptosis of mouse splenocytes at the studied concentrations (Figures 5.3 and 5.4). Similarly, the additive PCB

mixture B had no effect on the murine spleen cells at the studied concentrations (Figure 5.5).

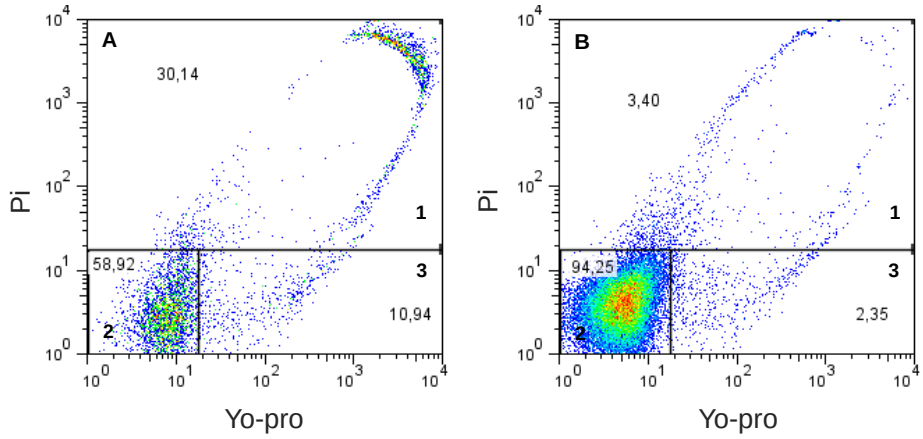


**Figure 5.2a**. Representative scattergram of apoptosis analysis in murine splenocytes. (A) Cells treated with 5 µmol/l staurosporine for 6h (positive control) and (B) corresponding untreated cells. The 3 quadrants represent necrotic cells (1), living cells (2) and apoptotic cells (3).

Previous studies on murine splenocytes reported an induction of apoptosis following PCB exposure. Yoo *et al.* (1997) observed that after treatment for 4h and 24h, Aroclor 1254 increased apoptosis (DNA fragmentation) in a dose-dependent manner in mice. However, the concentrations used in that study were 50 to 200 times higher than those tested in the present work and were thus not environmentally relevant. Similarly, treatment of murine spleen cells with 0.7 to 10 µg/ml (~2 to 25 µmol/l) of Aroclor 1254 for 4h induced significant DNA fragmentation (Jeon *et al.*, 2002). Nevertheless, the lowest tested concentration (0.5 µg/ml ~1.2 µmol/l), which corresponds approximately to the highest concentration tested in the present work, failed to induce apoptosis (Jeon *et al.*, 2002).

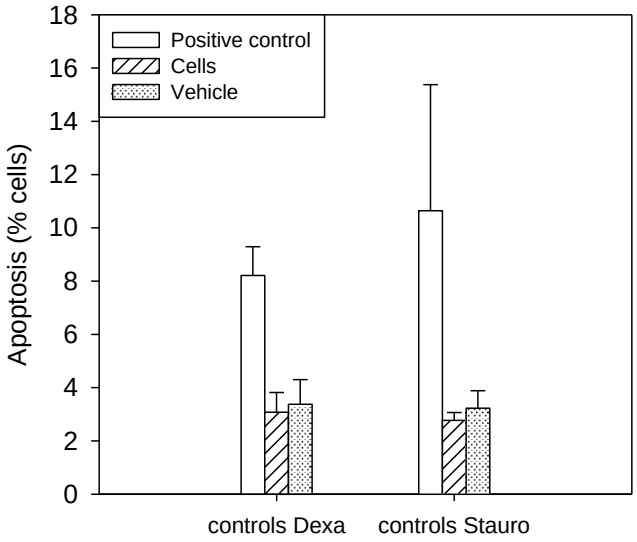


**Figure 5.2b**. Percentage of apoptotic cells after treatment with 10 µmol/l of dexamethasone (Dexa) or 5 µmol/l staurosporine (Stauro) for 6h (positive control) compared to untreated cells (cells) or cells+vehicle (vehicle).

The investigators showed that treatment of spleen cells with 5 µg/ml (~13 µmol/l, levels ~10 times higher than those used in our experiments) of the di-ortho forms of PCB congeners (CB-47, -52, -128, and -153) increased significantly DNA fragmentation, whereas the non-ortho forms (CB-77, -126, and -169) did not induce DNA fragmentation (Jeon *et al.*, 2002). The authors demonstrated that the apoptotic pathway was AhR-independent but involved the activation of caspases in mouse splenocytes (Jeon *et al.*, 2002). Di-ortho PCB congeners such as CB-138, -153 and -180 were also investigated in the present study (Figure 5.3) but no effect could be observed. This may be due to the relative low concentrations tested in the present work. It appears thus that, reassuringly, environmentally relevant concentrations of PCBs do not induce apoptosis in murine splenocytes.

Information regarding the apoptotic effects of PBDEs is scarce and, to the best of our knowledge, this is the first work to investigate the apoptotic properties of PBDEs on a primary immune cell culture in terrestrial mammals. A recent study has reported that exposure of BDE-47 at doses of 25 to 100 µmol/l for 48h induced apoptosis in Jurkat cells, a human T lymphocyte cell line (Yan *et al.*, 2011). In parallel a significant increase of ROS production and a decrease of mitochondrial membrane potential were observed, suggesting that ROS overproduction and mitochondrial dysfunction may be at the origin of the induced apoptosis (Yan *et al.*, 2011). Studies on other types of cell lines also showed that apoptosis was induced following PBDE exposure. A positive relationship was observed between BDE-47 exposure for 24h and apoptotic induction in human neuroblastoma SH-SY5Y cells after treatment with 4 and 8 µg/ml (~8 to 16 µmol/l) but not at lower levels (He W. *et al.*, 2008). Similarly, the percentage of apoptosis in primary cultured rat hippocampal neurons increased significantly after 24h-exposure to 41.2 µmol/l BDE-47, the highest concentration tested (He P. *et al.*, 2008). In human astrocytoma cells, exposure to BDE-99 (50-100 µmol/l, 24h) caused a small but significant increase in the number of apoptotic cells (Madia *et al.*, 2004). Therefore, PBDEs appear to induce apoptosis in several cell types but only at relatively high doses. As noted above for PCBs, the concentrations used in the present work were 8 to 100 times lower and periods of incubation were much shorter, which may explain the lack of effect of the tested congeners. However, our results are in agreement with the study of Frouin *et al.* (2010a) who reported no impact of BDE-47, -99 and -153 on apoptosis of seal immune cells (1.5-12 µmol/l, 24h). In addition, current environmental levels of BDE-47 and -99 did not induce apoptosis in lake trout thymocytes after 6h and 20h incubation (Birchmeier *et al.*, 2005). Thus the lack of apoptosis induction oberved here and in these 2 previous studies may be quite reassuring.

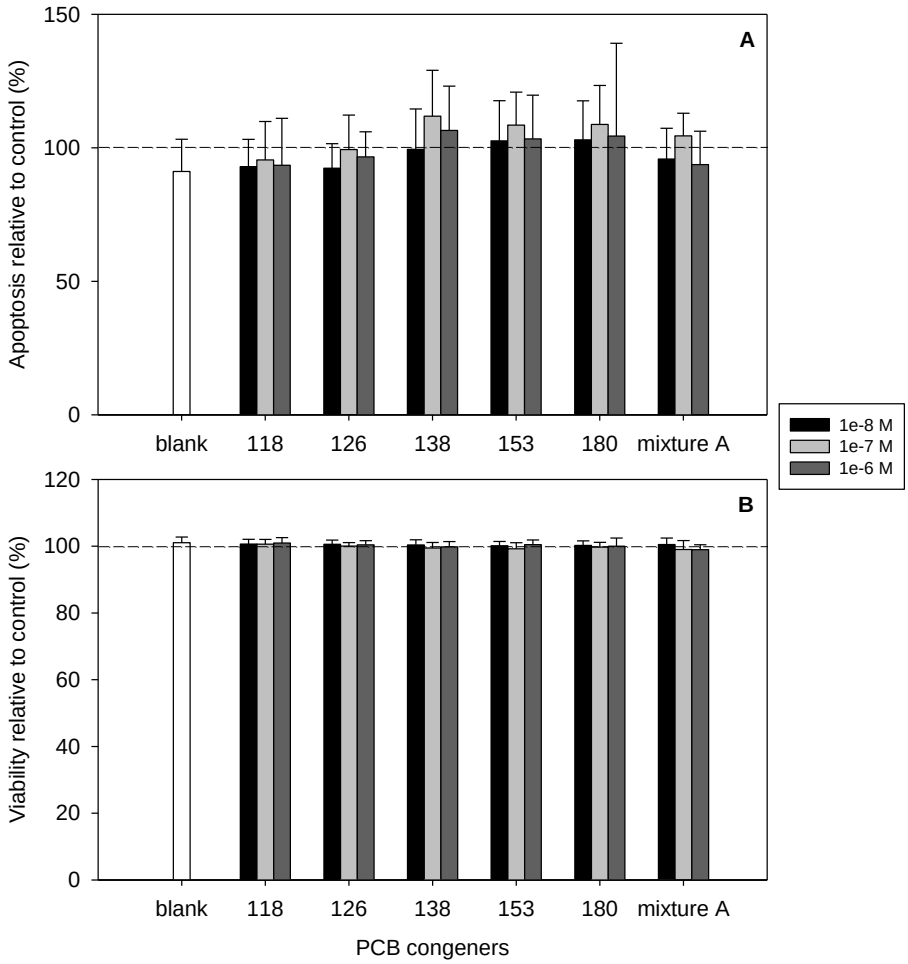


**Figure 5.3.** Effect of the 5 individual PCB congeners as well as their non additive mixture A (118+126+138+153+180) on the apoptosis (A) and viability (B) of murine spleen cells after 6h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± standard deviation (SD) of 6 independent experiments performed on a total of 13 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

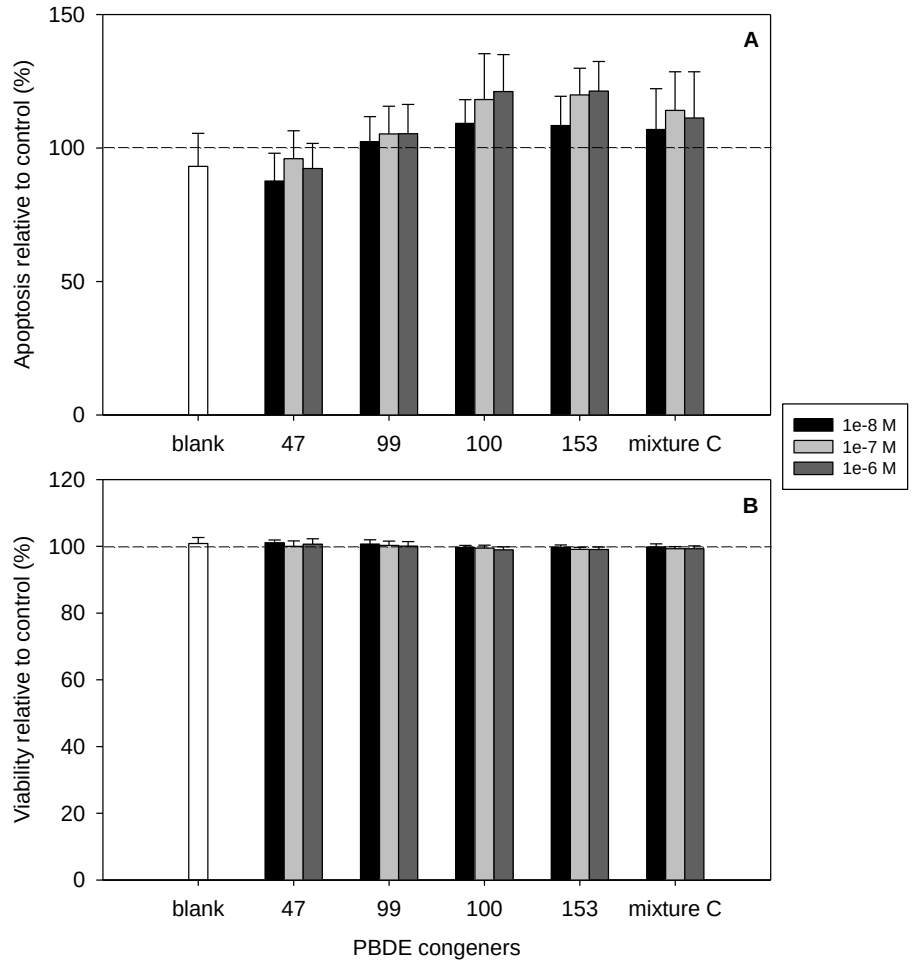


**Figure 5.4.** Effect of the 4 individual PBDE congeners as well as their non additive mixture (47+99+100+153) on the apoptosis (A) and viability (B) of murine spleen cells after 6h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 5 independent experiments performed on a total of 11 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

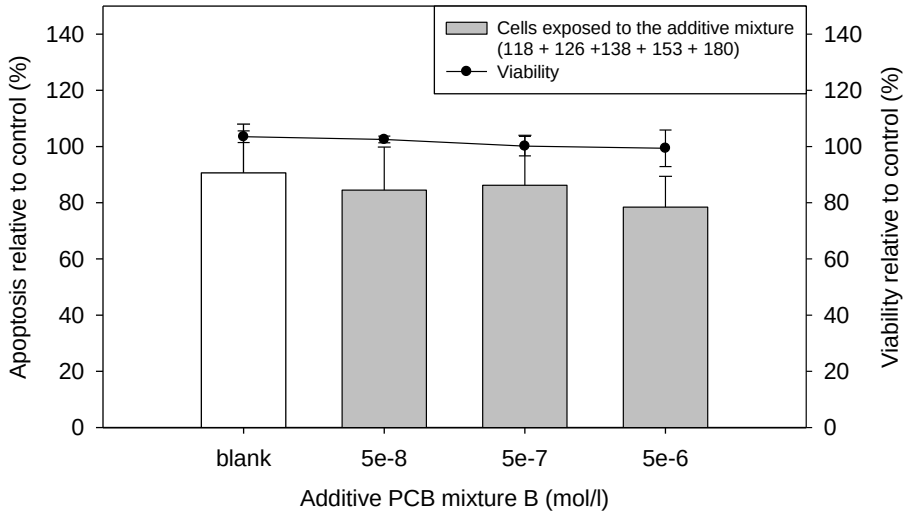


**Figure 5.5.** Effect of the additive PCB mixture B (118+126+138+153+180) on the apoptosis of murine spleen cells after 6h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 1 experiment performed on a total of 3 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

## Splenocyte proliferation and cell cycle analysis

*In vitro* exposure of murine splenocytes to individual PCB or PBDE congeners for 66h did not affect the LPS or Con A-induced splenocyte proliferation, measured by [$^3$H]-thymidine incorporation (Figures 5.6 and 5.7). Similarly, experiments on the cell cycle showed no effect of any PCB and PBDE congeners as well as their respective mixtures on LPS-stimulated splenocytes after 48h incubation (Tables 5.3a and b).

Several works have already investigated the effect of PCBs on mitogen-stimulated mouse splenocytes and the results varied among studies. For example, at comparable tested doses (0.01 to 1 µg/ml ~ 25 nmol/l to 2.5 µmol/l), Stack *et al.* (1999) reported no effect of the same individual congeners as well as CB-28 and -77 and several

commercial PCB mixtures (Aroclor 1221, 1242, 1254 and 1260) in LPS-induced murine splenocytes. Congeners CB-138 and -153 started to inhibit proliferation at 25 µg/ml (~63 µmol/l), while for mixtures, it started at 10 to 30 µg/ml (~25 – 76 µmol/l), depending on the Aroclor and the mouse strain (Stack *et al.*, 1999). It is thus possible that mouse splenocytes are insensitive to direct effects of low PCB levels, as suggested earlier for phytoheamaglutinin (PHA)- or pokeweed mitogen (PWM)-induced proliferation of human peripheral blood lymphocytes (Fernlöf *et al.*, 1997). On the other hand, Smithwick *et al.* (2003) observed a significant inhibition of LPS-induced splenocyte proliferation in mice after 72h-treatment with several individual PCB congeners at concentrations ranging from 0.01 to 40 ppm (~25 nmol/l to 100 µmol/l). Among those congeners, CB-180 significantly decreased LPS-induced proliferation at 0.01 ppm (~25 nmol/l), a concentration similar to those tested in the present work. They highlighted that congeners with two or more *ortho* chlorines significantly inhibited LPS-induced splenocyte proliferation at lower concentrations than required to induce a similar inhibition with non- or mono-ortho-substituted congeners (Smithwick *et al.*, 2003). The decrease of B cell proliferation following PCB exposure appeared to be due to an interruption of the cell cycle progression (accumulation of cells in the G0/G1 phase) and a decrease in cyclin D2 expression, a cell cycle protein that functions at the G1 checkpoint (Smithwick *et al.*, 2004). Similarly, *in vitro* exposures to CB-138, -153, -169 and -180 and their mixtures (5 ppm ~12 µmol/l) significantly decreased the LPS-induced proliferation of mouse splenocytes (Mori *et al.*, 2008). In addition, *in vitro* exposures to 5 ppm CB-138 and CB-180 as well as to the different mixtures tested with CB-138, -153, -169 and -180 also reduced the Con A–induced proliferation of mouse splenocytes (Mori *et al.*, 2006). However, the concentrations used by Mori and co-workers were more than 10 times higher than the ones used in the present study, which may explain the difference observed.

Regarding PBDEs, our results are consistent with those found by Fernlöf *et al.* (1997) who observed no significant change in PHA or PWM-induced lymphocyte proliferation in human lymphocytes after *in vitro* exposure of BDE-47 and -85 from $10^{-9}$ to $10^{-5}$ mol/l, a range of concentrations similar to those tested in the present study. Similarly,

when mice were orally exposed to high levels of penta-BDE and deca-BDE during gestation and lactation (up to 200 mg/kg for penta-BDE and 12.5 g/kg for deca-BDE), no significant change of B-spleen cell proliferation by LPS was observed in treated dams and their offspring (Hong, *et al.* 2012). However, penta-BDE appeared to increase the proliferation of spleen cells stimulated with Con A in both dams and offspring, whereas deca-BDE induced a significant T cell proliferation in dams only (Hong, *et al.* 2012). Finally, mitogen-stimulated T- and B-spleen cell proliferation in mice was increased following exposure to the 100 mg/kg of DE-71 (total administrated dose) compared to controls (Fair *et al.*, 2012).

In view of the present data and previous studies, PCBs and PBDEs appear thus to modulate the proliferation of murine splenocytes differently, varying from no effect for both to an inhibition of proliferation after PCB exposure and an overstimulation of proliferation after PBDE exposure. Why there is such a discrepancy between PCBs and PBDEs, it is still unknown. Although the immunosuppressive characteristics of PCBs is well known, the rise in B- or T-cell proliferation following PBDE treatment may also result in adverse health effects. Indeed, while immune-suppression may increase the susceptibility of infectious diseases, immune enhancement may lead to autoimmune diseases and anergy (inability to mount antigen-specific immune responses) (Schwartz, 2003).

The results found in the present study may be quite reassuring since no effect was observed on the investigated immune functions after incubation with current environmentally relevant PCBs and PBDEs concentrations and even with higher concentrations.

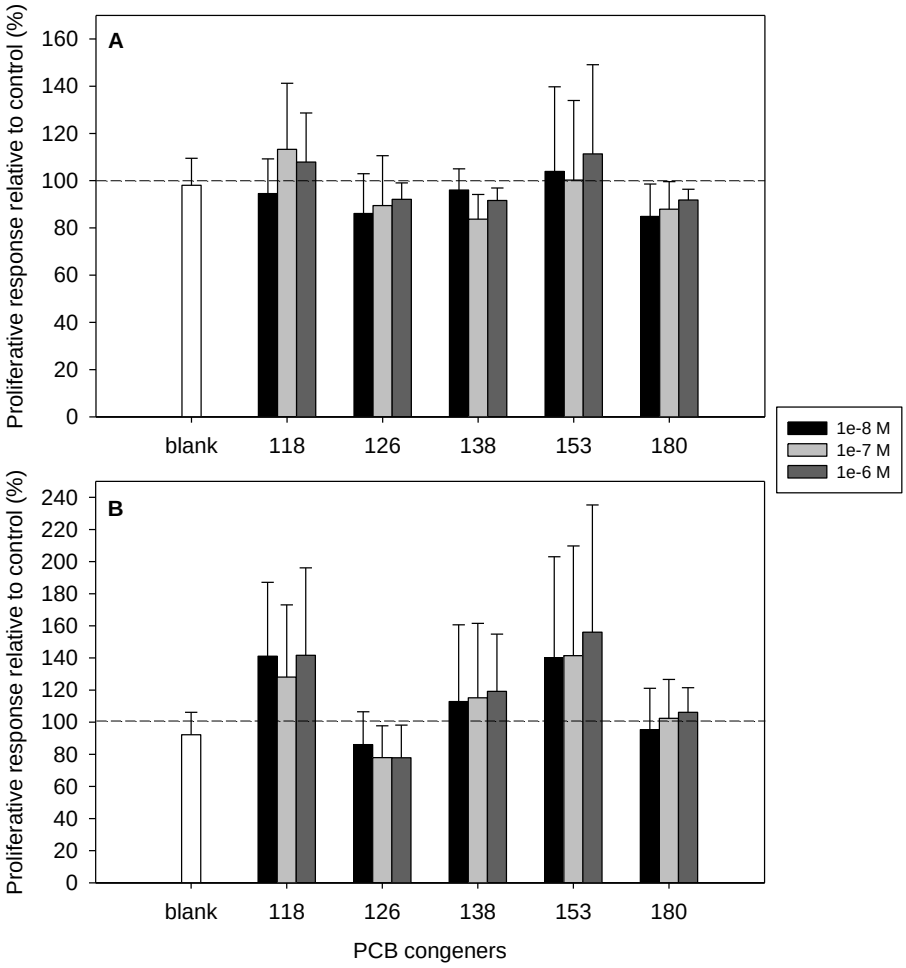


**Figure 5.6.** Effect of the 5 individual PCB congeners on the Con A (A)- or LPS (B)-induced murine splenocyte proliferation after 66h incubation, measured as [$^3$H]-thymidine incorporation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments performed on a total of 6 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

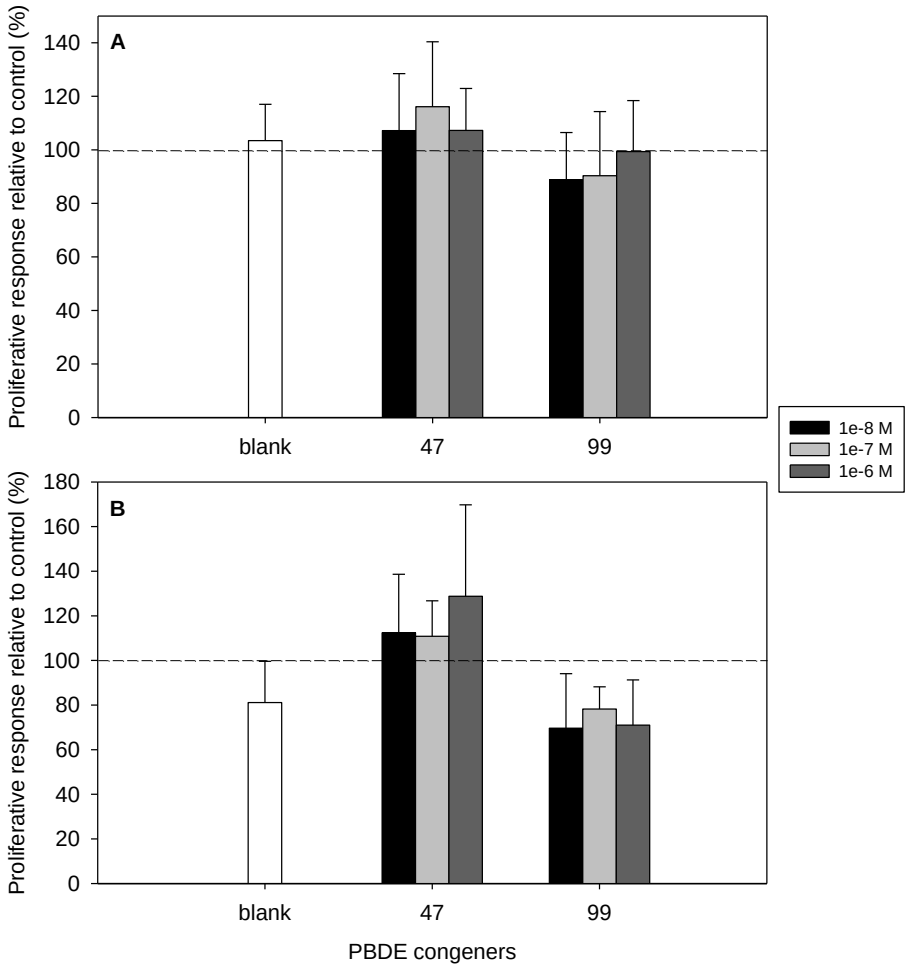


**Figure 5.7.** Effect of BDE-47 and -99 on the Con A (A)- or LPS (B)-induced murine splenocytes after 66h incubation, measured as [$^3$H]-thymidine incorporation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 2 independent experiments performed on a total of 4 individuals. BDE-100 and -153 could not be tested due to problem of precipitation in the stock solutions.

### 5.3.3. Effects of vitamin A in murine splenocytes

#### Apoptosis

Neither ATRA nor ReOH at concentrations ranging from $10^{-9}$ to $10^{-7}$ mol/l and $10^{-7}$ to $10^{-5}$ mol/l, respectively, prevented dexamethasone-induced apoptosis in murine spleen cells (Figure 5.8). Similarly, no change was observed for non-induced cells (Figure 5.9).

The fact that vitamin A did not prevent induced-apoptosis is in agreement with the study of Engedal *et al.* (2004) who observed that treatment with ATRA alone (100 nmol/l) over a time period of 11 days did not affect the spontaneous death of human blood T lymphocytes. Similarly, no apparent effect on apoptosis of anti-μ and anti-CD38 stimulated murine splenic B cells was detected after 5-day treatment with 10 nmol/l ATRA (Chen et Ross, 2005).

In contrast, physiological concentrations of ATRA appeared to prevent apoptosis in 12-O-tetradecanoyl phorbol 13-acetate (TPA)-activated human blood lymphocyte T via an enhancement of interleukin (IL)-2 production after 5- and 8-day treatment (Engedal *et al.*, 2004) as well as in human blood B-cells (3-day treatment) (Lømo *et al.*, 1998; Blomhoff *et al.*, 2004). Regarding ReOH, it has been demonstrated that a single neonatal treatment with 3 mg ReOH caused a significant elevation of the number of spontaneously apoptotic cells in rats at adult age (Gaál *et al.*, 1997). The discrepancies observed with some previous studies may be due to differences of sensitivity between species and/or strain, the type of investigated cells (e.g., blood cells vs splenic cells, purified B and T cells vs. non purified cells, stimulated cells vs. non-stimulated cells), the method (*in vitro* or *in vivo*) and the period of incubation.

#### Splenocyte proliferation and cell cycle analysis

We observed no modulation of the LPS- and Con A-induced murine splenocyte proliferation, measured by [$^3$H]-thymidine incorporation, after 66h-treatment with ATRA or ReOH (Figure 5.10).

**Table 5.3a.** Cell cycle progression of LPS-induced murine splenocytes exposed to individual PCB congeners and their non-additive mixture D for 48h[a].

| Treatment | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|
| *PCBs (n=2, N=3)* [b] | | | | |
| Control – (cells without LPS) | 96.3 ± 2.8 | 95.4 ± 1.7 | 2.5 ± 1.0 | 1.2 ± 1.0 |
| Cells | 95.1 ± 2.0 | 82.9 ± 4.2 | 9.1 ± 1.5 | 6.6 ± 2.9 |
| Vehicle | 91.9 ± 1.1 | 84.4 ± 3.3 | 8.1 ± 1.3 | 6.2 ± 1.8 |
| CB-126 | | | | |
| $1x10^{-8}$ M | 92.7 ± 3.1 | 85.6 ± 0.4 | 7.5 ± 0.4 | 5.6 ± 0.5 |
| $1x10^{-7}$ M | 93.3 ± 1.6 | 86.9 ± 1.0 | 6.7 ± 0.6 | 5.1 ± 0.7 |
| $1x10^{-6}$ M | 93.1 ± 1.6 | 84.1 ± 1.3 | 7.8 ± 0.8 | 6.8 ± 0.7 |
| CB-138 | | | | |
| $1x10^{-8}$ M | 94.1 ± 2.2 | 85.3 ± 2.0 | 7.7 ± 1.0 | 5.8 ± 0.9 |
| $1x10^{-7}$ M | 93.6 ± 2.5 | 84.0 ± 1.3 | 7.7 ± 1.1 | 6.7 ± 0.6 |
| $1x10^{-6}$ M | 93.4 ± 1.9 | 81.1 ± 3.0 | 9.7 ± 1.5 | 8.1 ± 1.3 |
| CB-180 | | | | |
| $1x10^{-8}$ M | 93.6 ± 1.5 | 84.1 ± 2.0 | 8.1 ± 0.9 | 6.3 ± 1.0 |
| $1x10^{-7}$ M | 94.3 ± 1.1 | 84.5 ± 1.5 | 7.7 ± 0.6 | 6.5 ± 1.0 |
| $1x10^{-6}$ M | 94.1 ± 1.4 | 81.8 ± 3.1 | 9.6 ± 1.7 | 7.8 ± 1.6 |
| Mixture D (126 + 138 + 180) | | | | |
| $1x10^{-8}$ M | 93.4 ± 1.4 | 83.4 ± 1.2 | 8.2 ± 1.0 | 7.0 ± 0.5 |
| $1x10^{-7}$ M | 93.5 ± 1.8 | 84.0 ± 1.2 | 8.0 ± 0.7 | 7.0 ± 1.2 |
| $1x10^{-6}$ M | 93.6 ± 1.4 | 84.2 ± 2.3 | 8.2 ± 1.7 | 6.4 ± 1.0 |

[a] Values represent mean % of cells in each phase of the cell cycle ± SD;
[b] n = number of independent experiments; N = number of tested individuals

**Table 5.3b.** Cell cycle progression of LPS-induced murine splenocytes exposed to individual PBDE congeners and their non-additive mixture C for 48h[a].

| Treatment | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|
| *PBDEs (n=3, N=9)* [b] | | | | |
| Control – (cells without LPS) | 96.8 ± 1.3 | N.A | N.A | N.A |
| Cells | 94.2 ± 2.5 | 76.6 ± 5.2 | 11.6 ± 3.2 | 9.2 ± 3.3 |
| Vehicle | 93.7 ± 1.8 | 78.0 ± 3.1 | 10.5 ± 2.2 | 8.6 ± 2.2 |
| BDE-47 | | | | |
| $1x10^{-8}$ M | 93.6 ± 2.0 | 74.8 ± 6.9 | 12.2 ± 3.2 | 9.7 ± 2.8 |
| $1x10^{-7}$ M | 93.1 ± 2.2 | 77.9 ± 3.9 | 10.7 ± 2.4 | 8.7 ± 2.6 |
| $1x10^{-6}$ M | 92.8 ± 2.5 | 76.7 ± 4.8 | 11.7 ± 3.3 | 9.6 ± 2.7 |
| BDE-99 | | | | |
| $1x10^{-8}$ M | 93.8 ± 1.3 | 76.3 ± 4.1 | 11.8 ± 3.1 | 9.4 ± 2.8 |
| $1x10^{-7}$ M | 94.4 ± 2.0 | 75.1 ± 4.5 | 12.4 ± 2.8 | 10.4 ± 2.9 |
| $1x10^{-6}$ M | 94.2 ± 2.2 | 76.3 ± 5.1 | 12.0 ± 3.0 | 9.5 ± 3.4 |
| BDE-100 | | | | |
| $1x10^{-8}$ M | 94.1 ± 1.8 | 77.1 ± 4.0 | 11.5 ± 2.7 | 9.3 ± 2.6 |
| $1x10^{-7}$ M | 94.5 ± 2.0 | 77.5 ± 4.3 | 11.1 ± 2.8 | 9.2 ± 2.5 |
| $1x10^{-6}$ M | 94.3 ± 1.8 | 76.0 ± 4.6 | 12.0 ± 3.5 | 9.8 ± 2.9 |
| BDE-153 | | | | |
| $1x10^{-8}$ M | 94.0 ± 2.1 | 75.4 ± 3.6 | 11.8 ± 2.6 | 10.1 ± 2.7 |
| $1x10^{-7}$ M | 94.3 ± 1.9 | 77.0 ± 4.8 | 11.0 ± 3.0 | 9.4 ± 2.8 |
| $1x10^{-6}$ M | 94.5 ± 2.1 | 77.8 ± 5.2 | 11.1 ± 3.1 | 8.9 ± 3.2 |
| Mixture C (47+99+100+153) | | | | |
| $1x10^{-8}$ M | 93.2 ± 3.4 | 78.0 ± 4.9 | 10.7 ± 2.9 | 8.8 ± 3.1 |
| $1x10^{-7}$ M | 93.2 ± 2.7 | 76.8 ± 4.7 | 11.4 ± 3.1 | 9.8 ± 3.4 |
| $1x10^{-6}$ M | 93.5 ± 2.6 | 76.2 ± 5.2 | 11.5 ± 3.6 | 10.1 ± 2.8 |

[a] Values represent mean % of cells in each phase of the cell cycle ± SD;

[b] n = number of independent experiments; N = number of tested individuals;

N.A = Not analysed

In contrast, a significant reduction of the G2/M phase of the cell cycle was detected in murine splenic cells stimulated with LPS after 48h-treatment with $5x10^{-8}$ and $10^{-7}$ mol/l ATRA ($p$ = 0.030 and 0.020, respectively) and $10^{-5}$ mol/l ReOH ($p$ = 0.048) (Table 5.4). This reduction was concomitant with an accumulation of cells in the G0/G1 phase ($p$ = 0.021 and 0.012 for $5x10^{-8}$ and $10^{-7}$ mol/l ATRA, respectively and $p$ = 0.025 for $10^{-7}$ mol/l ReOH) and a decrease of cells in the S phase ($p$ = 0.023 and 0.009 for $5x10^{-8}$ and $10^{-7}$ mol/l ATRA, respectively and $p$ = 0.048 for $10^{-7}$ mol/l ReOH) (Table 5.4). We did not show any effect of either ATRA or ReOH on the different phases of the cell cycle in the Con A-stimulated murine spleen cells (Table 5.4).

Blomhoff *et al.* (1992) showed that 72h-treatment with both ReOH and ATRA inhibited the proliferation of human blood B lymphocytes (0.3 to 300 nmol/l) as well as the cell cycle progression (1 µmol/l), which was inhibited in mid-G1 approximately 24h after the stimulation. Similar results were found for murine splenic B cells incubated in presence of a physiological concentration of ATRA (10 nmol/l) (Chen and Ross, 2005). In the present study, we also observed that the highest ATRA and ReOH concentrations appeared to significantly reduce the number of cells in the S and G2/M phase in murine splenic cells stimulated with LPS, while cells tended to accumulate in the G0/G1 phase. The key regulatory event for the transition from G1- to S-phase is the phosphorylation of the retinoblastoma protein (pRB) (Weinberg, 1995) and the inhibitory effect of retinoids on proliferation of B-cells appeared to result from the prevented phosphorylation of the pRB (Blomhoff *et al.*, 2004). However, when splenocyte proliferation was investigated by incorporation of [$^3$H]-thymidine, we failed to confirm the results (Figure 5.10). Although unexpected, the apparent discrepancy between the cell cycle data and cell proliferation assessed by the incorporation of [$^3$H]-thymidine has already been observed before and can be due to the different reading points measured by the two different methods (Marmer *et al.*, 1983; Sasvári-Székely *et al.*, 1983).

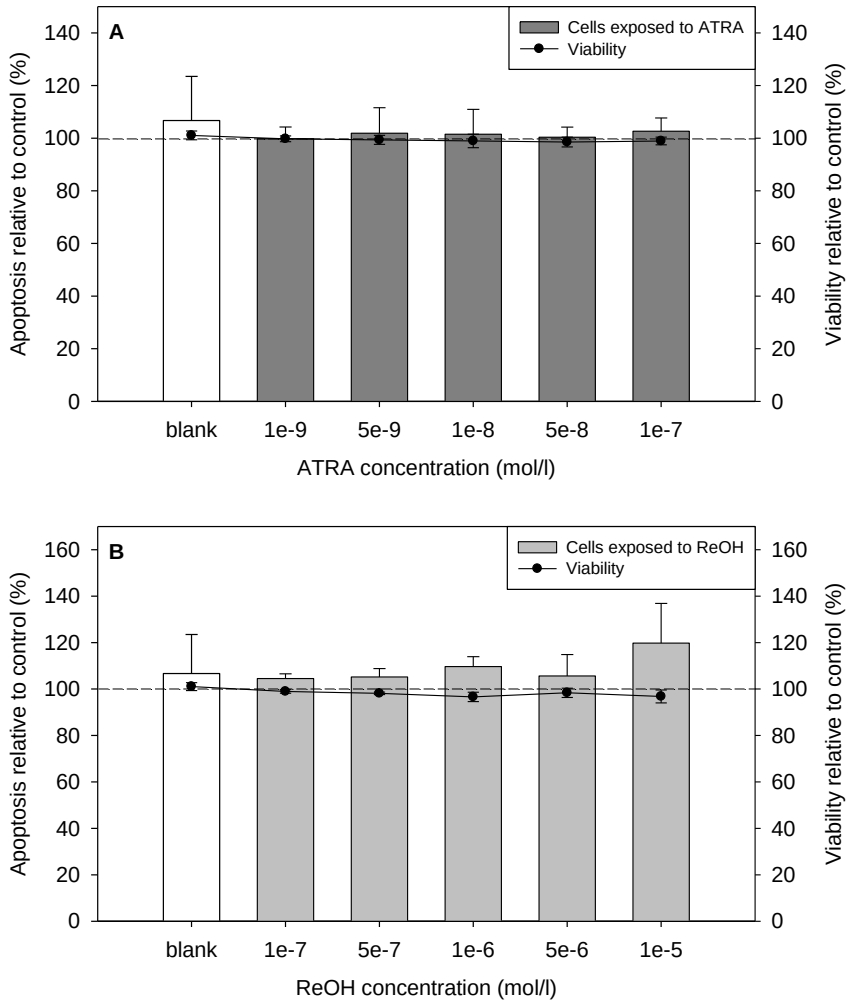


**Figure 5.8.** Effect of ATRA (A) and ReOH (B) on the dexamethasone-induced apoptosis of murine spleen cells after 6h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments performed on a total of 6 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

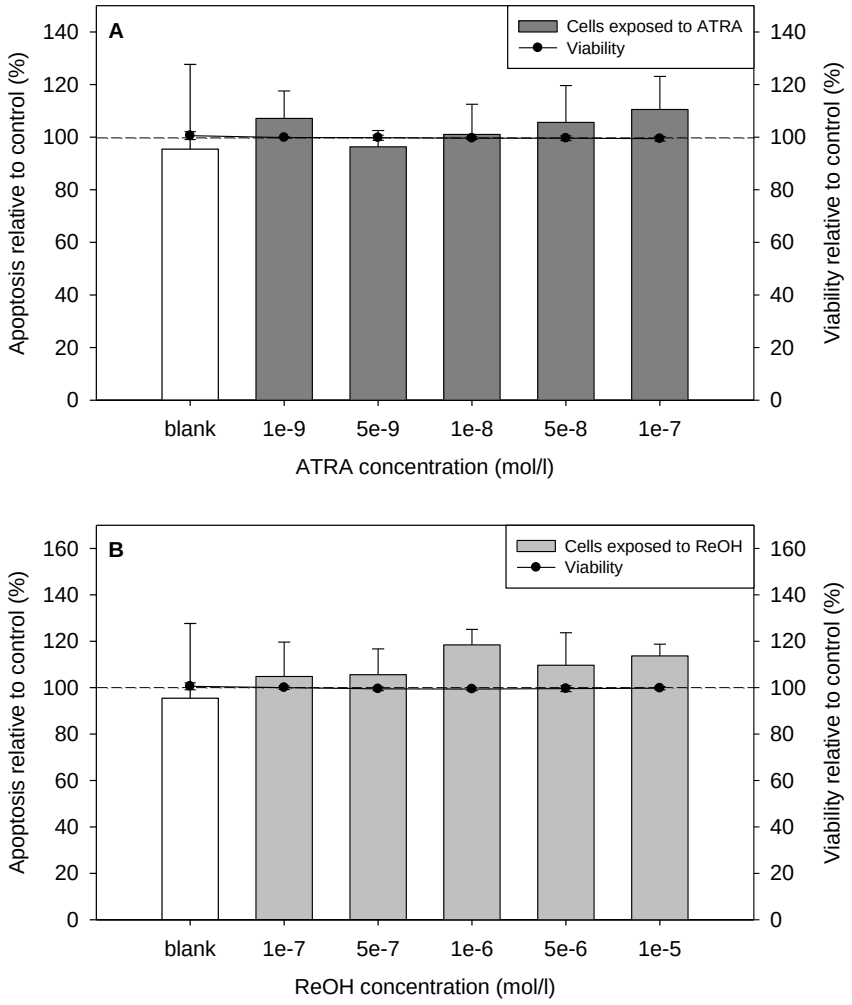


**Figure 5.9.** Effect of ATRA (A) and ReOH (B) on the non-induced apoptosis of murine spleen cells after 6h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments performed on a total of 6 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

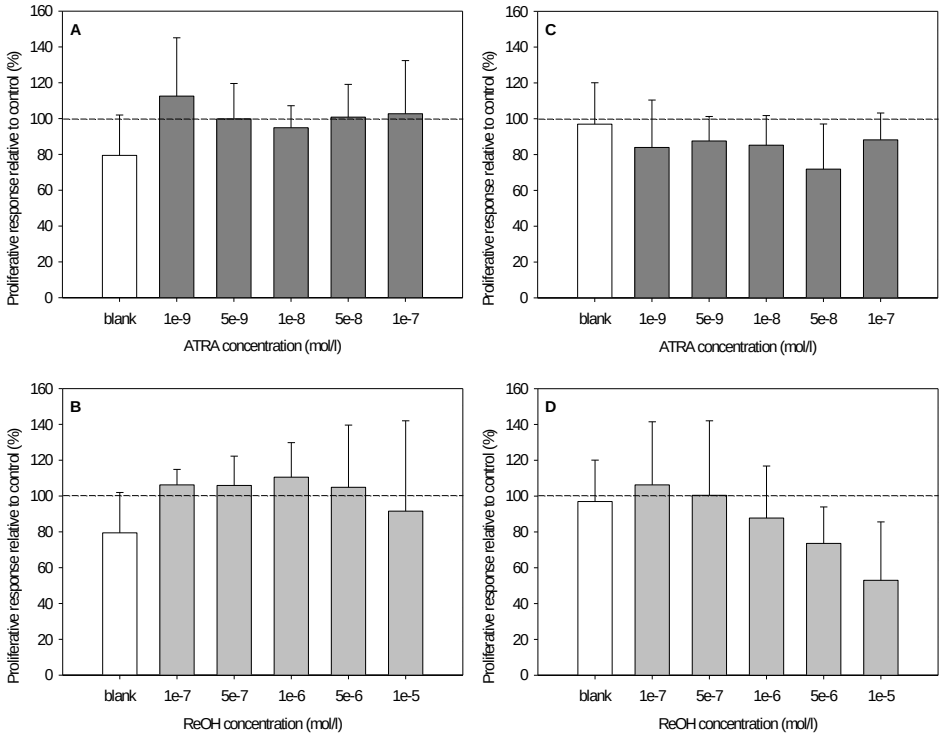


**Figure 5.10.** Effect of ATRA (in dark grey) and ReOH (in light grey) on the Con A (A, B)- or LPS (C, D)-induced murine splenocyte proliferation after 66h incubation, measured as [$^3$H]-thymidine incorporation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments performed on a total of 6 individuals. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

**Table 5.4.** Cell cycle progression of Con A and LPS-induced splenocytes exposed to ATRA and ReOH for 48h[a].

| Treatment | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|
| *Con A (n=3, N=6)* [b] | | | | |
| Control – (cells without con A) | 96.6 ± 0.8 | 97.7 ± 1.1 | 1.6 ± 0.8 | 0.4 ± 0.2 |
| Cells | 97.0 ± 0.6 | 73.3 ± 3.2 | 17.5 ± 1.4 | 8.7 ± 1.8 |
| Vehicle | 96.7 ± 0.5 | 77.1 ± 1.1 | 14.8 ± 0.8 | 7.5 ± 0.2 |
| ATRA | | | | |
| $1x10^{-9}$ M | 97.2 ± 0.3 | 79.6 ± 13.7 | 12.5 ± 8.5 | 7.3 ± 5.4 |
| $5x10^{-9}$ M | 97.6 ± 0.2 | 77.8 ± 14.5 | 13.7 ± 9.1 | 7.9 ± 5.7 |
| $1x10^{-8}$ M | 97.3 ± 0.7 | 78.9 ± 14.2 | 13.1 ± 8.8 | 7.4 ± 5.5 |
| $5x10^{-8}$ M | 97.6 ± 0.9 | 77.9 ± 14.9 | 13.9 ± 9.5 | 7.5 ± 5.6 |
| $1x10^{-7}$ M | 96.7 ± 0.4 | 77.5 ± 14.6 | 14.1 ± 9.3 | 7.7 ± 5.7 |
| ReOH | | | | |
| $1x10^{-7}$ M | 95.8 ± 0.7 | 79.5 ± 13.8 | 12.7 ± 8.8 | 7.0 ± 5.1 |
| $5x10^{-7}$ M | 95.8 ± 0.6 | 78.0 ± 14.2 | 13.8 ± 9.0 | 7.6 ± 5.4 |
| $1x10^{-6}$ M | 97.0 ± 0.9 | 79.9 ± 13.3 | 12.6 ± 8.2 | 7.0 ± 5.1 |
| $5x10^{-6}$ M | 96.9 ± 0.4 | 78.7 ± 14.7 | 13.6 ± 9.6 | 7.1 ± 5.5 |
| $1x10^{-5}$ M | 96.7 ± 1.0 | 77.1 ± 12.7 | 14.4 ± 8.5 | 7.5 ± 5.4 |
| *LPS (n=3, N=6)* [b] | | | | |
| Control – (cells without LPS) | 97.8 ± 0.1 | 95.9 ± 2.2 | 2.6 ± 1.2 | 0.7 ± 0.3 |
| Cells | 97.6 ± 0.2 | 81.4 ± 2.9 | 11.2 ± 1.4 | 6.8 ± 1.1 |
| Vehicle (DMSO) | 97.2 ± 0.2 | 84.1 ± 5.0 | 9.1 ± 2.6 | 6.4 ± 2.4 |
| ATRA | | | | |
| $1x10^{-9}$ M | 97.5 ± 0.4 | 86.8 ± 2.2 | 7.6 ± 1.2 | 5.1 ± 1.0 |
| $5x10^{-9}$ M | 98.0 ± 0.3 | 88.9 ± 2.2 | 6.6 ± 1.1 | 4.3 ± 1.2 |
| $1x10^{-8}$ M | 98.1 ± 0.5 | 89.2 ± 2.9 | 6.2 ± 1.6 | 4.3 ± 1.2 |
| $5x10^{-8}$ M | 96.8 ± 0.8 | 90.0 ± 2.5* | 6.0 ± 1.5* | 3.7 ± 1.1* |
| $1x10^{-7}$ M | 97.4 ± 0.2 | 90.4 ± 1.9* | 5.7 ± 1.2* | 3.6 ± 0.9* |
| ReOH | | | | |
| $1x10^{-7}$ M | 97.8 ± 0.4 | 86.0 ± 4.9 | 8.1 ± 2.7 | 5.5 ± 2.0 |
| $5x10^{-7}$ M | 97.6 ± 0.2 | 87.1 ± 3.6 | 7.5 ± 1.9 | 5.1 ± 1.5 |
| $1x10^{-6}$ M | 97.9 ± 0.3 | 88.8 ± 3.2 | 6.3 ± 1.8 | 4.5 ± 1.2 |
| $5x10^{-6}$ M | 97.5 ± 1.3 | 89.3 ± 2.2 | 6.4 ± 1.3 | 3.9 ± 1.0 |
| $1x10^{-5}$ M | 97.8 ± 0.1 | 90.5 ± 2.6* | 5.6 ± 1.5* | 3.5 ± 1.2* |

[a] Values represent mean % of cells in each phase of the cell cycle ± SD;
[b] n = number of independent experiments; N = number of tested individuals;
*Significantly different from the vehicle control ($p < 0.05$)

Regarding T-cells, the inverse tendency is generally observed. Physiological ATRA levels have been shown to enhance the proliferation of Con A-induced murine spleen cells and TPA-stimulated human blood T cells (Jiang *et al.*, 1993; Ertesvag *et al.*, 2002; Engedal *et al.*, 2006). In contrast to what has been found in B-cells, ATRA appeared to enhance the phosphorylation of pRB (Ertesvag *et al.*, 2002; Blomhoff *et al.*, 2004). Although our experiments respected comparable methods of culture, incubation period and detection analysis, we did not show any effect of either ATRA or ReOH on Con A-stimulated murine spleen cells (Figure 5.10 and Table 5.4). The differences may be species- and strain-related.

### 5.3.4. Effects of PCBs and PBDEs in the harbour seal cell line

#### Apoptosis

A representative scattergram of induced apoptosis with staurosporine (positive control) vs. non-induced apoptosis in the harbour seal cell line is illustrated in Figure 5.11. Treatment of the cell line with the different individual PCB and PBDE congeners as well as their non additive mixture for 24h had no impact on apoptosis (Figures 5.12 and 5.13).

To the best of our knowledge, the impacts of PCBs on apoptosis in marine mammal immune cells have never been investigated and only one study has recently reported the potential effects of PBDEs in harbour seal peripheral blood leukocytes and the 11B7501 cell line (Frouin *et al.*, 2010a). Similarly to our results, the authors reported that apoptosis was not affected by exposure to BDE-47, -99 and -153 at concentrations ranging from 1.5 to 12 µmol/l for 24h, neither in the fresh harbour seal leukocytes nor in the cell line (Frouin *et al.*, 2010a). Together these results may suggest that current PCBs and PBDEs concentrations reported in wild marine mammals are to low to induce apoptosis in these animals.

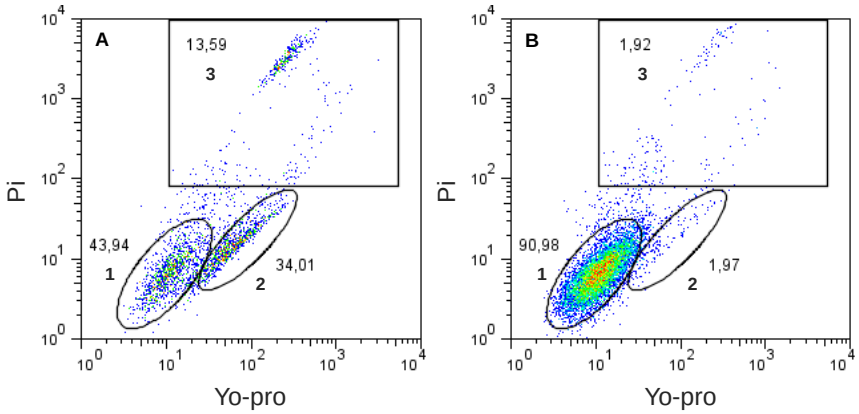


**Figure 5.11.** Representative scattergram of apoptosis analysis in the 11B7501 B lymphoma harbour seal cell line. (A) Cells treated with 5 µmol/l staurosporine for 24h (positive control) and (B) untreated cells. The 3 gates represent living cells (1), apoptotic cells (2) and necrotic cells (3).

However, as already presented in the previous section, several studies have already shown that PCBs and PBDEs were able to induce apoptosis in different mammalian cell types but generally at more important concentrations than those tested in the present experiments (Yoo *et al.*, 1997; Jeon *et al.*, 2002; Madia *et al.*, 2004; He P. *et al.*, 2008; He W. *et al.*, 2008; Yan *et al.*, 2011). The mechanisms involved in PCB and PBDE-induced apoptosis are still not clear but previous studies suggested that it could be related to activation of caspases and oxidative stress via AhR-independent mechanisms (Jeon *et al.*, 2002, Howard *et al.*, 2003; Yan *et al.*, 2011).

## Cell cycle analysis

No modulation of the different phases of the cell cycle could be detected following 48h-exposure of the harbour seal cell line to individual PCB and PBDE congeners as well as their non additive mixture (Tables 5.5a and b).

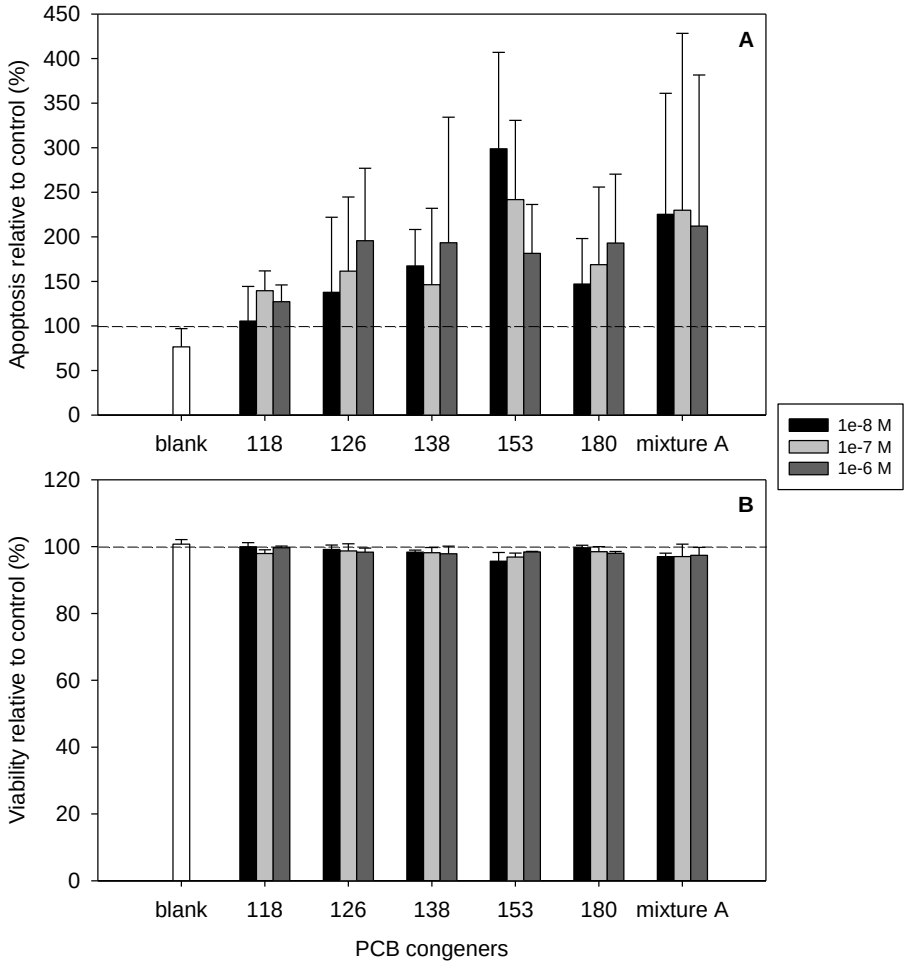


**Figure 5.12.** Effect of the 5 individual PCB congeners as well as their non additive mixture A (118+126+138+153+180) on the apoptosis (A) and viability (B) of the harbour seal cell line after 24h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.

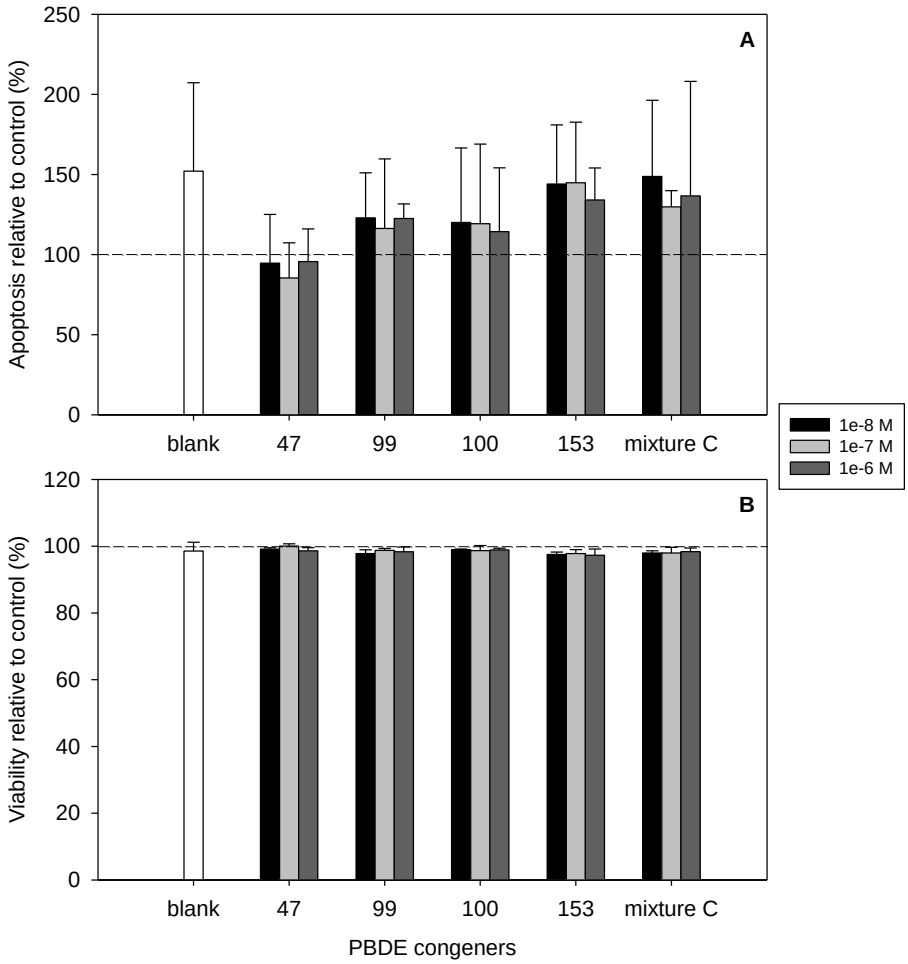


**Figure 5.13.** Effect of the 4 individual PBDE congeners as well as their non additive mixture C (47+99+100+153) on the apoptosis (A) and viability (B) of the harbour seal cell line after 24h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 3 independent experiments. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle

**Table 5.5a.** Cell cycle progression of the lymphoma B harbour seal cell line exposed to individual PCB congeners and their non-additive mixture for 48h[a].

| Treatment | | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|---|
| *PCBs (n=4)* [b] | | | | | |
| Cells | | 92.5 ± 1.1 | 67.8 ± 0.9 | 7.7 ± 0.7 | 22.8 ± 1.3 |
| Vehicle | | 91.2 ± 1.2 | 69.3 ± 1.8 | 8.0 ± 0.5 | 20.7 ± 1.3 |
| CB-118 | | | | | |
| | $1x10^{-8}$ M | 92.4 ± 1.4 | 66.1 ± 3.1 | 8.1 ± 0.3 | 24.1 ± 3.1 |
| | $1x10^{-7}$ M | 91.6 ± 0.8 | 66.0 ± 3.4 | 7.8 ± 0.6 | 24.0 ± 3.3 |
| | $1x10^{-6}$ M | 92.0 ± 1.0 | 66.0 ± 4.9 | 8.0 ± 0.1 | 24.1 ± 4.6 |
| CB-126 | | | | | |
| | $1x10^{-8}$ M | 92.3 ± 0.3 | 64.5 ± 4.1 | 8.5 ± 0.5 | 25.1 ± 4.0 |
| | $1x10^{-7}$ M | 92.1 ± 0.7 | 65.8 ± 2.3 | 7.7 ± 0.4 | 24.5 ± 2.5 |
| | $1x10^{-6}$ M | 92.1 ± 0.9 | 67.0 ± 2.6 | 7.8 ± 0.5 | 23.3 ± 2.5 |
| CB-138 | | | | | |
| | $1x10^{-8}$ M | 91.9 ± 1.1 | 66.7 ± 2.5 | 7.4 ± 1.1 | 24.0 ± 2.6 |
| | $1x10^{-7}$ M | 92.0 ± 0.9 | 66.6 ± 2.8 | 7.6 ± 1.2 | 23.9 ± 3.1 |
| | $1x10^{-6}$ M | 92.0 ± 0.9 | 67.9 ± 2.7 | 7.4 ± 0.5 | 22.7 ± 2.5 |
| CB-153 | | | | | |
| | $1x10^{-8}$ M | 92.2 ± 0.8 | 67.7 ± 3.3 | 7.8 ± 1.0 | 22.6 ± 3.1 |
| | $1x10^{-7}$ M | 91.9 ± 0.7 | 67.2 ± 3.1 | 7.2 ± 0.8 | 23.4 ± 2.6 |
| | $1x10^{-6}$ M | 92.2 ± 0.9 | 67.1 ± 3.0 | 7.3 ± 0.5 | 23.5 ± 3.1 |
| CB-180 | | | | | |
| | $1x10^{-8}$ M | 91.1 ± 1.6 | 67.1 ± 2.2 | 7.3 ± 0.8 | 23.5 ± 2.0 |
| | $1x10^{-7}$ M | 92.6 ± 1.2 | 66.9 ± 3.0 | 7.8 ± 0.5 | 23.6 ± 2.7 |
| | $1x10^{-6}$ M | 92.2 ± 0.9 | 68.5 ± 3.3 | 7.2 ± 0.5 | 22.5 ± 3.3 |
| Mixture A (118+126+138+153+180) | | | | | |
| | $1x10^{-8}$ M | 92.7 ± 1.4 | 67.4 ± 3.1 | 7.2 ± 1.0 | 23.6 ± 2.4 |
| | $1x10^{-7}$ M | 92.2 ± 0.9 | 69.3 ± 4.1 | 7.4 ± 0.5 | 21.2 ± 4.1 |
| | $1x10^{-6}$ M | 92.1 ± 0.7 | 68.8 ± 3.5 | 7.3 ± 0.4 | 22.0 ± 3.2 |

[a] Values represent % of cells in each phase of the cell cycle ± standard deviation;
[b] n = number of independent experiments

**Table 5.5b.** Cell cycle progression of the lymphoma B harbour seal cell line exposed to individual PBDE congeners and their non-additive mixture for 48h[a].

| Treatment | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|
| *PBDEs (n=4)* [b] | | | | |
| Cells | 97.0 ± 0.0 | 70.1 ± 7.9 | 7.5 ± 2.2 | 20.7 ± 5.9 |
| Vehicle | 97.4 ± 0.4 | 70.9 ± 6.8 | 7.4 ± 2.0 | 19.7 ± 4.9 |
| BDE-47 | | | | |
| $1x10^{-8}$ M | 96.8 ± 0.5 | 69.9 ± 6.7 | 7.7 ± 1.6 | 20.3 ± 5.3 |
| $1x10^{-7}$ M | 97.5 ± 0.6 | 69.7 ± 7.3 | 7.9 ± 2.0 | 20.2 ± 5.4 |
| $1x10^{-6}$ M | 97.2 ± 0.6 | 70.6 ± 6.9 | 7.6 ± 2.1 | 19.8 ± 5.3 |
| BDE-99 | | | | |
| $1x10^{-8}$ M | 96.4 ± 0.8 | 69.9 ± 7.9 | 8.0 ± 2.3 | 19.9 ± 6.0 |
| $1x10^{-7}$ M | 97.6 ± 0.7 | 69.7 ± 8.6 | 7.8 ± 2.2 | 20.5 ± 6.7 |
| $1x10^{-6}$ M | 97.3 ± 0.9 | 70.2 ± 6.9 | 7.4 ± 1.6 | 20.3 ± 5.6 |
| BDE-100 | | | | |
| $1x10^{-8}$ M | 97.3 ± 0.4 | 69.6 ± 9.1 | 7.8 ± 2.7 | 20.6 ± 6.8 |
| $1x10^{-7}$ M | 97.5 ± 0.8 | 69.7 ± 8.0 | 7.4 ± 2.0 | 20.7 ± 6.3 |
| $1x10^{-6}$ M | 96.7 ± 1.0 | 70.6 ± 7.4 | 7.3 ± 2.6 | 20.1 ± 5.6 |
| BDE-153 | | | | |
| $1x10^{-8}$ M | 97.0 ± 1.0 | 69.8 ± 8.0 | 7.4 ± 2.2 | 20.7 ± 6.3 |
| $1x10^{-7}$ M | 96.9 ± 0.7 | 69.5 ± 7.9 | 7.4 ± 2.2 | 20.9 ± 5.9 |
| $1x10^{-6}$ M | 97.1 ± 0.5 | 68.1 ± 9.6 | 8.3 ± 3.6 | 21.2 ± 6.9 |
| Mixture C (47+99+100+153) | | | | |
| $1x10^{-8}$ M | 97.0 ± 0.3 | 69.6 ± 8.6 | 7.8 ± 2.3 | 20.4 ± 6.6 |
| $1x10^{-7}$ M | 97.6 ± 0.5 | 69.3 ± 7.9 | 8.0 ± 2.3 | 20.9 ± 5.8 |
| $1x10^{-6}$ M | 96.6 ± 0.1 | 70.2 ± 7.8 | 7.2 ± 2.1 | 20.8 ± 5.5 |

[a] Values represent % of cells in each phase of the cell cycle ± standard deviation;

[b] n = number of independent experiments

In the literature, both field and semi-field studies generally showed that the proliferation of T lymphocytes was reduced in PCB contaminated marine mammals, while the proliferation of B lymphocytes was not significantly modulated (De Swart *et al.*, 1994; De Swart *et al.*, 1995; Lahvis *et al.* 1995; Mos *et al.*, 2006). Only one study reported that PCBs may induce the proliferative response of B and T lymphocytes in harbour seal pups (Levin *et al.*, 2005b). *In vitro* studies reported different findings, depending on the investigated species and the studied PCB congeners and/or mixtures.

The use of *in vitro* experiments in parallel with *in vivo* and/or environmental studies has allowed major advancements in the field of immunotoxicology and is very useful to study the direct characterisation of the toxic effects of specific contaminants on immune cells. Several *in vitro* experiments on beluga whale (*Delphinapterus leucas*), killer whale (*Orcinus orca*), bottlenose dolphin (*Tursiops truncatus*) and grey seal (*Halichoerus grypus*) reported a suppression of T lymphocyte proliferation following exposure to individual PCB congeners (such as CB-138 and -153), several Aroclors or mixtures including congeners CB-138, -153, -180, -169 and TCDD at concentrations ranging from ~4 µmol/l to 250 µmol/l (De Guise *et al.*, 1998; Mori *et al.*, 2006; Dufresne *et al.*, 2010; Frouin *et al.*, 2010c). Exposure to individual PCB congeners (such as CB-77, -126, -138, -153 and -169) or PCB and TCDD mixtures often resulted in no effect or even an increase of the T lymphocyte proliferation (De Guise *et al.*, 1998; Nakata *et al.*, 2002; Neale *et al.*, 2002; Mori *et al.*, 2006; Levin *et al.*, 2007) (see Table 1.1b in Chapter 1 for more details). Regarding B lymphocytes, Mori *et al.* (2008) showed that CB-138, -153 and -180 tested individually at a dose of 5 ppm (~12 µmol/l) failed to modulate B-cell proliferation in pilot whale (*Globicephala melaena*), beluga whale, bottlenose dolphin, harbour seal, Northern fur seal (*Callorhinus ursinus*) and sea otter (*Enhydra lutris*). Nevertheless, some of the tested mixtures stimulated B-cell proliferation in pilot whale, bottlenose dolphin, harbour seal and Northern fur seal. A similar study was conducted on captive sea otters and reported that LPS-induced lymphocyte proliferation was not affected by exposure to neither individual PCB congeners nor PCB mixtures at a final concentration of 5 ppm (Levin *et al.*, 2007).

Although we did not investigated proliferation by incorporation of [$^3$H]-thymidine, our findings resulting from the cell cycle experiments appear in agreement with the majority of those previous studies, since no modulation of the lymphoma B cell line could be observed after 48h-treatment with individual PCB congeners or their non-additive mixture.

This is the first report to analyse the impact of PBDEs on the cell cycle progression/proliferation of marine mammal immune cells and therefore, no comparison with the literature could be made. However, PBDEs have been reported to reduce the phagocytosis, increase ROS formation and decrease intracellular thiol levels in seal granulocytes *in vitro* (Frouin *et al.*, 2010a), indicating that some immune parameters may be at risk in marine mammals. Further research is thus needed regarding the potential immunotoxicity of PBDEs in marine mammals.

### 5.3.5. Effects of vitamin A in a harbour seal cell line

#### Apoptosis

To the best of our knowledge, this is the first study to investigate the effects of *in vitro* vitamin A exposure on immune functions in a marine mammal cell type.

To assess apoptosis, the cell line was exposed for 24h to several concentrations of ATRA or ReOH and staurosporine was used at 5 µmol/l as a positive control (see Figure 5.11). Only the highest concentration of ATRA induced significant apoptosis in the harbour seal cell line ($p$ = 0.041) (Figure 5.14). Although treatments of primary cultures of "healthy" mammal lymphocytes with physiological concentrations of ATRA showed either no effect or a reduction of apoptotic cells (Lømo *et al.*, 1998; Blomhoff *et al.*, 2004; Engedal *et al.*, 2004; this study), it has been demonstrated that ATRA induced apoptosis in B-cell chronic lymphocytic leukaemia at concentrations ranging from $10^{-8}$-$10^{-5}$ mol/l following 48h-treatment (Pepper *et al.*, 2002). Since the harbour seal cell line was derived from

an individual with malignant leukemia lymphoma, it is possible that the highest concentration of ATRA used in the present experiments has similar effects as those observed by Pepper *et al.* (2002).

### Cell cycle analysis

The cell cycle was unaffected after 48h incubation with ATRA or ReOH (Table 5.6). These results are in contrast with those found for murine splenocytes for which a significant decrease of the number of cells in the G2/M phase is usually observed (Blomhoff *et al.*, 1992; Chen and Ross, 2005; Blomhoff *et al.*, 2004; this study, see section 4.3.1.). It is possible that marine mammal lymphocytes react differently to vitamin A than rodents but since this is the only study to report such findings, comparison with the literature could not be made.

## 5.4. Conclusion

The PCB and PBDE concentrations used in the present work were representative or higher than those measured in human and marine mammal tissues. Since both the murine and the cell line models were unaffected by the tested concentrations, this may suggest that current detected levels may not adversely impact proliferation, apoptosis and cell cycle analysis in wildlife. The differences observed with previous studies may be species-, strain- and/or congener-related and may also depend on the dose and the duration of the exposure. Regarding vitamin A, our findings for mouse spleen cells and the harbour seal cell line appear to be contradictory. While neither ATRA nor ReOH had an effect on mouse splenocyte apoptosis, the highest concentration of ATRA increased the number of apoptotic cells in the cell line. One explanation could be that leukemia-lymphoma cells may be more sensitive to high ATRA levels than normal healthy cells.

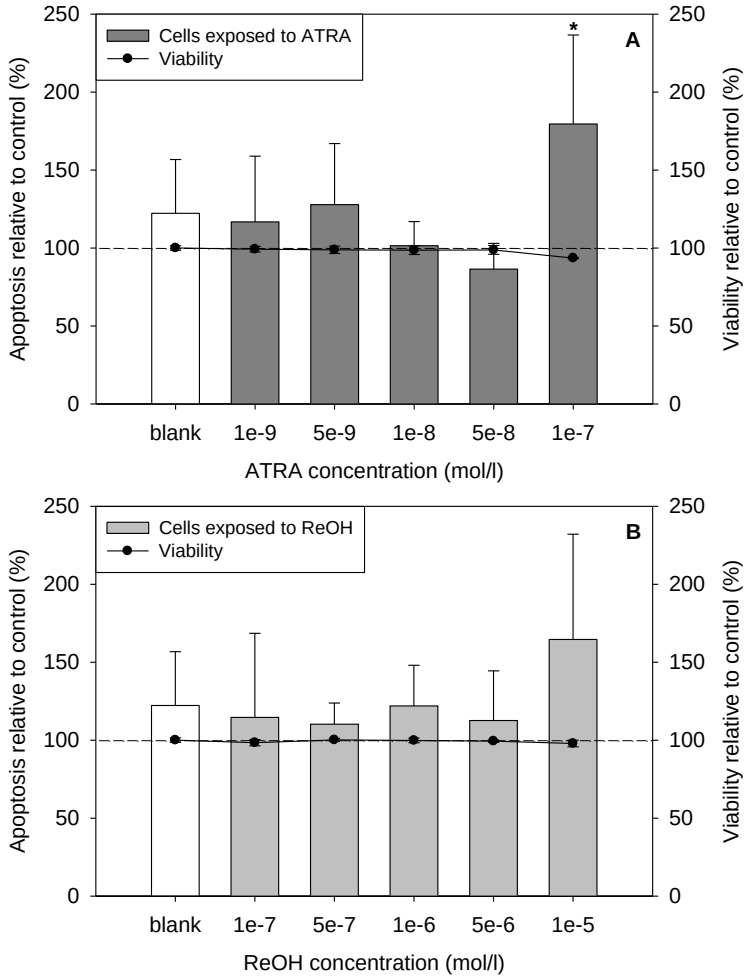


**Figure 5.14.** Effect of ATRA (A) and ReOH (B) on the apoptosis of the harbour seal cell line after 24h incubation. Results are expressed as a % of the vehicle control (unexposed cells + vehicle) and are presented as the mean value ± SD of 4 independent experiments. The dashed line indicates 100% of the vehicle control and the blank represents unexposed cells without vehicle.* indicates a significant difference from the vehicle control ($p < 0.05$).

**Table 5.6.** Cell cycle progression of the lymphoma B harbour seal cell line exposed to ATRA and ReOH[a].

| Treatment | Viability | G0/G1 | S | G2/M |
|---|---|---|---|---|
| Cells | 96.6 ± 0.3 | 78.7 ± 4.4 | 7.2 ± 1.6 | 13.0 ± 5.8 |
| Vehicle | 96.9 ± 0.3 | 78.2 ± 5.0 | 8.2 ± 2.0 | 12.5 ± 6.8 |
| ATRA (n=4)[b] | | | | |
| $1x10^{-9}$ M | 96.9 ± 0.2 | 77.3 ± 7.5 | 8.8 ± 1.6 | 12.9 ± 8.7 |
| $5x10^{-9}$ M | 96.8 ± 0.4 | 75.2 ± 7.8 | 9.0 ± 1.8 | 14.7 ± 9.3 |
| $1x10^{-8}$ M | 97.2 ± 0.6 | 76.2 ± 7.0 | 9.0 ± 1.6 | 13.6 ± 8.4 |
| $5x10^{-8}$ M | 97.1 ± 0.3 | 77.0 ± 8.5 | 8.6 ± 1.3 | 13.2 ± 9.9 |
| $1x10^{-7}$ M | 97.0 ± 0.7 | 76.1 ± 7.2 | 9.2 ± 2.1 | 13.5 ± 8.6 |
| ReOH (n=4)[b] | | | | |
| $1x10^{-9}$ M | 96.7 ± 0.6 | 78.9 ± 7.7 | 7.8 ± 0.7 | 12.2 ± 8.2 |
| $5x10^{-9}$ M | 97.1 ± 0.3 | 77.5 ± 8.4 | 8.0 ± 1.1 | 13.6 ± 9.1 |
| $1x10^{-8}$ M | 96.9 ± 0.6 | 78.6 ± 8.2 | 7.5 ± 0.6 | 12.9 ± 8.2 |
| $5x10^{-8}$ M | 96.3 ± 0.6 | 75.4 ± 6.8 | 7.0 ± 0.5 | 16.5 ± 6.1 |
| $1x10^{-7}$ M | 94.5 ± 3.3 | 76.2 ± 7.6 | 7.9 ± 1.3 | 14.8 ± 7.2 |

[a] Values represent % of cells in each phase of the cell cycle ± standard deviation;
[b] n = number of independent experiments

The cell cycle progression in mouse splenocytes was inhibited by the highest ATRA and ReOH concentration and these results seem to confirm some findings reported previously. In contrast, none of the ATRA and ReOH concentrations modulated the cell cycle in the cell line.

Since our data sometimes differ between the 2 models and from earlier studies performed on laboratory animals or wild marine mammals (from field or semi-field studies), we would thus find it premature to conclude that they are reliable immune models to investigate the mode of action of contaminants and vitamin A in wild mammals. Further research is needed and should involve, among other things, possible synergistic or antagonistic effects of PCBs, PBDEs and vitamin A. Indeed, in the environment, animals are exposed to a mixture of POPs and studies have already demonstrated the synergistic or antagonistic effects of PCB congeners in laboratory and wild animal immune functions. Therefore, the possible synergis-

tic or antagonistic effects of PCB and PBDE congeners together on immune parameters should be investigated. Moreover, since vitamin A is an important regulator of the immune system and has been demonstrated to be altered by PCBs and PBDEs, it would be interesting to investigate the effect of a vitamin A treatment together with a PCB and PBDE exposure *in vitro*.

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# Chapter 6

# General discussion and perspectives

## 6.1. General discussion

This thesis aimed at studying the lactational transfer of PBDEs, chlorinated POPs and their metabolites and evaluating their relationships with vitamin A status and dynamics in lactating grey seal females and their suckling pup. It also focused on the impact of PBDEs, PCBs and vitamin A on the immune system through the use of *in vitro* models. The experiments performed in the present work have provided novel information on these aspects and the major outcomes are listed in the next paragraphs.

### POPs and vitamin A concentrate in the maternal inner blubber at the end of the lactation period (Figure 6.1, point 1)

In order to find a physiological explanation to the origin of the similar dynamics of vitamin A and PCBs observed previously in grey seal milk (Debier *et al.*, 2004), we decided to examine the changes of PCBs and other POPs as well as vitamin A in maternal grey seal blubber during lactation (Chapters 2 and 3). Because outer blubber is usually considered as more stable in term of mobilisation as compared to inner blubber during periods of negative energy balance, both layers were analysed separately. As expected, the concentrations of PCBs, PBDEs and OCPs increased drastically in inner blubber at late lactation, while they remained stable or increased to a lesser extent in outer blubber. These findings confirm previous studies investigating the dynamics of PCBs in lactating grey seals (Debier *et*

*al.*, 2003a and 2003b). One explanation could be a less efficient mobilisation of POPs from maternal blubber than triacylglycerols, leading to increasing levels in the reduced blubber layer (Figure 6.2). It is also possible that POPs are released in similar proportions to lipids and, because of their higher affinity for the remaining blubber lipids, some of them may be directly redirected into the blubber layer (Figure 6.2). Interestingly, we have highlighted for the first time that vitamin A levels also increased in inner blubber, while they were more stable in outer blubber. A recent study on Northern elephant seals (*Mirounga angustirostris*) has also reported similar findings (Debier *et al.*, 2012). As postulated for POPs, vitamin A may also be less efficiently mobilised from blubber than triacylglycerols and thus concentrates in this tissue. The observed pattern in inner blubber supports thus the hypothesis of a similar mechanism of mobilisation from blubber that has been emitted to explain the parallelism between PCBs and vitamin A observed in grey seal milk (Debier *et al.*, 2004). Indeed, that previous work have shown that concentrations of both compounds in milk were relatively stable during the first half of lactation and then increased exponentially during the second half of lactation. The authors suggested that higher amounts of PCBs and vitamin A could be mobilised from blubber at late lactation (Figure 6.2). The present results seem to confirm that theory. Nevertheless, the mechanisms underlying such an abundant release of vitamin A and POPs from blubber into the circulatory system at late lactation are still unknown. It is possible that the mobilisation of those lipophilic compounds is linked to the selective mobilisation of fatty acids. As already explained in Chapter 2, the mobilisation of fatty acids from triacylglycerols stored in adipose tissue appears to be a selective process (Raclot, 2003). Short and unsaturated fatty acids are mobilised first, while less hydrophilic fatty acids are liberated later. Vitamin A is stored in the lipid droplets of adipocytes as retinyl esters (Reue *et al.*, 2011). Similarly, POPs are also localised in this intracellular organelle in adipose tissue (Bourez *et al.* 2012). As a consequence, it is possible that the mobilisation of both types of compounds is linked to the selective release of fatty acids stored in the lipid droplet as triacylglycerols.

In addition, the blubber layer, which gets thinner throughout lactation, may become saturated at late lactation and then releases greater amounts of vitamin A and POPs into the bloodstream. Nevertheless, a recent study investigating *in vitro* the accumulation of PCBs in mouse cultured adipocytes (Bourez *et al.*, 2012) has demonstrated that adipocytes were technically able to stock relatively huge concentrations of PCBs (up to 70 mg/g of lipids) that are more than 1000 times higher than those measured in our grey seals. The theory of saturation for POPs seems therefore unlikely. However, several parameters differed from the *in vivo*, lipolytic situation from the present study and such a comparison should be used with caution. Regarding vitamin A, other factors such as a higher expression of RBP and/or enzymes involved in the conversion of retinyl esters into retinol at late lactation may contribute to the increased mobilisation.

In Chapters 3 and 4, we were able to measure the percentage of lipids in maternal blubber. An interesting aspect that has been pointed out is the fact that the increase of the major POPs (PCBs, PBDEs and DDXs) and vitamin A levels appeared to be higher than the decrease in lipid content in inner blubber (Table 6.1), suggesting that the retention or direct re-absorption of these molecules in blubber might not be the only factors responsible for the increase of POPs and vitamin A in adipose tissue. An explanation can be found in other organs which can store POPs and vitamin A. Studies investigating the lactational transfer of PCBs in rodents observed a sharp decrease of the concentrations in adipose tissue but also in the liver, muscles, kidneys and brain of the mothers during lactation (Vodicnik *et al.*, 1980; Poul, 1991; Lee *et al.*, 2009). A similar mobilisation of POPs may also occur from these tissues in lactating seals. Once into the blood circulation, one part of these compounds may be quickly redirected into the blubber because of the larger amount of fat present in this tissue and their higher affinity for triacylglycerols, as mentioned previously. Regarding vitamin A, liver and kidneys represent other important storage sites. One hypothesis would be that one part of vitamin A released from the liver to enrich the milk may be redirected to blubber, even during an important lipolysis event as it occurs during lactation in seals. A specific RBP receptor

has been recently identified as STRA6, a multitransmembrane protein of previously unknown function (Kawaguchi *et al.*, 2007). STRA6 has a high affinity for RBP and is highly expressed in blood-organ barriers such as the retinal pigment epithelia, the placenta, the brain, the liver, the testis as well as the adipose tissue (Sun and Kawaguchi, 2011; Berry and Noy, 2012). Its expression seems to be increased in some particular cases such as in carcinoma cells but the significance of this increase in unknown (Berry and Noy, 2012). One can then assume that STRA6 is also expressed in blubber of seals and that the specific state of lactation may elevate its expression in this tissue. A thorough study investigating the expression of STRA6 in seal blubber and the possible variations during lactation is needed in order to validate or invalidate this hypothesis.

**Table 6.1.** Summary of the percentages of lipids, vitamin A and POP concentrations measured in maternal inner blubber at early and late lactation.

| | Early lactation | Late lactation |
|---|---|---|
| Lipids (%) | 84.6 ± 5.0 | 66.9 ± 12.9 |
| Vitamin A (µg/g ww) | 37 ± 9 | 92 ± 32 |
| ΣPCBs (ng/g ww) | 1308 ± 817 | 2537 ± 1645 |
| ΣPBDEs (ng/g ww) | 54 ± 38 | 90 ± 56 |
| ΣDDXs (ng/g ww) | 273 ± 89 | 438 ± 161 |

## POPs are selectively transferred from mother to their pup during the lactation period in grey seal (Figure 6.1, point 2)

Although this kind of information has already been reported for PCBs and OCPs in seals (Pomeroy *et al.*, 1996; Debier *et al.*, 2003b; Sørmo *et al.*, 2003; Miranda Filho *et al.*, 2009), this work is the first to present in details that PBDE congeners were also selectively transferred during lactation (Chapter 3).

We have confirmed that the lower chlorinated PCB congeners were released from maternal inner blubber to maternal serum more

efficiently than higher chlorinated ones. Similar observations were highlighted for PBDEs, with low brominated ones being preferentially transferred from maternal blubber into the circulatory system. The exact mechanisms underlying such a differential release are still not well understood but seem to be linked to the intrinsic physico-chemical properties of the congeners. We observed indeed that the transfer ratio of PCBs and PBDEs from maternal inner blubber to maternal serum decreased with the number of bromine atoms and that this transfer ratio was inversely related to their respective log $K_{ow}$ values. The selective release of POPs from maternal blubber during lactation may be also partly explained by the selective mobilisation of fatty acids according to their molecular structure. Indeed, as explained in the previous point, it is well known that during lipolysis, the rate of mobilisation of fatty acids from adipose tissue depends on their length and the number of double bonds (Raclot, 2003). As lipolysis occurs at the lipid–water interface of the lipid droplet, lipolytic enzymes may first hydrolyse the triacylglycerols present at this interface before starting to hydrolyse those present in the droplet core (Raclot, 2003). The only work that focused on the mobilisation of fatty acids in lactating seals also demonstrated that their release from maternal blubber was highly selective (Arriola, 2010). For a given fatty acid chain length, the mobilisation increased with the number of double bonds, and for a given double bond, mobilisation increased as chain length decreased (Arriola, 2010). In other words, more polar fatty acids appear to be more readily released from triacylglycerols in maternal blubber. It is thus possible that more polar POPs such as lower halogenated PCBs and PBDEs follow the flow of the more polar fatty acids, while the highly halogenated POPs remain in the blubber layer because of their higher affinity for the remaining less polar fatty acids, situated in the fat droplet core.

Previous studies have suggested that the presence of a biological barrier towards some highly halogenated POPs may exist at the mammary gland (Debier *et al.*, 2003; Sørmo *et al.*, 2003). In the present study, we found that the transfer ratios of PCBs and PBDEs from maternal serum to milk were relatively high, suggesting an efficient transfer of high halogenated compounds into the milk. In

addition, contrary to what was observed between maternal inner blubber and serum, no clear relationship could be underlined between transfer ratios from maternal serum to milk and the log $K_{ow}$ values of the different compounds. It is however important to highlight that physiological matrices are not static and that milk is constantly renewed, a phenomenon that is not taken into account by simple transfer ratios.

### The lactational transfer of HO-PCBs is limited in grey seals (Figure 6.1, point 3)

Another important finding that has been highlighted in Chapter 3 is the fact that only low amounts of HO-PCBs were transferred during lactation in grey seals. While several HO-PCB metabolites were detected in maternal and pup serum, only 4-HO-CB-107 could be detected at very low concentrations in milk. This finding corroborates data reported from human studies (Guvenius *et al.*, 2003; Fangström *et al.*, 2005). Interestingly, we observed that most of the HO-PCB metabolites measured in maternal serum were also found in pup serum and that their concentrations tended to increase at late lactation. In addition to a transplacental transfer, which has been reported for humans and rodents, this increase of concentrations might also result from the transfer of low or undetectable levels of HO-PCBs in milk. In addition, we also suspect that pups may be able to accumulate these metabolites following endogenous biotransformation. The presence of CYP activities in suckling harp and hooded seal pups (Wolkers *et al.*, 2002 and 2009) and the positive correlation between HO-PCB levels and the age of hooded seal pups reported by Gabrielsen *et al.* (2011) support this hypothesis.

None of the investigated HO-PBDEs could be detected in either serum or milk. Previous findings have reported no or only low concentrations of circulating HO-PBDEs in harbour and ringed seals (Routti *et al.*, 2009 and Weijs *et al.*, 2009c) and the authors suspected that biotransformation of PBDEs or retention of HO-PBDEs was low in those species. In the present work, PBDEs were present at low concentrations in the different tissues, so it is possible that HO-

PBDEs were formed at only low rates. Additionally, MeO-PBDEs which may be another source of HO-PBDEs (Wan *et al.*, 2009) were also detected at low concentrations in maternal tissues and were absent in pup serum. The low concentrations or absence of 2 potential sources of HO-PBDEs may explain the absence or undetectable levels of HO-PBDEs in the present study.

### The direction of the modulation of vitamin A status by POPs seems to depend on the physiological status of the animal and the investigated tissue (Figure 6.1, point 4)

Because POPs are well known disrupters of vitamin A homeostasis in laboratory and wild animals and that they seem to share similar mechanisms of mobilisation from adipose tissue in grey seals, we naturally focused an important part of our work on studying the potential impacts of POPs on the vitamin A status in lactating grey seal females and their suckling pup.

In Chapter 2 we observed that vitamin A levels in pup serum appear to be negatively affected by the PCB concentrations in their serum as well as in maternal milk (Figure 6.3b). Simms *et al.* (2000) also reported that harbour seal pups from Washington State (recently weaned), with higher total PCBs in their blubber, had significantly lower circulatory retinol levels as compared to pups from British Columbia (nursing and fasted) that were less contaminated. However, when the authors analysed separately the non-nursing (fasted and recently weaned) and the nursing pups, they observed that total circulatory retinol levels were positively correlated with PCB exposure in non-nursing pups, while no detectable effects of PCBs on total retinol levels could be found in nursing pups. The lower overall vitamin A levels observed in the Washington State group at first glance probably reflected their weaned status (Simms *et al.*, 2000). The differences with the present work may be due to the tissue in which PCBs were measured to establish correlations. Here, we compared PCBs in milk and serum with circulating vitamin A, while Simms *et al.* (2000) compared PCBs in blubber and vitamin A in serum.

No correlation between PCBs and vitamin A could be found in lactating females. In contrast, in the 4th chapter where only lactating females were examined, we highlighted positive relationships between vitamin A and most of the investigated POPs in inner blubber. In addition, vitamin A was positively related to PCB and PBDE levels in maternal serum. These findings suggest thus that more contaminated animals tend to have also higher concentrations of vitamin A in their serum and blubber. Since the period of lactation was fixed in our statistical model, the effects of time and thus adipose lipid loss associated with lactation could be excluded. This means that those relationships were also probably true before the onset of the lactation period. Together, these results may suggest an exacerbated mobilisation of vitamin A from the liver due to POP contamination. One can then suppose that vitamin A released by the liver may either end up in the kidneys, which are known to play an important role in the vitamin A homeostasis (Käkëla *et al.*, 2003) or in the blubber, as a result of the high affinity of vitamin A for blubber triacylglycerols (Figure 6.3a). It is also possible that POPs exert an indirect effect on the dynamics of vitamin A and its concentrations in tissues. For example, vitamin E has been shown to have a protective antioxidant effect against the impact of PCBs (Slim *et al.*, 1999). Vitamin E levels in the liver are usually reduced following PCB exposition (Palace *et al.*, 1996). Nyman *et al.* (2003) have reported that blubber and plasma vitamin E concentrations were positively related to PCB contamination in grey and ringed seals. Together those results suggest an enhanced oxidation of hepatic vitamin E as well as its mobilisation to other tissues which suffer from oxidative stress induced by PCBs. A similar mechanism recruiting vitamin A from the liver in order to prevent or protect physiological functions such as the immune system, that may be altered by POPs and for which vitamin A is essential, could also explain a greater amount of this nutrient in the bloodstream.

Several studies have already shown that PCB and PBDE contamination tend to deplete hepatic retinoid stores (Bank *et al.*, 1989; Zile, 1992; Hallgren *et al.*, 2001; Ellis-Hutchings *et al.* 2006), but the molecular mechanisms which may increase the mobilisation of liver vitamin A are still unknown. The possible mechanisms by

which POPs may disrupt the vitamin A physiology in vertebrates has been summarised in Figure 2.1. The decreased activity of LRAT or ARAT, two enzymes involved in the hepatic storage of vitamin A or an increased release of retinol bound to RBP from the liver into the bloodstream may be at the origin of an enhanced mobilisation and redistribution in other tissues. That last mechanism has, however, never been examined in details.

**Table 6.2.** Results of the linear mixed models performed on the 2006 dataset in order test the relationships between total vitamin A concentrations (on a wet weight basis) and the total PCB levels (on a wet weight basis) in the serum of lactating grey seals. The period of lactation and vitamin A in inner blubber were included as confounding variables.

| Explanatory variables | Estimate | STD error | t | P value |
|---|---|---|---|---|
| Period | 0.088 | 0.100 | 0.883 | 0.390 |
| Vit A inner blubber | 0.325 | 0.166 | 1.960 | 0.066 |
| Σ PCBs serum | 0.065 | 0.093 | 0.698 | 0.497 |

All LMM are made on ln-transformed values.

The fact that both studies investigating the relationships between PCBs and vitamin A in lactating females showed different results (no relationship in Chapter 2 and a positive relationship in Chapter 4) remains unclear. Several hypotheses may explain those differences. The first study was carried out on 2 different Scottish grey seal populations, but since the number of observations for each population was low (10 individuals from the Isle of May and 7 individuals from North Rona), the statistics were performed on all animals without any population distinction. In addition, in the first study, some individuals could not be sampled at each period. The number of samples was thus sometimes unequal for some animals and tissues which might have lowered the statistical power analysis. The statistical methods used were also different, which might be at the origin of these discrepancies. However, we attempted to perform the same LMM on data of Chapter 2 but again, no relationship between vitamin A and PCBs in female serum could be observed (Table 6.2).

The fact that, for some explanatory variables, especially vitamin A levels in blubber, we did not have data for some individuals in Chapter 2 may have lowered the statistical power of the test. In order to have a more global statistical treatment, we run our LMM on the whole grey seal data of 2006 and 2008. Reassuringly, we found a strong positive relationship between vitamin A and PCBs in maternal serum (Table 6.3). It thus seems that the positive relationship between both types of compounds is the conclusion to keep in mind for lactating grey seal females.

**Table 6.3.** Results of the linear mixed models performed on the combined 2006 and 2008 dataset in order to test the relationships between total vitamin A concentrations (on a wet weight basis) and the total PCB levels (on a wet weight basis) in the serum of lactating grey seals. The period of lactation and vitamin A in inner blubber were included as confounding variables.

| Explanatory variables | Estimate | STD error | t | P value |
|---|---|---|---|---|
| Period | 0.146 | 0.086 | 1.694 | 0.097 |
| Vit A inner blubber | 0.332 | 0.114 | 0.291 | 0.772 |
| Σ PCBs serum | 0.434 | 0.036 | 1.925 | 0.000 |

All LMM are made on ln-transformed values.

Contrary to our expectations, vitamin A levels in serum were positively related to HO-PCBs. Since HO-PCBs have been shown to reduce circulating vitamin A concentrations via a disruption of TTR-RBP-retinol in human and laboratory animals, this result is quite surprising. In addition, studies on other marine mammal species have reported negative correlations between HO-PCBs and TTR or thyroid hormones (Gutleb *et al.*, 2010; Gabrielsen *et al.*, 2011). It is possible that other unknown molecular mechanisms are at the origin of those positive relationships in lactating marine mammals.

In the light of the present results and the previous works, it appears that POPs are able to induce either an increase or decrease of the levels of vitamin A in a particular tissue or have no effect at all. It is

important to keep in mind that, although the vitamin A status may be modulated by POPs, it does not necessarily mean that total body concentrations are changing. As explained above and more in details in Chapter 4, the increase of vitamin A concentrations following POP contamination may be due to a redistribution of existing vitamin A.

Environmental data are often difficult to interpret due to the numerous external factors that cannot always been controlled. In the present work, although we have taken the lactation period into account, this particular physiological state probably plays an important role in the relationships between POPs and vitamin A. For example, the fact that POPs and vitamin A appear to share similar mechanisms of mobilisation from blubber may contribute to the observed relationships. Another important point to highlight is that, although the effects were attributed to the investigated POPs, wild animals are exposed to a wide and complex mixture of chemical compounds. The observed effects may then be due to other molecules that have not been measured in the present work.

In view of the different associations reported between vitamin A status and POPs in the present work and elsewhere, the question may be raised whether vitamin A is a good biomarker of POP contamination and/or effects. Several factors should be considered when assessing the appropriateness of a particular biomarker (Gil and Pla, 2001): 1) Can it be quickly and easily measured? 2) Is it sensitive? 3) Is it specific? 4) Is it non invasive? The last point is of particular importance when dealing with endangered or protected species such as many marine mammal sepcies. Vitamin A can be easily and quickly analysed for relatively low costs. In addition, it can be measured in blood or blubber and is thus ethically acceptable. Besides, it is highly sensitive to POP contamination since its homeostasis has been shown to be altered in both laboratory and wild animals (Simms and Ross 2000; Novák *et al.*, 2008). However, although vitamin A can be considered as a sensitive biomarker regarding the effect of POP contamination, it cannot be used to identify the specific chemical causing the effect, since a wide range of contaminants such as PCBs, PBDEs and DDT are able to disrupt vitamin A metabolism. Nevertheless, such a non-specific biomarker is

useful to evaluate the overall effect of chlorinated and brominated organic pollutants on an organism (Gil and Pla, 2001). Another difficulty when using vitamin A as a biomarker is related to the variability of the response to POPs in function of a variety of factors such as the physiological state, the gender, the age, etc…, as observed in the present thesis and previous works. It is thus of great importance to take all these parameters into account prior to use vitamin A as a biomarker.

## Scottish grey seals do not seem to suffer from POP contamination

In order to see whether grey seal populations of the Scottish waters were at risk, the concentrations of dioxin-like PCB congeners (Table 6.4) were compared with those measured in other seal species from different areas (Table 6.5). TEQ values were calculated for mono-ortho PCBs found in our grey seal tissues (CB-105, -118 and -156) by multiplying the concentration of individual dioxin-like congeners by their respective TEF value, which is 0.00003 for the 3 congeners (Van den Berg *et al.*, 2006). These values are however given for information only. Their comparison with literature are indeed not relevant since ΣTEQs from other studies often includes a larger number of dioxin-like PCBs and other dioxin-related compounds such as PCDDs or PCDFs which were not measured in the present work.

**Table 6.4**. Concentrations and ΣTEQs of mono-ortho PCBs (in pg/g lw) measured in grey seal maternal blubber, serum, milk and pup serum sampled on the IOM at early and late lactation during the 2008 breeding season.

| Tissues | CB-105 | | CB-118 | | CB-156 | | ΣTEQs | |
|---|---|---|---|---|---|---|---|---|
| | *Early* | *Late* | *Early* | *Late* | *Early* | *Late* | *Early* | *Late* |
| Inner blubber | 6020 | 12060 | 14920 | 34870 | 9060 | 24910 | 0.90 | 2.16 |
| Outer blubber | 6700 | 7840 | 16790 | 20120 | 15540 | 20000 | 1.17 | 1.44 |
| Maternal serum | 4340 | 5490 | 9910 | 15020 | 4620 | 9190 | < 0.01 | 0.01 |
| Milk | 3750 | 5610 | 9960 | 14800 | 3820 | 7140 | 0.53 | 0.83 |
| Pup serum | 4830 | 5970 | 12790 | 15920 | 6010 | 9100 | 0.01 | 0.01 |

**Table 6.5**. Concentrations of CB-105, -118 and -156 (in pg/g lw) measured in several pinniped species of different regions.

| Species | Tissues | CB-105 | CB-118 | CB-156 | References |
|---|---|---|---|---|---|
| **Grey seal** | | | | | |
| Sable Island, CA | Maternal blubber | 51700 | 98400 | 59600 | Addison *et al.* 1999 |
| | Milk | 44000 | 83100 | 37000 | |
| | Pup blubber | 30100 | 50700 | 20500 | |
| Baltic | Blubber | 82000 | 276000 | 306000 | Harju *et al.* 2003 |
| | Liver | 79000 | 398000 | 328000 | |
| **Harbour seal** | | | | | |
| Baltic and Atlantic | Blubber | 73920 | 518940 | 599560 | Kannan and Petrick, 2009 |
| **Baïkal seal** | Male blubber | 110000-840000* | 300000-2300000* | 29000-240000* | Imaeda *et al.* 2009 |
| | Female blubber | 51000-170000* | 150000-450000* | 19000-76000* | |
| **North. Fur Seal** | Blubber | 31500 | 55300 | 6200 | Wang *et al.* 2010 |
| Bering Sea | Brain | 1500 | 6600 | ND | |
| | Kidney | 13500 | 43200 | ND | |
| | Liver | 17500 | 43700 | ND | |

*Ranges

Levels of CB-105, -118 and -156 found in our grey seals are generally low compared to other pinniped species (Table 6.5). Nevertheless, as discussed above, other PCB congeners which were not analysed may account for the TEQ toxicity. An earlier study that analysed CB-77, -126 and -169 in the milk of grey seals from the same Scottish colony 20 years ago reported that ΣTEQs for those 3 congeners was 22 pg/g lw (Green *et al.*, 1996). Since PCBs have been banned for several decades, current concentrations should logically have decreased or at least, remained similar. Other dioxin-related compounds such as PCDDs or PCDFs are generally found at lower concentrations than dioxin-like PCBs and therefore, ΣTEQ concentrations should not be much higher than those reported by Green *et al.* (1996). This means that our grey seal population is exposed to TEQ values that are below those causing immunosuppression in harbor seals fed with Baltic herrings in which the mean TEQ concentrations in blubber were 209 pg/g lw (De Swart *et al.*, 1995; Ross *et al.*, 1995; Ross *et al.*, 1996). Besides, another study carried out again on the same grey seal colony found no relationship between the cumulative lactational dose of organochlorine contaminants (as measured by dioxin-like PCBs) and prevalence of infection or alterations in blood haematology or serum chemistry in newborns (Hall *et al.*, 1997). These findings suggest that the Scottish grey seal populations may not suffer from immunodeficiency disorders. However, in the present work, negative relationships between vitamin A and PCBs were highlighted in pups and positive correlations were detected in lactating females. The biological importance of these impairments is still unknown, but the fact that vitamin A levels are highly regulated and important for growth and development in mammals may raise concerns of potential harmful and severe effects of these compounds in the exposed grey seal pups. Moreover, despite the ability of the TEQ approach to predict the toxicity of dioxine-like compounds, non dioxin-like chemicals contained in complex environmental mixtures can also exert adverse health effects via AhR-independent mechanisms (Giesy and Kannan, 1998; Stack *et al.*, 1999; Levin *et al.*, 2005). The fact that such non-dioxin-like effects are ignored could lead in an underestimattion of the potential adverse effects of environmental mixtures (Giesy and Kannan, 1998). The TEQ approach also assumes that toxic effects are additive and thus, it may not be valid for

predicting toxic effects of compounds in a mixture if interactions of chemicals in the mixture are antagonistic or synergistic (Giesy and Kannan, 1998).

### Environmental concentrations of PCBs and PBDEs did not modulate apoptosis, cell cycle and proliferation in mice and the harbour seal cell line (Figure 6.1, point 5)

Another sensitive biomarker for POP contamination is the immune system. Therefore, in the last experimental chapter, we attempted to investigate *in vitro* the potential effect of several PCB (CB-118, -126, -138, -153 and -180) and PBDE (BDE-47, -99, -100 and -153) congeners on 3 essential parameters of the immune system: apoptosis, cell cycle and proliferation.

Our preliminary results seem to indicate that environmentally relevant concentrations of these specific PCB and PBDE congeners do not affect the 3 investigated parameters. Regarding mouse splenocytes, when looking in the literature, we noticed that when similar PCB concentrations were used, no effect was generally detected. Some exceptions were found and the origin of the discrepancies between studies may lie in differences of protocol (strain, tested congeners, time of exposure, method of measurement,...). Concerning the harbour seal cell line, our findings confirm previous results that reported no induction of apoptosis after PBDE treatment in fresh harbour seal leukocytes as well as in the same cell line (Frouin *et al.*, 2010). Similarly, the fact that no modulation of the cell cycle was observed corroborates results of Mori *et al.* (2008) who reported no impact of individual PCB congeners in B lymphocytes of several species of marine mammals.

When studying the toxicology of a molecule, it is important to test concentrations representative to those found in the environment in order assess its "real" toxicity. Additionally, individuals in the environment are exposed to a complex mixture of contaminants. It is thus essential to examine the potential toxic effects of combined exposure of pollutants. It has recently been observed that interactions

between PCBs and PBDEs existed *in vitro* in eliciting the cytotoxicity in human neuroblastoma cells (Pellacani *et al.*, 2012). Therefore, the combined effects of PCBs and PBDEs on immune functions should be assessed.

### Vitamin A seems to modulate the cell cycle machinery in LPS-induced mouse splenocytes but not in the harbour seal cell line (Figure 6.1, point 6)

Vitamin A is an essential nutrient for the immune system homeostasis. Indeed, vitamin A deficiency leads to the alteration of several immune functions and is thought to be associated to the emergence of infectious diseases. In the last chapter, in addition to assess the immunotoxic effects of PCBs and PBDEs, we also examined the impact of vitamin A on apoptosis, cell cycle and proliferationof mouse splenocytes and the harbour seal cell line. Although the effects of vitamin A have already been well studied in mice, this is the first study to report its potential impact on the immune system of marine mammals.

Our preliminary *in vitro* experiments on LPS-induced mouse splenocytes have confirmed previous findings reporting that vitamin A inhibited the cell cycle progression of B lymphocytes, likely by preventing the phosphorylation of pBR (Blomhoff *et al.*, 2004). In contrast, the cell cycle of the harbour seal cell line was unaffected by vitamin A, which suggests that marine mammal immune cells may be less sensitive to variations of concentrations of this nutrient.

**Final view summarising the main results and interpretations of the present work**

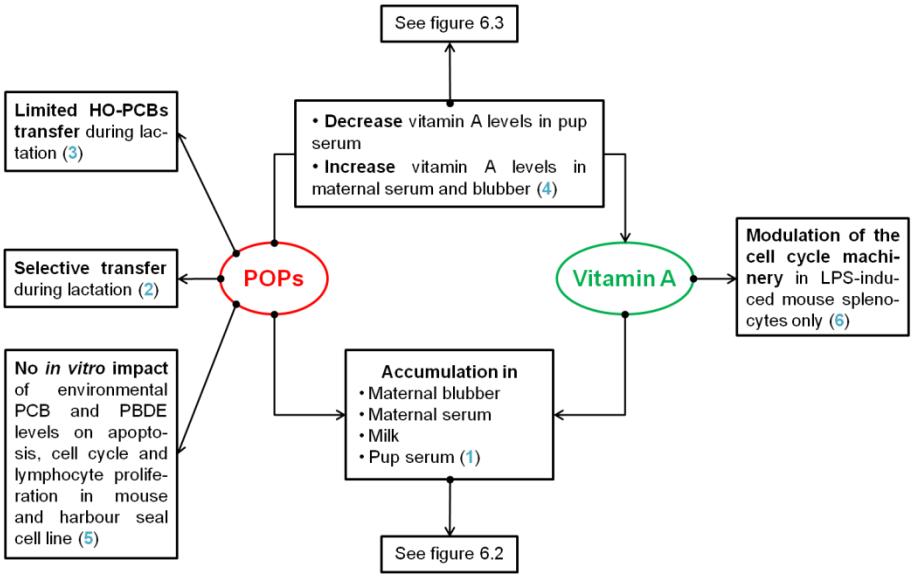


**Figure 6.1.** Chart summarising the different topics covered by this thesis.

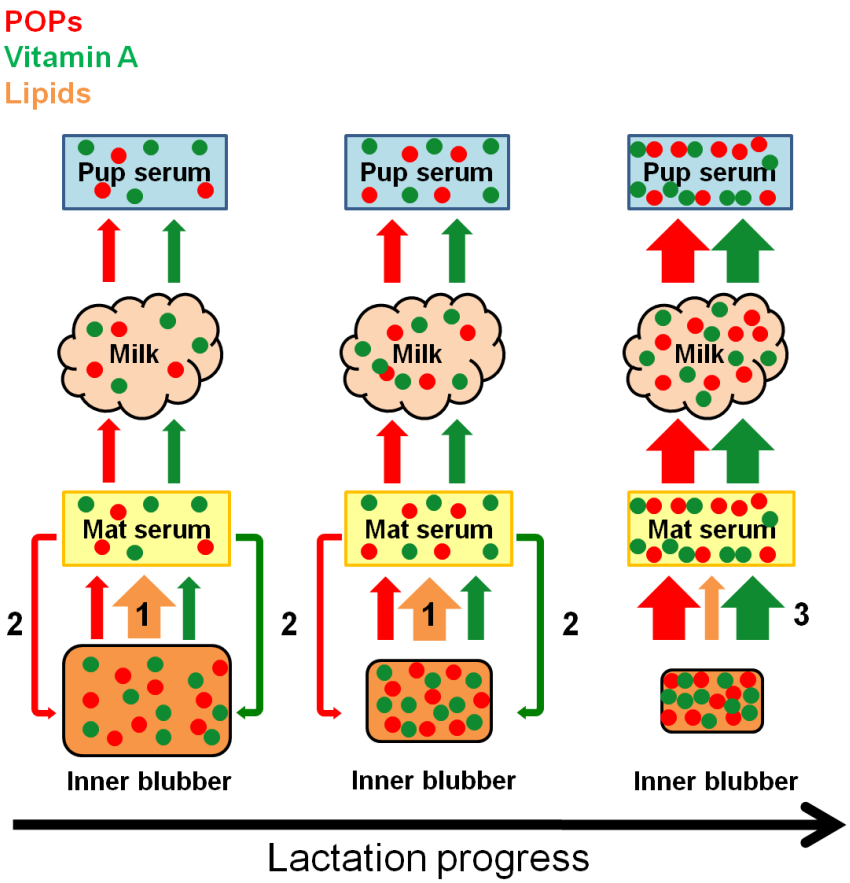


**Figure 6.2.** Possible mechanisms involved in the dynamics of vitamin A and POPs in maternal inner blubber and transfer into the different investigated tissues. Vitamin A and POPs may be less efficiently mobilised than blubber lipids (**1**) or mobilised in a similar proportion and quickly redirected into blubber due to a higher affinity for blubber triacylglycerols than for blood lipids (**2**). At late lactation, vitamin A and POPs appear to be released more abundantly from maternal blubber (**3**), leading to increasing concentrations in the next tissues of transfer. Mechanisms which may be involved in such an enhanced mobilisation include, among others, the selective mobilisation of lipids from blubber or the limit of retention of the reduced inner blubber layer.

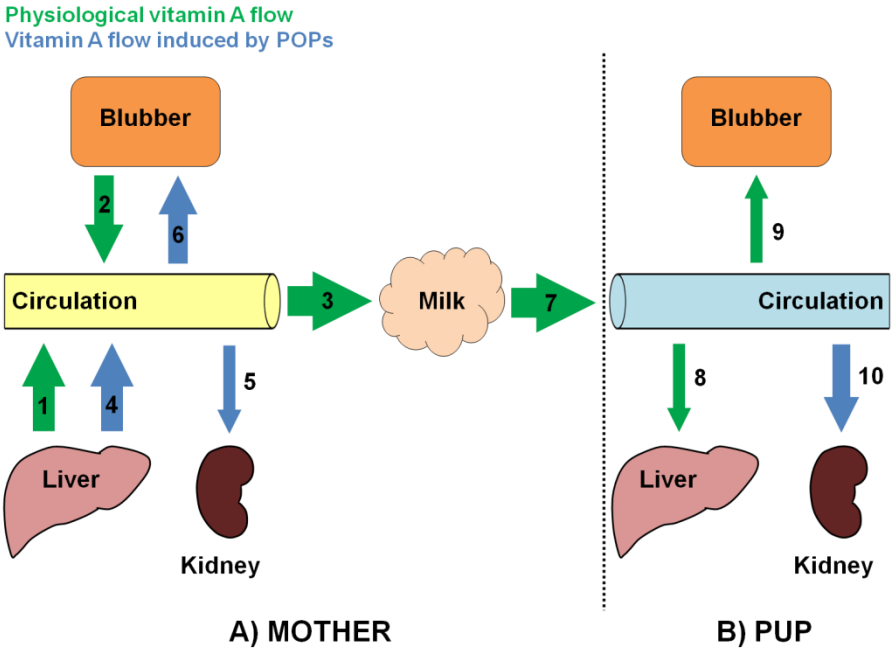


**Figure 6.3.** Possible flows of vitamin A in mother and pup during lactation. In the mother (**A**), vitamin A is mobilised from the liver (**1**) and blubber (**2**) in order to enrich milk (**3**). Our results reported higher vitamin A concentrations in maternal serum and inner blubber in more contaminated females, suggesting that POP contamination may exacerbate vitamin A mobilisation from the liver (**4**), leading to increasing circulating vitamin A levels which may either end up in the kidneys (**5**) or in the blubber (**6**), as a result of its high affinity for blubber triacylglycerols. In the pup (**B**), vitamin A ingested via the milk (**7**) may be stored in the liver (**8**) or the blubber (**9**). Our results suggest that more contaminated pups tend to have less vitamin A in their serum, probably resulting from a higher excretion (**10**).

## 6.2. Perspectives

Besides the new interesting data that this thesis has brought, it also opened the door to new questions. Some of the research perspectives opened through this work are proposed hereafter:

### 6.2.1. *In vivo* studies

- On the overall, a higher number of samples should be collected in the future in order to determine more thoroughly the dynamics of POPs and vitamin A during lactation in grey seals.

- We showed that concentrations of vitamin A differed between maternal inner and outer blubber, particularly at early lactation. In a previous study on ringed seals (*Pusa hispida*), Strandberg *et al.* (2008) have suggested that the vertical distribution of vitamin A could exhibit stratification patterns similar to the vertical profile of fatty acids. Since vitamin A is stored as retinyl esters in adipose tissue, it would be interesting to analyse the distribution of the different forms of retinyl esters in both blubber layers and to compare it to the pattern of the fatty acids. The analyse of CRBP which are intracellular proteins that specifically bind retinol and LRAT and ARAT which are involved in the esterification of retinol into retinyl esters could also be carried out in order to examine whether different levels of expression exist between inner and outer blubber.

- STRA6 is a specific RBP receptor which has been recently identified and it has been shown to be expressed in white adipose tissue of mice. It may be therefore worth investigating the presence of STRA6 in grey seal blubber and its behaviour during the lactation period, especially in inner blubber. Indeed, this receptor could be involved in the increased concentrations of vitamin A observed in inner blubber as well as in its possible redistribution in this tissue following POP exposure.

- In order to complete our study on the transfer of POPs in grey seals, it would be interesting to obtain serum and blubber samples of pups from early lactation until the end of the post-weaning fast. However, such samplings, especially blubber ones, cannot be done easily on grey seals. An interesting alternative is elephant seals. The study of the mobilisation of POPs during the post-weaning fast in elephant seals is the purpose of a current thesis in progress in our laboratory and will likely bring new additional information on the mobilisation and transfer of POPs in fasting animals.

- We observed increasing concentrations of HO-PCBs in both maternal and pup serum at late lactation. An analyse of CYP activities and their modulation in lactating females as well as in their suckling pups would allow to verify the hypothesis of an enhanced biotransformation in both mothers and pups.

- Interactions between HO-PCBs and the TTR-RBP-retinol complex have often been suggested but never been thouroughly investigated in seals. In addition, our results showed an unexpected positive relationship between circulating vitamin A and HO-PCBs in lactating grey seals. Therefore, the impact of HO-PCBs on vitamin A blood transport should be examined in grey seals. Experiments should involve competitive assays for binding sites on TTR with labelling $T_4$ and HO-PCBs.

- We demonstrated that circulating vitamin A of pups was adversely affected by PCBs in the milk and in pup serum. The implication of a possible disruption in vitamin A homeostasis by other POPs such PBDEs and HO-PCBs in grey seal pups should be further investigated.

### 6.2.2. *In vitro* studies

- *In vitro* adipocyte models are currently carried out in our laboratory in order to get a better understanding of the dynamics of POPs in adipose tissue. These experiments involve, among others, lipolysis of adipocytes after PCB treatment. It would be

worth using the same model to examine the mobilisation of vitamin A during lipolysis. Additionally, a treatment of PCBs and/or vitamin A during lipopysis should be investigated in order to verify whether these lipophilic compounds can still enter the cells when lipids are abundantly released.

- We reported that environmentally relevant concentrations of PCBs or PBDEs do not modulate apoptosis, proliferation and/or cell cycle of murine splenocytes and the harbour seal cell line. The combined effects of PCBs and PBDEs on these 2 models should be assessed in order to highlight possible interactions between the 2 types of POPs.

- In order to evaluate the influence of vitamin A on the immunotoxic effects of PCBs and PBDEs, murine splenocytes and the cell line should be incubated together with PCBs/PBDEs and vitamin A. Since the present PCB and PBDE concentrations did not affect the investigated parameters, higher concentrations should be first tested in order to detect some immunotoxic effects. For the same purpose, it would be interesting to investigate the effect of a modulation of vitamin A status *in vivo* on the immunotoxic effects of PCBs and PBDEs *in vitro* in mice.

- Similar *in vitro* experiments involving treatment with PCBs, PBDEs and vitamin A should be conducted on freshly isolated seal lymphocytes.

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