BIOTRANSFORMATION AND ENDOCRINE DISRUPTIVE EFFECTS OF CONTAMINANTS IN RINGED SEALS
–implications for monitoring and risk assessment

by

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are indicated in the text by their Roman numerals I-VI:


VI Routti H., Nyman M., Jenssen B.M., Bäckman C., Koistinen J., Gabrielsen G.W. 2008. Bone related effects of contaminants in seals may be associated to vitamin D and thyroid hormones. Environmental Toxicology and Chemistry. 27, 873-88

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“Etsin ankarasti totuutta, päästäkseni siitä eroon”

”I strive for truth in order to get rid of it”

- Risto Ahti –

(Translated by Laura Routti)
ABSTRACT

Marine mammals are exposed to persistent organic pollutants (POPs), which may be biotransformed to metabolites some of which are highly toxic. Both POPs and their metabolites may lead to adverse health effects, which have been studied using various biomarkers. Changes in endocrine homeostasis have been suggested to be sensitive biomarkers for contaminant-related effects. The overall objective of this doctoral thesis was to investigate biotransformation capacity of POPs and their potential endocrine disruptive effects in two contrasting ringed seal populations from the low contaminated Svalbard area and from the highly contaminated Baltic Sea.

Biotransformation capacity was studied by determining the relationships between congener-specific patterns and concentrations of polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polybrominated diphenyl ethers (PBDEs) and their hydroxyl (OH)- and/or methylsulfonyl (MeSO$_2$)-metabolites, and catalytic activities of hepatic xenobiotic-metabolizing phase I and II enzymes. The results suggest that the biotransformation of PCBs, PBDEs and toxaphenes in ringed seals depends on the congener-specific halogen-substitution pattern. Biotransformation products detected in the seals included OH-PCBs, MeSO$_2$-PCBs and –DDE, pentachlorophenol, 4-OH-heptachlorostyrene, and to a minor extent OH-PBDEs. The effects of life history state (moulting and fasting) on contaminant status and potential biomarkers for endocrine disruption, including hormone and vitamin homeostasis, were investigated in the low contaminated ringed seal population from Svalbard. Moulting/fasting status strongly affected thyroid, vitamin A and calcitriol homeostasis, body condition and concentrations of POPs and their OH-metabolites. In contrast, moulting/fasting status was not associated with variations in vitamin E levels. Endocrine disruptive effects on multiple endpoints were investigated in the two contrasting ringed seal populations. The results suggest that thyroid, vitamin A and calcitriol homeostasis may be affected by the exposure of contaminants and/or their metabolites in the Baltic ringed seals. Complex and non-linear relationships were observed between the contaminant levels and the endocrine variables. Positive relationships between circulating free and total thyroid hormone concentration ratios and OH-PCBs suggest that OH-PCBs may mediate the disruption of thyroid hormone transport in plasma. Species differences in thyroid and bone-related effects of contaminants were studied in ringed and grey seals from low contaminated references areas and from the highly contaminated Baltic Sea. The results indicate that these two species living at the same environment approximately at the same trophic level respond in a very different way to contaminant exposure.

The results of this thesis suggest that the health status of the Baltic ringed seals has still improved during the last decade. PCB and DDE levels have decreased in these seals and the contaminant-related effects are different today than a decade ago. The health of the Baltic ringed seals is still suggested to be affected by the contaminant
exposure. At the present level of the contaminant exposure the Baltic ringed seals seem to be at a zone where their body is able to compensate for the contaminant-mediated endocrine disruption. Based on the results of this thesis, several recommendations that could be applied on monitoring and assessing risk for contaminant effects are provided. Circulating OH-metabolites should be included in monitoring and risk assessment programs due to their high toxic potential. It should be noted that endogenous variables may have complex and highly variable responses to contaminant exposure including non-linear responses. These relationships may be further confounded by life history status. Therefore, it is highly recommended that when using variables related to endocrine homeostasis to investigate/monitor or assess the risk of contaminant effects in seals, the life history status of the animal should be carefully taken into consideration. This applies especially when using thyroid, vitamin A or calcitriol-related parameters during moulting/fasting period. Extrapolations between species for assessing risk for contaminant effects in phocid seals should be avoided.
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ABBREVIATIONS

Br  Bromine
BROD  Benzyloxyresorufin-O-dealkylase
Cl  Chlorine
CYP  Cytochrome P450
CHL  Chlordane
DDT  1,1,1-trichloro-2,2-bis[p-chlorophenyl]ethane
DDE  1,1-dichloro-2,2-bis[p-chlorophenyl]ethylene
DIO  Deiodinase
EROD  Ethoxyresorufin-O-deethylase
FT₃  Free triiodothyronine
FT₄  Free thyroxine
GHR  Growth hormone receptor
GST  Glutathione S-transferase
H  Hydrogen
HCB  Hexachlorobenzene
HpCS  Heptachlorostyrene
MeSO₂  Methylsulfonyl
MROD  Methoxyresorufin-O-demethylase
OCP  Organochlorine pesticide
OH  Hydroxyl
PBDE  Polybrominated diphenyl ether
PCA  Principal component analysis
PCB  Polychlorinated biphenyl
PCP  Pentachlorophenol
PCR  Polymerase chain reaction
POP  Persistent organic pollutant
PROD  Pentoxyresorufin-O-dealkylase
RAR  Retinoic acid receptor
RDA  Redundancy analysis
T₄  Thyroxine
T₃  Triiodothyronine
TH  Thyroid hormone
TOX  Toxaphene
TR  Thyroid hormone receptor
TSH  Thyroid stimulating hormone
TT₃  Total triiodothyronine
TT₄  Total thyroxine
TTR  Transthyretin
UDPGT  Uridine-diphosphate glucuronosyltransferase
1. INTRODUCTION

1.1 ASSESSING THE RISK OF CONTAMINANT EFFECTS IN WILDLIFE

Assessing the risk of chronic exposure to environmental contaminants is challenging. Two approaches have been used when assessing risk for contaminant effects in wildlife (de Wit et al. 2004). The first approach is based on comparisons between contaminant levels in wildlife and threshold levels of effects obtained from experimental or semi-field studies. In this method, wide extrapolations between species are made without taking into account species-specific accumulation profiles, metabolic capacity and inherent sensitivities to toxicological action of contaminant. Until recently, both wildlife and experimental studies have focused mainly on the parent (precursor) compounds that animals are exposed to via their diet. However, following exposure, contaminants may be biotransformed in the body to highly toxic hydroxylated (OH) and methysulfonyl (CH$_3$SO$_2$=MeSO$_2$) metabolites (Figure 1). Formation/retention of toxic metabolites may be species-specific and it may depend on the physiological state of the animal. Therefore, comparisons of exposure and effect threshold concentrations of contaminants within a wide range of exposure (concentrations) in different species at a different physiological state may lead to highly biased estimates of risk for possible effects of contaminants. Risk for contaminant effects in wildlife population has also been assessed by relating the concentration of environmental contaminants to biomarkers for effects. Biomarkers are defined as measurable changes or responses in biochemical processes or (endogenous) compounds induced by xenobiotics and are therefore indicative of potential pollution-mediated effects (Peakall 1992). Changes in endocrine functions may be sensitive biomarkers for contaminant-related effects because several environmental contaminants have endocrine disruptive potencies (Figure 1) (Tyler et al. 1998, Boas et al. 2006). However, these biomarkers are regulated by physiological processes and their response may often vary greatly depending on the species and the life history state of the animal (Figure 1). The knowledge gaps in contaminant-mediated mechanisms leading to changes in biomarker responses cause difficulties to assess causality between contaminant levels and effects. Therefore mechanisms of action of contaminants (Figure 1), which may also vary between the species due to genetic variation, should be of concern in order to strengthen the links between biomarkers and contaminant effects. Formation/retention of toxic metabolites, physiological regulation of potential biomarkers and effects and mechanisms of action of contaminants, in species, which may be exposed to high levels of contaminants, are thus necessary information in order to assess the health risk associated with contaminant exposure in wildlife.
Figure 1. Interactions between contaminant and life history status, and endocrine systems. Interactions investigated in the present thesis are indicated with black arrows.
1.2 **PERSISTENT ORGANIC POLLUTANTS**

Persistent organic pollutants (POPs) contain several classes of the major environmental contaminants is (Figure 2). POPs consist of a wide range of man-made chemicals produced for several purposes and by industrial by-products. The main attention has focused on polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs). PCBs may theoretically be formed of 209 different congeners, but PCB mixtures used for various industrial purposes are mainly consisted of a minority of these congeners. OCPs, produced mainly for agricultural purposes, consist of several groups of chemicals including 1,1,1-trichloro-2,2-bis[p-chlorophenyl]ethane (DDT), chlordanes (CHLs), toxaphenes (TOXs) and hexachlorobenzene (HCB). Recently, also polybrominated diphenyl ethers (PBDEs) and other brominated flame retardants have received increasingly attention. Although several POPs have been banned decades ago, their threat to the environment is still of concern. PBDEs (e.g., deca-BDE technical mixture) are still produced worldwide (Betts 2008), DDT production and usage continues in developing countries, and PCBs are constantly released in the environment by leakage and degradation (de Wit et al. 2004).

![General molecular structures of selected POPs.](image)

**Figure 2.** General molecular structures of selected POPs.
Contaminants accumulate in the species at the higher trophic levels through dietary exposure. The bioaccumulation of POPs and their chemical profile in the food web depends on the physicochemical properties of the compounds and the biotransformation capacity of the organisms (Fisk et al. 2001). Therefore the contaminant pattern at the higher levels of the food chain does not reflect the mixture of the compounds produced.

There is rising concern that POPs are potential endocrine disruptors. Several reports indicate that the endocrine disruptive potential of POPs may significantly increase after their biotransformation to OH- or MeSO$_2$-metabolites (Boas et al. 2006; Bondy et al. 2003; Gauger et al. 2007; Legler 2008; Lund et al. 1988; Mercado-Feliciano and Bigsby 2008; Ucán-Marín et al. 2009), which rises the need to define biotransformation capacity and formation/retention of metabolites in wildlife.

1.3. BIOTRANSFORMATION

Levels and patterns of contaminants and their metabolites in biota at higher trophic levels are ultimately defined by biotransformation processes. POPs induce xenobiotic-metabolizing phase I (cytochrome P450, i.e. CYP) and conjugating phase II enzymes. CYPs belong to a large superfamily of enzymes which catalyze the synthesis/metabolism of endogenous compounds and the biotransformation of exogenous compounds. Phase II enzymes catalyze the conjugation of e.g. glutathione, sulphate and glucuronic acid to endo- and exogenous compounds.

Biotransformation of POPs includes numerous complex processes and pathways. PCB biotransformation (Figure 3) is initiated by CYP enzymes, which catalyze the direct insertion of an OH group to meta or para position of PCB molecule (Letcher et al. 2000). Alternatively, CYP enzymes may catalyze the formation of arene oxide intermediates in meta-para or in ortho-meta position of a PCB congener. Arene oxide may be further metabolized to OH-PCBs by epoxide hydroxylase (EH) or to MeSO$_2$-metabolites via mercapturic acid pathway involving for example glutathione-S-transferase (GST). OH-PCBs may be further biotransformed by uridine-diphosphate glucuronosyltransferase (UDPGT), which is involved in glucuronidation of OH-PCBs. Both oxidation mediated by CYPs and debromination may be involved in biotransformation of PBDEs (Hakk and Letcher 2003). CYPs also catalyze the dechlorination of DDT to 1,1-dichloro-2,2-bis[p-chlorophenyl]ethylene (DDE) (Kitamura et al. 2002), which may be further metabolized to MeSO$_2$-DDE. Multiple steps and pathways catalyzed by phase I and II enzymes have been suggested to be involved in the biotransformation of chlordanes, which favours the formation of persistent oxychlordane and heptachlorepoxide (Nomeir and Hajjar 1987). Biotransformation of toxaphenes may occur via multiple processes including oxidation, dechlorination and dehydrochlorination mainly catalyzed by CYPs (De Geus et al.)
In addition, CYPs catalyze HCB biotransformation to pentachlorophenol (van Ommen et al. 1985). OH-containing POPs retained in the body are mainly found in blood (Bergman et al. 1994; Gebbink et al. 2008a). More lipophilic MeSO₂-metabolites accumulate in more lipid-rich tissues such as liver and adipose tissue (Gebbink et al. 2008a; Larsson et al. 2004). OH- and MeSO₂-metabolites have been detected in various wildlife species including for example seals, whales, polar bears and seabirds (Gebbink et al. 2008b; Hoekstra et al. 2003; Letcher et al. 2000; McKinney et al. 2006a; Sandala et al. 2004; Verreault et al. 2005).

*In vitro* and *in vivo* studies indicate that OH- and MeSO₂-metabolites of POPs show high toxic potential towards mammalian endocrine system (Lund et al. 1988; Kato et al. 1999; Lund et al. 1999; Boas et al. 2006; Legler 2008). Therefore, endocrine related effects of POPs and their metabolites are of concern in free-ranging animals exposed to high concentrations of POPs.
1.4. ENDOCRINE DISRUPTIVE EFFECTS OF CONTAMINANTS

Chronic exposure to contaminants may affect the health of an animal at various levels (Figure 4). Contaminant effects may be detected from molecular, physiological, histological to pathological level. First, effects may be compensated to some extent at the physiological level. At this compensative stage the health status of the animal can be defined as stressed. With increased exposure, contaminants may lead to the disease status, when the effects are first reversible and then irreversible. The most dramatic irreversible effect of contaminants is death.

A wide range of endocrine disruptive effects, including failures in reproduction, thyroid hormone and vitamin homeostasis, adrenal function and bone and eggshell formation, have been associated with POP exposure (Boas et al. 2006; Giesy et al. 2006; Guillette et al. 2006; Jenssen 2006; Letcher et al. 2009; Novák et al. 2008; Rolland 2000). Numerous in vivo, in vitro and correlative, free-ranging wildlife studies suggest that thyroid and vitamin A homeostasis are target systems of contaminat-
mediated endocrine disruption in experimental and free-ranging animals (Boas et al. 2006; Jenssen 2006; Letcher et al. 2009; Novák et al. 2008; Rolland 2000; Simms and Ross 2000). Disruption of these systems has been suggested to be of high ecological significance in marine mammals (Brouwer et al. 1989; Jenssen 2006; Mos et al. 2007). Hormones controlling calcium homeostasis are also suspected to be disrupted in highly contaminated animals, because bone deformities and reduced bone mineral density have been linked to high levels of contaminants in wildlife and laboratory rodents (Bergman et al. 1992; Lind et al. 2000; Lind et al. 2003; Lind et al. 2004; Sonne et al. 2004).

Thyroid hormones (THs), including thyroxine (T\textsubscript{4}) and triiodothyronine (T\textsubscript{3}) play a key role in controlling the metabolism, growth and development from cellular to whole organism level (McNabb 1992). The synthesis of T\textsubscript{4} in the thyroid gland is regulated by the hypothalamic thyrotropin-releasing hormone and thyroid-stimulating hormone (TSH) secreted from the anterior pituitary (Chiamolera and Wondisford 2009). The transport of THs in plasma is mediated by carrier proteins including transthyretin (TTR), thyroxin binding globulin and albumin, the composition and TH-binding capacity of which may vary between species (McNabb 1992). T\textsubscript{4} is further converted to the biologically active T\textsubscript{3} by outer ring deiodinizing enzymes (DIO1 and DIO2) mainly in peripheral tissues (Gereben et al. 2008). T\textsubscript{3} generated by DIO1 in plasma membrane is rapidly distributed in plasma and the main function of intracellular DIO2 is to hold T\textsubscript{3} level constant in plasma and at cellular level (Gereben et al. 2008). THs act via nuclear thyroid hormone receptors (TR\textalpha and TR\textbeta), which control the expression of numerous genes e.g. growth hormone (McNabb 1992). Inactivation/metabolism of THs is mediated by conjugating enzymes, including UDPGT, and by inner ring deiodinase DIO3 (Gereben et al. 2008). Numerous studies have reported associations between altered thyroid hormone levels and POP concentrations in wildlife (Braathen et al. 2004; Debier et al. 2005; Hall et al. 2003; Hall and Thomas 2007; Jenssen 2006; Rolland 2000; Sormo et al. 2005; Tabuchi et al. 2006; Verreault et al. 2004). In vitro studies indicate that thyroid homeostasis may be interfered by xenobiotic compounds via various mechanisms including the synthesis, transport, metabolism and receptor-mediated actions (Cheek et al. 1999; Boas et al. 2006; Marchesini et al. 2008). Function of transport-proteins and receptors have been of particular concern in POP-mediated effects on thyroid homeostasis. Several OH-POPs formed by xenobiotic-metabolizing enzymes show high structural similarity to thyroid hormones. OH-POPs show higher affinity to human and avian transthyretin and avian albumin than T\textsubscript{4} and T\textsubscript{3} (Cheek et al. 1999; Hamers et al. 2008; Lans et al. 1993; Sandau et al. 2000; Ucán-Marín et al. 2009; van den Berg 1990; Ucán-Marín, F., Arukwe, A., Mortensen, A. Gabrielsen, G.W., Letcher, R.J. unpublished) and they may have both agonistic and antagonistic effects on TR (Gauger et al. 2007; Miyazaki et al. 2008). OH-PBDEs have been demonstrated to have a strong binding capacity to thyroxing binding globulin (Marchesini et al. 2008). MeSO\textsubscript{2}-PCBs have been suggested
to influence thyroid hormone metabolism resulting to altered thyroid hormone levels (Kato et al. 1999).

Retinoids (vitamin A and its metabolites) have been defined as “dietary hormones”, because they have a vital role in regulating development and growth, antioxidative functions, epithelial maintenance, immune function, vision and reproduction (Debier and Larondelle 2005; Novák et al. 2008; Simms and Ross 2000). Various forms of vitamin A or its precursors are ingested from diet. Vitamin A metabolism in small intestine and liver involves several enzymes and binding proteins (Debier and Larondelle 2005; Novák et al. 2008). Several species store vitamin A in the form of retinyl esters in liver, while in marine mammals a large proportion of retinyl esters is stored in blubber (Debier and Larondelle 2005). In several mammal species, including seals, retinoids are transported in blood circulation as retinol bound to retinol-binding protein, which forms a transport protein complex with TTR (Debier and Larondelle 2005). The most bioactive forms of vitamin A, retinoic acids, induce their effects through nuclear retinoic acid receptor (RARα, RARβ and RARγ) and retinoic X receptor. Altered levels of vitamin A have been related to contaminants in several wildlife studies (Brouwer et al. 1989; Jenssen et al. 2003; Letcher et al. 2009; Mos et al. 2007; Nyman et al. 2003; Rolland 2000; Simms and Ross 2000). Lately, also altered mRNA expression of RARα in blubber has been related to POP concentrations in seals (Mos et al. 2007). Contaminant-mediated disruption of retinoid homeostasis and function may be mediated by retinoid metabolism, transport or signal transduction (Novák et al. 2008). Also OH-PCBs have been reported to have vitamin A disruptive properties in vivo in rodents (Brouwer et al. 1988).

Calcitriol (1,25-(OH)₂-vitamin D₃), an active metabolite of vitamin D₃, has an essential role in calcium homeostasis (Horst. et al. 2005). Therefore vitamin D is categorized as a hormone rather than as a vitamin (Horst. et al. 2005). Vitamin D₃ is absorbed in the intestine or produced in skin from 7-dehydrocholesterol. It is metabolism to 25-OH-vitamin D₃ in liver and further to calcitriol in kidney is mediated by CYP enzymes (Vieth 2005). Calcitriol plays a major role in regulating serum calcium homeostasis together with parathyroid hormone and calcitonin (Yasuda et al. 2005). Calcitriol stimulates intestinal absorption and renal reabsorption of calcium and phosphate, which further stimulates bone mineralization (Faibish and Boskey 2005; Yasuda et al. 2005). The action of calcitriol is mediated by nuclear vitamin D receptor (Norman 2006). Decreased levels of vitamin D metabolites have been reported in PCB-exposed rodents (Alvarez-Lloret et al. 2009; Lilienthal et al. 2000), but the mechanisms are still unclear.
1.5. CONTAMINANT EFFECTS IN SEALS

Marine mammals are exposed to high levels of persistent organic pollutants through their diet. Although POPs are transported to remote arctic areas (de Wit et al. 2004), it should be kept in mind that for example PCBs and DDT have been detected at tens of times higher concentrations in a given marine mammal species from industrialized areas compared to those from Arctic areas (McKinney et al. 2006a; Nyman et al. 2002).

One of the most prominent examples for environmental pollution is the Baltic Sea, which is surrounded by a heavily industrialized drainage area. Extremely high levels of POPs have been detected in ringed (*Phoca hispida*) and grey seals (*Halichoerus grypus*) inhabiting the Baltic Sea (Jensen et al. 1969; Nyman et al. 2002). The high levels of POPs, mainly PCBs and DDTs, have been associated with numerous adverse effects and drastic population decline in the Baltic seals. In late 1970s, the majority of the ringed seal females suffered from uterine occlusions (Helle 1980) leading to life-long sterility. A disease syndrome including lesions in skin, claws, kidney, intestinal, reproductive and circulatory systems observed in grey seals, and to a lesser extent in ringed seals, has been associated to adrenocortical hyperplasia (Bergman and Olsson 1985). That interpretation has been based on the similarities between the morphological lesions observed in the Baltic seals and those associated to adrenocortical hyperplasia in other species. In grey seals also bone deformities and uterine tumours have been common problems (Bergman 1999). The prevalence of these pathological problems and POP levels have decreased significantly during the last decades (Bergman 1999; Helle et al. 2005; Olsson et al. 1994; Nyman et al. 2002). Today, the growth is 4.3% for the Baltic ringed seal population and 8% for the the Baltic grey seal population (Karlsson et al. 2007). Although the pathological disorders observed in the Baltic seals have not yet been linked to the contaminant burdens by any specific toxic mechanisms, high levels of POPs have been related to alterations in xenobiotic-metabolizing enzyme activities, vitamin A and E concentrations, haematology and blood chemistry (Nyman et al. 2003).

1.6 STUDY SPECIES AND AREAS

Ringed seal has been recommended as a model species for studying contaminant effects in Arctic marine mammals (Arctic Monitoring and Assessment Program (AMAP) 1999; Letcher et al. 2009). This species has a circumpolar distribution in the Arctic, and some populations live in highly polluted southern areas such as the Baltic Sea, the Lake Saimaa in Finland and Lake Ladoga in Russia (Kostamo et al. 2000; Nyman et al. 2002). This small seal species (length 120-150 cm, weight 60-100 kg) is the most abundant seal in the northern hemisphere. In the Arctic, ringed seals give birth to a single pup and mate in late spring (March/April) and moulting takes place in early summer (May/June). In the Baltic, reproduction and
moulting occur approximately a month earlier than in the Arctic. During the period of reproduction and moulting their energy requirements, energy consumption and food intake vary widely (Ryg and Oritsland 1991), and they are generally in a state of negative energy balance, losing 30-35% of their blubber stores (Ryg et al. 1990). The Svalbard ringed seal population is considered to be a healthy population (Krafft et al. 2006; Tryland et al. 2006) that is subjected to low contaminant levels compared to the populations found in industrialized areas (Nyman et al. 2002); it is thus an interesting reference population for ecotoxicological studies of ringed seals.

The ringed seal population in the Baltic Sea provides a unique opportunity to study contaminant biotransformation, effects, biomarkers and mechanisms of toxicity of contaminants for several reasons. Pathological failures and xenobiotic-metabolizing enzyme system have been well described in these seals (Bergman and Olsson 1985; Helle 1980; Hyyti et al. 2001; Nyman et al. 2000; Nyman et al. 2001). Although PCB and DDT levels have decreased in the Baltic ringed seals during the last decades, the levels are still high enough to potentially cause adverse health effects to the seals (Nyman et al. 2002). The occurrence of uterine occlusions in ringed seal females sampled in 1995-2004 is a bit over 20% (Helle et al. 2005). The present growth rate of the Baltic ringed seal population suggests that their reproduction capacity is still below the potential reproduction capacity of the species (Karlsson et al. 2007). In addition, associations between several health parameters, including vitamin A and E levels, hematology and blood chemistry, with POPs have been studied in these seals (Nyman et al. 2003). In ringed seals, increased CYP1A activity, high vitamin E concentrations in blubber and plasma, decreased retinyl palmitate levels in liver, and altered levels of parameters for haematology and clinical chemistry have been related to their individual contaminant exposure (Nyman et al. 2003; Routti et al. 2005).
2. **OBJECTIVES OF THE STUDY**

The overall objective of this thesis was to investigate the biotransformation capacity and endocrine disruptive effects of persistent organic pollutants in ringed seals from two contrasting areas: the low-contaminated Svalbard and the highly contaminated Baltic Sea.

In the biotransformation part (papers I-III) the specific objectives were to investigate:

- Current status of PCB, PBDE and OCP levels and the activities of xenobiotic-metabolizing phase I and II enzyme activities in the two seal populations

- Biotransformation capacity by determining the relationships between congener-specific patterns and concentrations of PCBs, OCPs, PBDEs and their OH- and/or MeSO₂-metabolites in liver and/or plasma, and the catalytic activities of hepatic phase I and II enzymes of the low- and highly contaminated ringed seals.

In the endocrine disruption part (papers IV-VI) the specific aims were to investigate:

- The effects of life history state (moulting/fasting) on contaminant status and potential biomarkers, including hormone and vitamin status, in the low-contaminated ringed seal population from Svalbard (IV)

- Endocrine disruptive effects of contaminants and their metabolites at multiple endpoints in the two contrasting ringed seal populations from Svalbard and the Baltic Sea (V)

- Species differences in thyroid and bone-related effects of contaminants in ringed and grey seals from low-contaminated references areas and from the highly contaminated Baltic Sea (VI)
3. MATERIALS AND METHODS

3.1 FIELD SAMPLING

The seal samples from the west coast of Svalbard (Figure 5), Norwegian Arctic, were obtained in May and June 2007 (I-V) and 1996-98 (IV-VI). At Svalbard, the seals were sampled with special permission granted to the Norwegian Polar Institute by the Governor of Svalbard and during the local hunting season under local hunting law in Svalbard. Ringed seals from the Baltic Sea (Figure 5) were sampled in April-May 1997-98 (V-VI) and in April 2002-2007 (I-III, V) from the Bothnian Bay. Also grey seals were sampled from the Bothnian Bay in May 1997-1998 (VI). Sampling in the Baltic was conducted according to the special permission granted by the Ministry of Forestry and Agriculture in Finland to the Finnish Game and Fisheries Research Institute. Reference grey seals were sampled from Sable Island, eastern Canada, in June 1998 (VI) with special permission granted to the Marine Institute by the Bedford Institute of Oceanography in Canada (host institute for sample collection). All samples were collected during the seals’ moulting season at approximately the same phase of their annual reproductive cycle. Detailed information of age determination, condition indexes, sampling procedures and sample storage are given in the papers I-VI.

3.2 CHEMICAL ANALYSIS

Chemical data used in this thesis was obtained from three laboratories. Ringed seals from 2002-2007 were analyzed for hepatic and circulating PCBs and OCPs (p,p’-DDE, Σ-CHL, Σ-TOX, HCB), and hepatic PBDEs at the University of Örebro (B. van Bavel, Örebro, Sweden). The method for analysis, which is described in detail in papers I-III, was based on previously described methods with some modifications (van Bavel et al. 1995; van Bavel et al. 1996). The same individuals were analyzed for circulating OH-PCBs, OH-PBDEs, PCP, 4-OH-HpCS and PBDEs, and hepatic MeSO₂-PCBs and 3-p,p’-DDE at the National Wildlife Research Centre (R.J. Letcher, Ottawa, Canada) as completely described in the papers I-III. MeSO₂-PCBs and –DDE in liver sample were analyzed according to previously described methods with some modifications (Gebbink et al. 2008b; McKinney et al. 2006a). The extraction and clean-up of plasma (or serum) for OH-POPs and PBDEs were based on procedures described elsewhere with some modifications (Gebbink et al. 2008b; Verreault et al. 2007). The chemical data used in
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paper VI were obtained from the National Public Health Institute (Jaana Koistinen, Kuopio, Finland). This method has been described in detail elsewhere (Vartiainen et al. 1995). Congener-specific analyses and quantification was performed using gas chromatography – mass spectrometry.

3.3 ENZYME ASSAYS

Phase I enzyme activities in liver microsomes were studied using ethoxyresorufin-O-deethylase (EROD), benzyloxyresorufin-O-dealkylase (BROD), methoxyresorufin-O-demethylase (MROD) and pentoxyresorufin-O-dealkylase (PROD) activity assays based on the end point method according to Burke and Mayer (1974). EROD is catalyzed by CYP1A in seals (Nyman et al. 2000). In dogs, catalyzation of BROD is mediated by CYP1A1/2 (Jayyosi et al. 1996) and CYP2B11 (Kleotka and Halpert, 1995). In rodents, MROD and PROD have been used as model substrates for CYP1A2 (Nerurkar et al. 1993) and CYP2B (Burke et al. 1985), respectively. Methods for analysis of activities of Phase II enzymes, microsomal uridine-diphosphate glucuronosyltransferase (UDPGT) and cytosolic glutathione S-transferase (GST), were based on previously described methods (Andersson et al. 1985; Habig et al. 1974). The assays were adapted to 96-well plate reader and measured spectrophotometrically using a microplate reader for absorbance reading. Total amount of protein was determined with the method of Bradford (1976). Detailed information of preparation of microsomal and cytosolic fractions and enzyme assays is given in paper I.

3.4 THYROID HORMONES, CALCITRIOL, CALCIUM AND PHOSPHATE

Analyses of plasma concentrations of total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), free triiodothyronine (FT3) and calcitriol were conducted using radioimmunoassay as described in papers IV-VI.

Circulating calcium and phosphate were analyzed from frozen serum of ringed and grey seals using an automatic analyser (Nyman et al. 2003).

3.5 mRNA EXPRESSIONS

Messenger RNA (mRNA) expressions of DIO1, DIO2, TRα and β, TSH, GHR and RARα were analyzed in the liver of ringed seals from Svalbard and the Baltic Sea using real-time PCR as described in detail in papers IV-V.

3.6 VITAMIN A, E AND D

A₁ and A₂ forms of retinol, retinyl palmitate (16:0), -linoleate (18:2), -oleate (18:1), and stearate (18:0), and α-tocopherol were identified in liver and plasma of ringed seals (IV-V). The samples were extracted using diisopropyl ether and separated and quantified by high performance liquid chromatography (HPLC) as described in
Materials and Methods

detail in paper IV. Analytical methods for retinol in ringed and grey seal plasma used in paper VI have been described elsewhere (Nyman et al. 2003).

The extraction, clean-up and quantification of vitamin D$_3$ in liver are described in detail in paper VI. Following the hydrolyzation and column-extractions, the samples were first pre-cleaned and then separated and quantified by HPLC.

3.7 STATISTICAL ANALYSIS

Geographical differences of contaminant levels and enzyme activities in ringed seals were studied using linear models (I-III). Multivariate descriptive methods, including principal component analysis (PCA) and principal component analysis for instrumental variables, also named redundancy analysis (RDA), and Pearson correlation coefficients, were used to investigate aspects regarding contaminant patterns (I-III) and their relationships with enzyme activities and concentrations of POPs (I, III). PCA and RDA for contaminant composition studies were derived from the covariance matrix of centered log-ratio of proportions.

The differences in body condition, vitamin, hormonal and contaminant status between pre-moulting and moulting ringed seals from Svalbard were studied using Wilcoxon rank sum test (IV). Geographical differences between the low and high contaminated seal populations for hormone and vitamin levels, and gene expressions were studied using linear models, Wilcoxon rank sum test and Welch test (V-VI). Influence of moulting status, age, gender and condition on the levels of hepatic vitamin D$_3$, calcitriol and THs in ringed and grey seals were studied using linear models (V-VI). Relationships between biological and chemical variables were investigated using PCA derived from correlation matrix and Pearson/Spearman correlation coefficients (V-VI).
4. RESULTS AND DISCUSSION

4.1 GEOGRAPHICAL AND TEMPORAL TRENDS IN CONTAMINANT LEVELS AND ENZYME ACTIVITIES (I-III)

PCBs and DDTs, measured as liver lipid weight, have been reported to be 60 and 95 times higher, respectively, in ringed seals from the Baltic Sea compared to those from Svalbard (Nyman et al. 2002). In these seals sampled a decade ago, the high levels of contaminants were associated to elevated activities of CYP1A enzymes (Nyman et al. 2003). Although the presence of phase II enzymes have been reported in ringed seals (Wolkers et al. 1998a), a contaminant-mediated induction of these enzymes has not been reported in pinnipeds. In order to assess the current status of contaminant burden and the xenobiotic-metabolizing enzymes in ringed seals from Svalbard and the Baltic Sea, we investigated the concentrations of PCBs, PBDEs and OCPs, and the catalytic activities of phase I and II enzymes in ringed seals sampled in 2002-2007.

Levels of hepatic PCBs, PBDEs and OCPs (lipid weight) were still 15, 5 and 6 times higher, respectively, in the ringed seals from the Baltic Sea compared to those from Svalbard. Contaminant pattern in the seals from both areas were dominated by PCBs, followed by \( p,p' \)-DDE and chlordanes, while the contribution of \( \Sigma \)-PBDEs, toxaphenes and HCB was minor. The geographical differences of hepatic POP concentrations were greater for \( \Sigma \)-PCBs, \( \Sigma \)-PBDEs, \( p,p' \)-DDE and \( \Sigma \)-CHLs compared to \( \Sigma \)-TOXs and HCB, which is possible related to the long-range transport potential of these contaminants. The concentrations of the main PCB congener, CB153, and \( p,p' \)-DDE in ringed seals from the present study were 20 and 13 %, respectively, of the levels detected in the ringed seals sampled in the Baltic in 1997-98 (Nyman et al. 2002). Although different analytical methods have been used in these studies, the results indicated that PCB and DDE levels have significantly decreased over this time period in the Baltic seals.

Activities of xenobiotic metabolizing phase I enzyme activities measured as catalytic activities of EROD, PROD, BROD and MROD, were induced in the ringed seals from the Baltic compared to those from Svalbard. Of the phase II enzymes, GST activity correlated positively with contaminant levels, while UDPGT activity did not differ between the seal populations.

4.2 BIOTRANSFORMATION (I-III)

The process of biotransformation ultimately defines the levels and patterns of contaminants and their metabolites in the biota. In order to study the biotransformation of various groups of POPs in ringed seals, we examined congener-specific patterns and concentrations of PCBs, PBDEs and OCPs and their metabolites in liver and/or plasma in the two contrasting ringed seal populations. Further, the relationships between the
levels and patterns of contaminants and the catalytic activities of phase I and II enzymes were investigated. In general, our findings indicated that PCBs and toxaphenes, depending on their Cl-substitution pattern molecular configuration, and p,p'-DDE and HCB are partly biotransformed in ringed seals. In addition, our results indicated the formation of OH- and MeSO₂-metabolites via phase I and II enzymes.

4.2.1 PCBs (I)

Biotransformation capacity PCBs and their affinity to the hepatic CYP isoenzymes have been shown to vary widely between and among species and populations. Chlorine (Cl) substitution pattern of a PCB compound strongly influences PCB biotransformation and their affinity to specific CYP isoforms. Biotransformation of PCBs has been studied in pinnipeds using several approaches including comparisons of contaminant patterns in seals and their diet (Boon et al. 1997; Wolkers et al. 1998b), in vitro metabolism/inhibition assays (Li et al. 2003) and measurements of residues of PCB metabolites in seal tissues (Jensen and Janson 1976; Troisi et al. 2000; Larsson et al. 2004; Letcher et al. 2000; Verreault et al. 2008). Initial pharmacokinetic studies (Boon et al. 1997), which are further supported by other studies (Li et al. 2003; Wolkers et al. 1998b), suggest that PCBs can be divided into five metabolic groups in marine mammals. PCB biotransformation in the present ringed seals was studied by the compositional changes of these five metabolic groups (Table 1) in liver in relation to contaminant exposure, catalytic activities of phase I and II enzymes and OH- and MeSO₂-metabolite formation.

In general, these results indicated metabolism of PCBs having one or no ortho-Cl (CB III) and those having two ortho-Cl and vicinal meta-para H atoms (CB IV) to OH- and MeSO₂-PCBs in ringed seals. Non-planar PCBs with no vicinal hydrogen (H)-atoms (CB I) and with vicinal H-atoms only in ortho and meta-positions in combination with ≥ 2 ortho-Cl (CB II) appeared to be persistent. These findings are in accordance with bioaccumulation (Boon et al. 1997; Wolkers et al. 1998b) and in vitro studies (Li et al. 2003).

Relative concentration of CB III to Σ-PCB decreased with increasing contaminant exposure and this change was strongly related to phase I enzyme, in particular CYP1A, activities and the formation of OH-PCBs. Group III PCBs acquire easily a planar configuration and are thus preferred substrates to CYP1A-like enzymes in seals (Boon 2001). In the Baltic seals, more than a half of Σ-OH-PCB concentrations determined in plasma consisted of 4-OH-CB107/4’OH-CB108 (co-eluting congeners), which potential precursors belong to the CB III group including among other congeners CB118 and CB105. Concentration ratio of CB IV to Σ-PCBs also decreased with increasing contaminant levels, which was explained by contaminant-mediated induction of phase I enzymes and GST. The metabolism of non-planar group IV PCBs, which are preferred
Table 1. Structural characteristics of five metabolic groups of polychlorinated biphenyls (adapted from Boon et al. 1997).

<table>
<thead>
<tr>
<th>PCB-group</th>
<th>Vicinal hydrogen atoms at ortho-meta position</th>
<th>Vicinal hydrogen atoms at meta-para position</th>
<th>Number of ortho-chlorine atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB I</td>
<td>No</td>
<td>No</td>
<td>0 - 4</td>
</tr>
<tr>
<td>CB II</td>
<td>Yes</td>
<td>No</td>
<td>≥ 2</td>
</tr>
<tr>
<td>CB III</td>
<td>Yes</td>
<td>No</td>
<td>0 - 1</td>
</tr>
<tr>
<td>CB IV</td>
<td>No</td>
<td>Yes</td>
<td>≤ 2</td>
</tr>
<tr>
<td>CB V</td>
<td>No</td>
<td>Yes</td>
<td>≥ 3</td>
</tr>
</tbody>
</table>

substrates of CYP2B isoenzymes (Lewis et al. 1998), suggest that CYP2B-like enzymes may be involved in PCB metabolism in ringed seals. Majority of the MeSO₂-PCBs detected in the Baltic ringed seals originated from group IV PCBs. Congeners of CB IV, such as CB101 and 110 are known precursors of 3- and 4-MeSO₂-substituted PCBs of the same chlorination pattern (Letcher et al. 2000). Relative concentrations of hepatic group I and II PCBs to Σ-PCB increased with increasing contaminant levels suggesting the persistence of these congeners. Only small amounts of metabolites of these PCBs compared to other PCB metabolites were detected in ringed seals. Interestingly, the formation of 3'-OH-CB184 (parent CB from groups I and V) was observed only in the ringed seals from Svalbard. This may be explained by higher CYP3A activity in the Svalbard ringed seals compared to the seals from the Baltic (Nyman et al. 2001).

4.2.2 PBDEs (II)
Biotransformation of PBDEs has been suggested to depend on the bromine (Br) substitution pattern of a PBDE molecule, comparable to PCBs (McKinney et al. 2006b). PBDE biotransformation has been observed using in vitro models with beluga whale and rat liver microsomes (McKinney et al. 2006b; Hamers et al. 2008) and in vivo in rodents (Marsh et al. 2006). OH-PBDEs have been detected in marine biota, including beluga and minke whales, and ringed seals (Kelly et al. 2008; McKinney et al. 2006a; Verreault et al. 2008), and they may originate either from CYP-mediated
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biotransformation (Hamers et al. 2008; Marsh et al. 2006) or from bioaccumulation via natural sources (only ortho OH-substituted PBDEs) (Teuten et al. 2005; Malmvärn et al. 2008). In order to assess PBDE biotransformation in ringed seals, levels and patterns of PBDEs in liver and OH-PBDEs in plasma were determined in the two ringed seal populations, which are contrasted by their contaminant exposure and the activities of xenobiotic enzymes.

Levels and patterns of OH-PBDEs in the differentially contaminated ringed seal populations indicated low formation and/or plasma retention of OH-PBDE metabolites. OH-PBDE pattern in the Baltic ringed seals was dominated by ortho OH-substituted PBDEs, which have been reported to occur as natural products in the Baltic Sea (Malmvärn et al. 2008). Small amounts of 3-OH-BDE47 and 4’-OH-BDE49 were detected in the majority of the Baltic seals. These compounds are probably products of biotransformation, since meta and para substituted OH-PBDEs have not been detected at lower trophic levels from the Baltic Sea (Malmvärn et al. 2008) and 3-OH-BDE47 has been reported to be the main OH-metabolite of BDE47 in phenobarbital-induced rat microsomes (Hamers et al. 2008). In contrast to our findings, PBDEs biotransformation was not reported in liver microsomes of harbour seals in vitro (de Boer et al. 1998). Formation of 3-OH-BDE47 and 4’-OH-BDE49 in ringed seals may be mediated by CYP enzymes. In rodents, both CYP2B and 3A induction has been related to PBDE exposure (Pacyniak et al. 2007; Richardson et al. 2008).

Observed patterns of PBDEs in the Baltic seals suggested the persistence of BDE153 and -154 and the metabolism of BDE28. Relative concentrations of BDE153 and 154 to Σ-PBDE increased with increasing Σ-POP levels, while BDE28 showed the opposite pattern. This finding is supported by the hypothesis of McKinney et al. (2006b), who suggested that the metabolic capacity of beluga whales is greater towards congeners with increasing number of ortho-meta Br-unsubstituted sites. BDE153 and -154 have no ortho-meta Br-unsubstituted sites in contrast to BDE28, which has two pairs of vicinal ortho-meta hydrogen pairs.

4.2.3 ORGANOCORINE PESTICIDES (III)

Biotransformation of OCPs has been studied relatively little in seals, although OCPs is a major group of POPs in many seal populations followed by PCBs. Tissue residues of MeSO₂-DDE, a known metabolite for DDE, have been detected in seals (Haraguchi et al. 1992; Jensen and Janson 1976; Larsson et al. 2004; Letcher et al. 1998; Troisi et al. 2000). In vitro biotransformation studies suggests that metabolism of toxaphenes in seals depends on the Cl-substitution pattern of a congener (Boon et al. 1998). Similar studies have also been reported the involvement of CYP3A in toxaphene metabolism in seal microsomes (van Hezik et al. 2001). Biotransformation of OCPs in the present ringed seals was investigated by assessing the levels and patterns of various
groups of OCPs in liver and their metabolites in plasma/liver in relation to xenobiotic-metabolizing enzyme activities in the two contrasting seal populations.

The concentrations of 3-MeSO$_2$-p,p$'$-DDE were several times higher in the ringed seals from the Baltic compared to the seals from Svalbard, which suggested that p,p$'$-DDE is partly biotransformed to 3-MeSO$_2$-p,p$'$-DDE. Our findings are supported by the studies reporting tissue residues of 3-MeSO$_2$-p,p$'$-DDE in seals (Haraguchi et al. 1992; Jensen and Janson 1976; Larsson et al. 2004; Letcher et al. 1998; Troisi et al. 2000).

Positive correlations between the concentrations of PCP, 4-OH-HpCS and the activities of phase I enzymes support the hypothesis that these compounds may be products of phase I metabolism. Also previous reports have suggested that PCP and 4-OH-HpCS detected in marine mammals result from biotransformation of HCB or pentachloroanisole, and octachlorostyrene, respectively (Hoekstra et al. 2003; Sandau et al. 2000).

Toxaphene patterns in the seals from the less and more contaminated areas suggested that biotransformation of toxaphenes is dependent on the Cl-substitution pattern of a compound as suggested by Boon et al. (1998). Relative concentrations of Parlar-44 to Σ-TOX in liver decreased with increasing hepatic contaminant concentrations, while the opposite pattern was observed for Parlar-26 and -50. Parlar-44 has Cl-unsubstituted sites at the positions C-3 and C-6 of the lateral carbon ring, while Parlar-26 and -50 possess Cl-substituents at each carbon atom positioned at the lateral ring.

The chlordane pattern was surprising in the ringed seals from Svalbard and the Baltic Sea. Relative concentrations of oxychlordane and cis-heptachlor epoxide to Σ-CHL were higher in the seals from Svalbard compared to the seals from the Baltic, while the trend was opposite for the parent compounds trans-nonachlor/MC6 and cis-nonachlor. Oxychlordane and cis-heptachlor epoxide are metabolites of nonachlors (Nomeir and Hajjar 1987). As suggested by an experimental study on Greenland sledge dogs (Verreault et al. 2009a), elevated levels of other POPs in the more contaminated animals may result in reduced formation of oxychlordane compared to the lower contaminated animals. Another possibility is that CYP3A, which had higher activity in the ringed seals from Svalbard compared to those from the Baltic Sea (Nyman et al. 2001), may play a role in chlordane metabolism. Geographical differences of the chlordane composition in ringed seals may also be related to the dietary exposure, because chlordane metabolites have been detected in ringed seal diet items (Borgå et al. 2007; Strandberg et al. 1998; Wolkers et al. 2000; Wolkers et al. 2006). However, bioaccumulation factors were not investigated in the present study.
4.2.4 BIOTRANSFORMATION CAPACITY OF RINGED SEALS AND OTHER SPECIES (I, III)

Biotransformation capacity in a given species has been shown to depend on the compound-specific characteristics, such as molecular structure and physicochemical properties, and on the contaminant exposure (Letcher et al. 2000). Our results suggest that the level of contaminant exposure influences the order of susceptibility of compounds being biotransformed. In the present ringed seals a wide range of parent POPs and their OH-metabolites were detected at several times higher levels in the Baltic ringed seals than in the seals from Svalbard. Interestingly, compositional changes in the metabolite formation were observed. In the seals from Svalbard OH-POPs were dominated by PCP (51 %) followed by OH-PCBs (32 %). In contrast, OH-PCBs were the major OH-POP group (85 %) in the ringed seals from the Baltic Sea. In both populations PCBs accounted more than half of hepatic Σ-POP levels. These findings suggest that the less contaminated seals from Svalbard biotransform HCB more readily, while in the more contaminated ringed seals from the Baltic PCBs are more susceptible for biotransformation.

Geographical comparison of the ratios of sum of metabolites to sum of parent compounds in a given body compartment indicate that biotransformation capacity increases with increasing level of contaminant exposure. Similar comparisons between species suggest that the formation of OH-PCBs and MeSO₂-metabolites decreased from polar bear (Gebbink et al. 2008b) to ringed seal to Glaucous gull (Verreault et al. 2005). Also composition of metabolites varies between species, suggesting a species differences in xenobiotic metabolizing enzyme systems.

4.3 ENDOCRINE DISRUPTION (IV-VI)

Contaminant-mediated disruption of endocrine systems has been suggested to be of ecological significance in marine mammals (Brouwer et al. 1989; Jenssen 2006; Letcher et al. 2009). Endocrine disruption in wildlife is often studied using biomarkers, which may vary greatly depending on the life history state of the animal and on the species. We studied the effects of life history state, contaminants and species on endogenous parameters in seals. Our results indicated that the variation of endogenous parameters in seals depends on contaminant levels, life history state and species.

4.3.1 EFFECTS OF LIFE HISTORY STATE ON BIOMARKERS (IV)

Phocid seals moult and fast simultaneously in spring (Ryg et al. 1990). Fasting periods are believed to be the most susceptible times for contaminant-mediated effects due to POP mobilization from blubber stores (Lydersen et al. 2002). However, biomarkers used to study contaminant effects, such as hormones and vitamins, are regulated in endogenous physiological processes and their concentrations often vary greatly depending on the physiological state of the animal. Therefore, we investigated
body condition, concentrations of plasma total and free T₄ and T₃, and calcitriol, vitamin A and E in liver and plasma, hepatic mRNA expressions for TRβ, DIO1, DIO2 and RARα, hepatic POPs and their circulating OH-metabolites in pre-moultung and moultung adult ringed seals from the low-contaminated Svalbard area.

Our results indicated that hormone, vitamin and contaminant status are related to moultung and the concomitant fasting period in ringed seals. Concentrations of THs, vitamin A, POPs and their OH-metabolites were higher in moultung seals compared to pre-moultung seals. The opposite trend was observed for body condition, plasma calcitriol levels and hepatic mRNA expression of TRβ.

THs play a major role in controlling development, growth, thermogenesis and basal metabolic rate (McNabb 1992). The higher levels of THs in moultung seals may thus be related to the hair replacement process (Ramot et al. 2009) and thermoregulation. Elevated TH levels during the moultung period have also been observed in other phocid seals (Boily 1996; John et al. 1987). Low hepatic mRNA expression of TRβ may be a compensatory mechanism for the high levels of bioactive FT₃ in the plasma in the moultung seals. However, it should be noted that the present study investigated only mRNA expressions, which may not correlate with the protein activity.

Lower calcitriol levels in the moultung seals compared to the pre-moultung seals could be associated with calcium metabolism. Low calcitriol levels in the moultung seals may reduce calcium uptake by bone (Faibish and Boskey 2005), which may lead to increased serum calcium levels in the seals at low body condition (Tryland et al. 2006). Low calcitriol levels in the moultung seals may also be related to hair growth (Ramot et al. 2009).

Higher levels of vitamin A in the liver and plasma of moultung seals in comparison to the pre-moultung seals may be related to mobilization of vitamin A compounds from blubber into other body compartments during fasting (Debier et al. 2002a; Schweigert et al. 2002). The observed changes in plasma retinol levels could also be associated with an altered metabolic rate (Bonet et al. 2003) or the hair replacement process (Ramot et al. 2009). No differences were observed in hepatic or circulating vitamin E concentrations between moultung and pre-moultung seals. The different associations of vitamin A and E levels with moultung in the present ringed seals could indicate that vitamin A and E are not mobilized from storage tissues by similar mechanisms, as suggested by previous seal studies (Debier et al. 2002a; Debier et al. 2002b; Schweigert et al. 2002).

The elevated levels of POPs and their OH metabolites during moultung period support the hypothesis that animals are more sensitive to adverse effects of
contaminants during fasting period (Letcher et al. 2009). The higher concentrations of POPs in moulting seals in comparison to pre-moulting seals is likely a result of mobilization of POPs from blubber stores during fasting (Debier et al. 2006; Hall et al. 2008; Lydersen et al. 2002). The increased levels of circulating OH-POPs in moulting versus pre-moulting seals are probably a consequence of increased bioavailability of POPs in the liver, which would induce the activity of xenobiotic enzymes and the formation of metabolites. Hepatic CYP1A activity was not significantly higher in moulting than in pre-moulting ringed seals. However, CYP3A has been suggested to catalyze the formation of 3’-OH-CB184, which is one of the major OH-PCBs detected in these animals (I).

4.3.2 CONTAMINANT-MEDITATED ENDOCRINE DISRUPTION IN RINGED SEALS (V-VI)

Endocrine systems, including thyroid, vitamin A and calcitriol homeostasis may be affected by environmental contaminants (Boas et al. 2006; Jenssen 2006; Lilienthal et al. 2000; Novák et al. 2008). Both POPs and their OH- and MeSO₂-metabolites may disrupt endocrine systems through various mechanisms including synthesis, transport, cellular uptake, receptor function and metabolism of the hormones (Boas et al. 2006; Kato et al. 1999). First, in order to investigate possible effects of contaminants at multiple biological levels in seals, we analyzed circulating THs, calcitriol, calcium and phosphate, plasma and hepatic vitamin A components, hepatic vitamin D₃ and thyroid- and vitamin A related hepatic mRNA expressions in ringed seals from two areas: low-contaminated Svalbard area and more contaminated Baltic Sea. Second, we examined the relationships between the biological variables and the concentrations of POPs and their OH- and/or MeSO₂-containing metabolites in the seals.

The results suggested that contaminant-mediated endocrine disruption occurs both at the level of circulating hormones/vitamins and hepatic mRNA expressions in the Baltic ringed seals. OH-PCBs may play an important role in the disruption of thyroid homeostasis.

Free and total TH concentrations and the ratios between free to total THs, and expression of hepatic mRNA of DIO1, TRβ and its indirect target gene, GHR, were higher in the ringed seals from the Baltic Sea than in those from Svalbard, which suggests that disruption of thyroid system by POPs in ringed seals from the Baltic Sea. The positive relationships between circulating FT₄:TT₄ ratios and Σ-OH-PCB concentrations in the Baltic seals indicate that persistent OH-PCBs may be having an effect on the transport mechanisms of T₄ in plasma. Several OH-PCBs including 4-OH-CB107/108, which is the major OH-PCB detected in the present Baltic ringed seals (I), as well as 4-OH-CB187, have been shown to have higher affinity to human and avian transthyretin (TTR) than T₃ or T₄ (Brouwer et al. 1998; Ucán-Marín et al. 2009). 4-OH-
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CB187 has also been reported to have higher affinity to avian albumin than T4 and T3 (Ucán-Marín, F., Arukwe, A., Mortensen, A., Gabrielsen, G.W., Letcher, R.J. unpublished). The higher FT4:TT4 and FT3:TT3 ratios in the Baltic ringed seals than in the reference seals support the hypothesis of contaminant-mediated disruption of TH-transport system. Positive correlations between plasma levels of THs and albumin (Nyman et al. 2003; Hall et al. 2003) suggest that albumin may be involved in TH plasma transport in seals. The role of TTR or thyroxin binding globulin in ringed seals is still to be recovered.

The higher concentrations of T4 and T3, and mRNA expressions of TRβ and DIO1 in the Baltic seals may be compensative mechanisms for the contaminant-mediated disruption of the T4 transport system in plasma. Alternatively, POPs or their metabolites may directly induce T4 synthesis, and TRβ and/or DIO1 mRNA expression. 4-OH-CB-107 has been suggested to be an important agonist to TR (Gauger et al. 2007). The higher DIO1 mRNA expressions in the Baltic seals in comparison to the seals from Svalbard could be caused by FT3, which is an inducer of DIO1 (Bianco et al. 2002), or by contaminants via constitutive androstane receptor (Tien et al. 2007). Concentrations of T4 and T3, and FT4:TT4 ratio, were negatively related to hepatic contaminants in the highly contaminated Baltic ringed seals sampled in 1990s (VI), while FT4:TT4 showed positive correlations hepatic POPs and their circulating OH-PCBs in the less contaminated Baltic ringed seals sampled in 2002-2007 (V). This suggests that ringed seals may have a bell-shape response curve (Figure 4B) for the response of TH levels to contaminant exposure. Different mechanisms may possibly play a role at different levels of contaminant exposure and/or formation of OH-POPs leading to either positive or negative relationship between THs and contaminants.

Circulating retinol levels were lower in the highly contaminated Baltic seals than in the seals from Svalbard, while opposite trend was observed for hepatic expression of RARα mRNA. Elevated mRNA levels of RARα could be a compensative mechanism for the low plasma retinol levels (Mos et al. 2007). However, the pathway from mRNA expression to protein activity may be interrupted by several factors. Hepatic vitamin A concentrations were no more related to contaminants in the Baltic ringed seals in contrast to the more contaminated Baltic ringed seals sampled in 1990s (Nyman et al. 2003). Interestingly, differences of plasma retinol level were not observed in the seals sampled a decade ago (Nyman et al. 2003).

Circulating calcitriol levels were significantly lower in the highly contaminated Baltic ringed seals sampled in 1990s compared to the reference population from Svalbard, while hepatic vitamin D3 levels showed the opposite trend. These seals did not show any population difference for circulating calcium or phosphate levels. In contrast, the population difference for calcitriol was opposite in the seals sampled a decade later.
4.3.3. **SPECIES DIFFERENCES** (VI)

Pathological problems associated with high levels of contaminants have been reported to differ between the Baltic seal species. Ringed seals have suffered mainly from reproductive problems, while bone lesions and various problems linked to adrenocortical hyperplasia have been widely observed in Baltic grey seals (Bergman and Olsson 1985; Bergman 1999; Helle 1980). In order to investigate species differences of contaminant effects on bone and thyroid system at biomarker level, we investigated circulating THs, calcitriol, calcium, phosphate and vitamin A, and hepatic POPs and vitamin D$_3$ in ringed and grey seals from the low-contaminated reference areas and from the highly contaminated Baltic Sea sampled in 1990ies.

Species-specific difference in contaminant-mediated endocrine responses was observed between ringed seal and grey seal. Population differences for THs, calcitriol and vitamin A between the ringed seals from the Baltic Sea and from the reference area are described in the previous chapter. No inter-correlations were observed between these variables in Baltic ringed seals. In contrast, THs, calcitriol, calcium, phosphate and vitamin A showed both population differences and strong inter-correlations in grey seals.

Concentrations of circulating THs, calcium and phosphate were clearly higher in the Baltic grey seals compared to the reference grey seals from Canada, while calcitriol showed the opposite trend. These bone-related parameters were strongly correlated to hepatic POPs, which could be explained by various mechanisms. First, contaminants might depress circulating calcitriol levels by depressing calcitriol formation or enhancing its renal clearance (Lilienthal et al. 2000) leading to decreased serum calcium and phosphate levels. Alternatively, high levels of POPs may lead to hyperthyroidism, which has been suggested to be the reason for positive correlations between POPs and THs in grey seals from the British waters (Hall et al. 2003). Decreased calcitriol levels and hyperthyroidism may compensate each other in order to maintain serum calcium concentration stable (Mohan et al. 2004). Elevated TH levels have been associated with a dramatic increase in bone resorption and a reduction of bone mineral density, thus increasing serum calcium levels (Lakatos 2003), which further leads to a suppression of parathyroid hormone secretion and a decrease in calcitriol production (Epstein and Schneider 2005). TH levels showed a strong positive correlation with circulating retinol in the grey seals. This could be related to the amount of transthyretin–retinol-binding protein complex. Because vitamin A stores in the liver and blubber are decreased in Baltic grey seals (Nyman et al. 2003), mobilization of vitamin A from the storage tissues may increase the amount of carrier proteins.
5. CONCLUSIONS

This thesis provides novel information on biotransformation of POPs, contaminant-mediated endocrine disruption and effects of the life history status on potential biomarkers in ringed seals. Detailed findings are listed below.

- Biotransformation of PCBs, PBDEs and toxaphenes in ringed seals depends on the halogen-substitution pattern of a compound. PCBs having one or no ortho-Cl are potential substrates to CYP1A-mediated formation of OH-PCBs. MeSO₂-PCBs and 3-MeSO₂-p,p’-DDE are suggested to be formed from PCBs having two ortho-Cl and vicinal meta-para H atoms, and p,p’-DDE, respectively, by CYP2B-like and GST enzymes. Formation of PCP of HCB and/or pentachloroanisole, and 4-OH-HpCS of octachlorostyrene may also be catalyzed by phase I enzymes, while the formation of OH-PBDEs is minor in ringed seals. The level of contaminant exposure may influence the biotransformation capacity and the order of susceptibility of compounds being biotransformed. In comparison to other species, formation and/or retention of OH-PCBs and MeSO₂-metabolites in ringed seals is higher compared to Glaucous gulls and lower compared to polar bears. Also the metabolite composition varies between the species.

- Increased levels of bioavailable POPs and their metabolites during fasting/moulting period, in addition to the enhanced physiological stress associated with this period, strengthen the hypothesis that fasting/moulting period increases the animal’s susceptibility for adverse effects of contaminants in phocid seals.

- Moulting/fasting status was strongly related to thyroid, vitamin A and calcitriol homeostasis, and the concentrations of POPs and their OH-metabolites indicating that contaminant effect studies using biomarkers related to thyroid, vitamin A or calcitriol homeostasis may be confounded by the life history state of the animal. Vitamin E homeostasis was not influenced by moulting status, and it may thus be a robust biomarker through the moulting season in phocid seals.

- Alterations in thyroid and vitamin A homeostasis at multiple levels were associated with contaminant exposure, which indicates that ringed seals are susceptible to contaminant-mediated endocrine disruption. Positive relationships between circulating free and total thyroxine concentration ratios and OH-PCBs suggest that OH-PCBs may mediate the disruption of thyroid hormone transport in plasma in ringed seals.
Conclusions

- Thyroid hormone levels showed contrasting associations with contaminant concentrations in the more and less contaminated Baltic ringed seals suggesting that thyroid hormone levels has a bell-shaped response curve to contaminant exposure in ringed seals (Figure 4B).

- Ringed seals are suggested to compensate for POP-mediated effects (Figure 4A) between circulating hormone/vitamin concentrations and tissue mRNA expressions of receptors and/or activating enzymes. Disruption of thyroid transport in plasma was associated to increased levels of plasma THs and hepatic expressions of mRNA of thyroid hormone receptors and of thyroxine activating enzymes (DIO1). Decreased levels of plasma retinol in the contaminated seals were associated with increased retinoic acid receptor mRNA expressions in liver.

- Contaminant-related effects on thyroid and bone related biomarkers were different in ringed seals and grey seals. Bone lesions observed in Baltic grey seals could be mediated by disruption of thyroid hormone and vitamin D homeostasis by contaminants. Observed effects in Baltic ringed seals could not be related to any pathological effects.

- This study suggests that the health status of Baltic ringed seals has still improved during the last decade. PCB and DDE levels have decreased significantly over the last decade in the Baltic ringed seals, although the levels of hepatic PCBs, PBDEs and OCPs were still several times higher in the ringed seals from the Baltic Sea compared to those from Svalbard. In the Baltic seals, sampled mainly in 2006-07, decreased concentrations of hepatic vitamin A and circulating calcitriol were no longer observed in contrast to the seals sampled a decade earlier. However, the levels of contaminants in the Baltic ringed seals are still high enough to affect the health of the animals. Based on the results of this thesis, at the present level of the contaminant exposure the Baltic ringed seals seem to be at a zone where their body is able to compensate for the contaminant-mediated endocrine disruption (Figure 4A).
6. RECOMMENDATIONS

This thesis strengthens the importance to continue research on bioactive metabolites of contaminants. Species-specific metabolite formation/retention should be assessed in wildlife species, particularly among those that are likely to be exposed to high levels of contaminants. Due to their high toxic potential, circulating OH-metabolites should be included in monitoring and risk assessment programs. In ringed seals, most importantly OH-PCBs and PCP should be monitored of the OH-metabolites. In addition, the significance of metabolites in endocrine disruption should be investigated using in vitro experiments parallel to correlative free-ranging wildlife studies.

It is recommended that when planning free-ranging wildlife studies, risk assessment and monitoring programs, time window of sampling should be carefully planned. Moulting/fasting period is probably the most sensitive period to the effects of POPs in phocid seals. However, studying endocrine disruptive effects of contaminants during that period is challenging, because endogenous biomarkers may be largely confounded by physiological processes during different life history states. In order to study correlations between contaminant levels and biomarkers within a single population, a large enough sample size is needed within a short time period. These animals should be at similar life history state, and the range of contaminant concentrations should be wide enough. Sometimes logistical conditions make this kind of sampling impossible. Therefore a better possibility would be to compare contaminant-exposed populations to low-contaminated healthy reference populations sampled during a short period of time.

Choosing biomarkers for monitoring or assessing risk of contaminant effects is demanding. This study showed complex relationships between variables related to thyroid, vitamin A or calcitriol homeostasis, and contaminants, in ringed seals. It is highly recommended that when using variables related to endocrine homeostasis to investigate/monitor or assess the risk of contaminant effects in seals, life history status of the animal should be carefully controlled. This applies especially when using thyroid, vitamin A or calcitriol related parameters during moulting/fasting period. It should also be kept in mind that the endocrine parameters may have complex responses to contaminant exposure. Calcitriol and thyroid hormone homeostasis may be potential biomarkers for bone-related effects on grey seals.

Extrapolations between species for assessing risk for contaminant effects in wildlife should be avoided. The example of ringed and grey seals in the Baltic Sea shows that species living at the same environment approximately at the same trophic level respond in a very different way to contaminant exposure. Whether this is caused by species-specific biotransformation capacity of contaminants or other aspects of
physiology is not known. Possible differences in biotransformation capacity and other aspects of physiology between species should also be taken into consideration when studying other groups of animals.

Future research should also focus to a difficult but very important question: what is the ecological relevance of contaminant-mediated effects observed at the current levels of contaminant exposure in wildlife?
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