Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyroid hormone and vitamin A concentrations

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PREFACE AND ACKNOWLEDGEMENTS

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LIST OF PAPERS

The thesis is based on the following papers that will be referred to in the text by their Roman numerals:

Paper I Sørmo EG, Skaare JU, Lydersen C, Kovacs KM, Hammill MO, Jenssen BM (2003). Partitioning of persistent organic pollutants in grey seal (*Halichoerus grypus*) mother-pup pairs. *The Science of the Total Environment* 302:145-155.

Paper II Sørmo EG, Skaare JU, Jüssi I, Jüssi M, Jenssen BM (2003). Polychlorinated biphenyls and organochlorine pesticides in Baltic and Atlantic gray seal (*Halichoerus grypus*) pups. *Environmental Toxicology and Chemistry* 22: 2789-2799.

Paper III Sørmo EG, Jüssi I, Jüssi M, Braathen M, Skaare JU, Jenssen BM (2005). Thyroid hormone status in Baltic and Atlantic grey seal (*Halichoerus grypus*) pups in relation to polychlorinated biphenyls and organochlorine pesticides. *Environmental Toxicology and Chemistry* 24: 610-616.

Paper IV Jenssen BM, Haugen O, Sørmo EG, Skaare JU (2003). Negative relationship between PCBs and plasma retinol in low-contaminated free-ranging gray seal pups (*Halichoerus grypus*). *Environmental Research* 93: 79-87.

CONTENTS

BACKGROUND

Studies on laboratory animals suggest that developing and newborn mammals are more vulnerable than the adults with respect to the harmful effects of organochlorine (OC) pollutants such polychlorinated biphenyls (PCBs) and OC pesticides (Brouwer et al. 1995; 1998a; Colborn 2004). These effects include neurobiological and neurochemical effects, reproductive effects, reduced brain and circulatory thyroid hormone levels, impaired immune functions and vitamin A homeostasis (Brouwer et al. 1995; Colborn 2004; Safe et al. 1994; Tryphonas 1994). In humans, several of these negative effects of OCs have been reported in children of mothers with high pollutant burdens (Jacobson and Jacobson 1996, 1997; Koopman-Esseboom et al. 1994; Sandau et al. 2002; ten Tusscher et al 2003; Weisglas-Kuperus et al. 2000, 2004). Most organochlorines are highly lipophilic and resistant against degradation, and biomagnify as a function of the trophic level in food webs. Due to long food chains and the lipid richness of marine ecosystems, marine apex predators, such as seals, often accumulate very high burdens of these compounds (Aquilar et al. 2002), exemplifying the particular concern about toxic effects in these species. Studies of newborn phocid seals may be particularly interesting, because seals have lipid rich milk, resulting in the nursed grey seal pups being exposed to relatively high concentrations of OCs (Addison and Brodie 1977, 1987: Addison et al. 1999; Debier et al. 2003; Green et al. 1996; Pomeroy et al. 1996; Schweigert and Stobo 1994) during a period of their life when their endocrine and neural systems are still under development, and under the influence of the potentially disruptive properties of these pollutants (Hall et al. 1998; Simms et al. 2000b).

Particularly high concentrations of OCs have been found in seals from the Baltic Sea as compared to seals from the North-Atlantic Ocean (Jenssen 1996; Nyman et al. 2002), and it has been suggested that these high exposure levels severely affects the health of Baltic seals (Bergman 1999; Bergman et al. 2001; Helle et al. 1976a,b; Nyman et al. 2001, 2003; Olsson et al. 1994). Given that the developing mammal might be particularly susceptible to the effects of OCs, it is of special interest to examine possible OC related effects that newborn seals may suffer from. Results from such a study may also predict the effects of OCs on marine mammals in general and possibly on humans (Jenssen 2003).

Organochlorines

Due to their resistance against degradation in the environment, OCs like many other organic compounds are referred to as persistent organic pollutants (POPs). OCs belong to a group of predominately man-made pollutants, which are substances of many different uses and origins, including agricultural and technical uses, and have mainly been manufactured and used from the 1930s and onwards. Following evidence that emerged in the 1950s and 1960s of their occurrence and toxicity in non-target species including humans, their production and usage have been restricted or banned in many countries since the 1970s and 1980s. This effort to ban these chemicals has intensified recently through a global treaty signed in 2001 by 122 countries covering the phase-out of 12 POPs, referred to as the 'Dirty Dozen'. This treaty became legally binding from 2004 (UN Environment Programme, www.unep.org), and includes eight OC pesticides (i.e., dichlorodiphenyl-trichloroethane [DDT], hexachlorobenzene [HCB], toxaphene, chlordane, aldrin, mirex, dieldrin, pentachlorophenol and endrin), two industrial chemicals (i.e., polychlorinated biphenyls [PCBs] and hexabromobiphenyl) and two unwanted by-products of combustion and other industrial processes (i.e., polychlorinated dibenzo-*p*-dioxins and –furans [PCDD/Fs]) (Kaiser and Enserink 2000). Many of these compounds are dealt with in the present thesis.

Despite the reduction in the use of many of these OCs over the last two decades, their levels in biota have declined relatively slowly and high levels are still reported in marine top-

predators, possibly still increasing in concentrations in populations inhabiting the open Oceans (Aquilar et al. 2002). Taking into consideration that exposure to OCs will remain high in marine top-predators in the decades ahead, and that these animals may be exposed to a great variety of other anthropogenic stressors, such as over-fishing, climate change and exposure to new pollutants, it is still important to expand our knowledge on how OCs may affect wildlife, and subsequently biodiversity and ecosystem functioning. One group of new chemicals worth mentioning in this context is the brominated flame-retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs), hexabromocyclo-dodecane (HBCD), which are still used in large quantities and reported in steadily increasing concentrations in the environment (Watanabe and Sakai 2003).

Polychlorinated biphenyls (PCBs)

The PCBs have a general structure of a biphenyl molecule into which varying numbers of chlorine atoms are substituted. The physical properties of technical PCB mixtures range from oily liquids to waxy solids, with no smell or taste (Erickson 1997). PCBs were first synthesised in the 1880s but were not produced commercially until 1929 (Safe et al. 1994; Schecter et al. 1994); and are known by trade names such as Aroclor, Clophen, Phenoclor and many more (Erickson 1997; Safe 1994). Due to their non-flammability, chemical stability, high boiling point and electrical insulating properties, PCBs are/were used in hundreds of industrial and commercial applications including dielectric liquids in capacitors, cooling liquids in electrical transformers, as plasticisers in paints, plastics and rubber products, and as hydraulic fluids (Erickson 1997; U.S. Environmental Protection Agency, www.epa.gov; Safe 1994; Tanabe 1988). The environmental occurrence of PCBs were first reported in 1966 by Sören Jensen (Jensen 1969), who found high levels of PCBs when analysing environmental samples for DDT residues. Although the use of PCBs has been banned or restricted to closed systems in Western Europe and North America since the 1970s, they are still released into aquatic systems by leaking and evaporation from, or dumping and burning of PCB containing products (Muir et al. 2000; Tanabe et al. 1988). Of the more than 1.2 million tonnes of PCBs that have been manufactured worldwide prior to the cessation of production in 1984, one third has so far been released into the environment (Aquilar et al. 2002).

The mechanism of toxicity is particularly known for some PCB congeners, which make up a very small proportion of the total quantity of the PCBs usually detected in biological samples. These congeners, which are highly toxic even in extremely low concentrations, belong to the group of *non*-ortho (e.g., PCB-77, -126, -169) and *mono*-ortho (e.g., PCB-105, -118, -156, -157) PCBs that acquire a planar configuration to elicit their dioxin-like toxicity through the nuclear aryl hydrocarbon receptor (AhR) (Ahlborg et al. 1992; Safe 1994). The binding of PCBs to the receptor result in the changed expressions of AhR-dependent genes in a large variety of cells causing a wide range of toxic actions, including acute lethality, body weight loss, dermal toxicity, thymic atrophy, immunosuppression and increased susceptibility to infections, reproductive and developmental toxicity, endocrine disruption, neurotoxicity, carcinogenesis, heptomegaly, fatty liver and porphyria (see reviews by Brouwer et al. 1995, 1998b; Safe 1994; Tryphonas 1994). Less toxicological knowledge is available for the non-planar PCB congeners, although they are the most abundant occurring congeners in environmental samples. The toxic effects of these PCB congeners (e.g., PCB 153) have, however, been related to neurotoxic and behavioural effects, carcinogenesis, developmental effect and endocrine disruption (Brouwer et al. 1995; Holene et al. 1998, 1999; Mariussen et al. 2002). Hydroxy- and methylsulfonyl PCB metabolites formed by the actions of detoxifying enzymes have been shown to exert a variety of toxic effects, including reduced plasma vitamin A and thyroid hormone levels, altered adrenal function, and to cause hepatic and developmental toxicity (Brouwer et al. 1998a).

The developing mammal may be exposed to PCBs, both through transplacental transfer and via the milk. Studies in humans and experimental animals suggest that the developing animal is more vulnerable than the adult is with respect to the harmful effects of PCBs (see reviews by Brouwer et al. 1995, 1998a). Women with a high intake of PCB contaminated marine food have been shown to give birth to children with impaired responses in behavior, impaired motor skills and decreased short-term memory. The alterations have been found to persist in these children for several years during their early life (Jacobson and Jacobson 1996, 1997). Impairments of thyroid hormone- and the immune system have been reported in children of mothers of high PCB body burdens (Koopman-Esseboom et al. 1994; Osius et al. 1999; Sandau et al. 2002; ten Tusscher et al 2003). Noteworthy in this context are reports of up to 10-fold higher cord blood concentrations of PCBs in delivering women of communities with a traditionally high intake of highly contaminated and fatty marine foodstuffs, (e.g., Inuit's and Indians, fisherman communities) as compared to the general population (Bjerregaard et al. 1998). It should be stressed that for the general human population in the industrialised world the benefits of breast-feeding are considered to outweigh any risks from exposure to PCBs (and other OCs) in the mother's milk (Brouwer et al. 1998a). However, due to the possibility of the negative effects that PCBs may have on the developing child, both children and women of reproductive age are often advised to limit their intake of marine and inland lake food items with a high OC content (e.g., eggs from seabirds, fatty fish meat and blubber from marine mammals) (Dewailly et al. 1992; Kamrin and Fischer 1999; The Norwegian Food Safety Authority, http:\snt.mattilsynet.no\).

Dichlorodiphenyltrichloroethane (DDT)

DDT is the abbreviation for an obsolete chemical name (4,4'-dichloro-diphenyltrichloroethane), whereas the current name is 1,1,1-trichloro-2,2(4chlorophenyl)-ethane. DDT was first synthesised in 1874 and is a white crystalline solid with no smell or taste. Its insecticide property was discovered in 1939, and the Swiss chemist Paul Müller received the Noble prize in Physiology and Medicine in 1948 for this discovery (U.S. Environmental Protection Agency, www.epa.gov). DDT was therefore in early use and extensively used to control disease-carrying insects (e.g., malaria mosquito), and commercialised as an agricultural pesticide back in 1945. In the 1950s the environmental disadvantages of DDT became known, including their biomagnification in food chains, and its ability to cause eggshell thinning and to impair the reproduction of many raptor and fish-eating birds (Carson 1962; Lundholm et al. 1997; Peterle 1991; Ratcliffe 1967); leading to the cancellation of its agricultural use in the US and most European countries in the early 1970s (U.S. Environmental Protection Agency, www.epa.gov). However, DDT is still used by many tropical countries for vector control, and due to this the human intake of DDTs in some communities in developing countries is up to 100 times greater than in the more industrialised parts of the world (Turusov et al. 2003). Such high maternal body burdens of DDTs have been associated with an increased risk of delivery of preterm and small-for-gestational-age babies at birth (Longnecker et al. 2001; Torres-Arreola et al. 2003). Similar to reports on PCBs, high concentrations of DDT have been reported in communities (e.g., Inuit's) that consume large amounts of fatty foodstuffs of marine origin (Dallaire et al. 2003).

Most of the DDT in the environment exists as *p,p'*-DDE and to a lesser extent other metabolites such as *o,p'*-DDE, *p,p'*-DDD and *o,p'*-DDD. In particular, DDE is extremely persistent against degradation in soils and sediments and has a long half-life of several years in most organisms (Parkinson 1996). A number of reports indicate that DDT and many of the metabolites induce endocrine disruption, including effects on thyroid and adrenal functions (Jefferies 1969; Longnecker et al. 1997; Scollon et al. 2004). High levels of DDTs in lakes

have been shown to cause reproductive failure in fish and there is experimental and human evidence of carcinogenicity of DDTs (Turusov et al. 2003).

Chlordane

Chlordane was used as an insect pesticide, mainly in the USA, and it is estimated that more than 70.000 tonnes of technical chlordane has been produced from 1946 to 1983 when most use was banned (Dearth and Hites 1991; U.S. Environmental Protection Agency, www.epa.gov). In Norway as in most other European countries, chlordane has not been used since the late 1960s and early 1970s (De March et al. 1998). The different chlordane mixtures may consist of more than 140 different components; among the most important are *cis*-chlordane, *trans*-chlordane, heptachlor and trans-nonachlor, where the latter is particularly persistent in the biota (Bondy et al. 2003; Dearth and Hites 1991). Toxic effects of high-level chlordane exposure predominately relates to neurotoxic alterations (U.S. Environmental Protection Agency, www.epa.gov). Health effects of chronic exposure to low levels in the environment are poorly understood (De March et al. 1998). In higher vertebrates, the metabolite oxychlordane is found in significant concentrations, and found to be more bioaccumulative and toxic than the parent compounds (Bondy et al. 2003). Noteworthy, in glaucous gulls (*Larus hyperboreus*) from Svalbard, oxychlordane together with HCB were found to be the most prominent OCs to alter thyroid hormone levels (Verreault et al. 2004), suggesting a potential endocrine disruptive effect of this compound in wildlife species.

Hexachlorohexanes (HCHs)

HCHs are used worldwide in insecticides, and technical HCH consists of eight different chemical forms or isomers. The three most common isomers are α-, β-, and γ-isomers, constituting 55-80%, 8-14% and 8-15%, respectively, of the total of various technical HCH mixtures. However, only γ-HCH is effective against insects, and has therefore been refined and produced with the common name Lindane, and has a long record of use as a pesticide in the USA and Western Europe (De March et al. 1998). Technical HCH, consisting of minor amounts of γ-HCH, was in the past used in large quantities as an insecticide in the former Soviet Union, Poland, Romania, India, China, Mexico and Brazil. However, since both α-and β-HCH degrades relatively slowly and have low insecticide properties, they are now banned in most of the industrialised world (Voldner and Li 1995; Willett et al. 1998). The global usage of Lindane and technical HCH is estimated to be 720 000 and 550 000 tonnes, respectively, between 1950 and 1993 (Voldner and Li 1995). HCHs are characterised as relatively volatile and capable of long-range transport. Furthermore, because of their relatively low lipophilicity and short half-lives in the biota, HCHs are less bioaccumulative than most of the other OCs dealt with in this thesis (De March et al. 1998). HCH primarily affects the central nervous system; however, the toxicological mechanisms behind the neurotoxic actions are unknown. Little is known concerning the possible endocrine disruptive potential of HCH, but β-HCH has been suggested to be an environmental oestrogen (Willett et al. 1998).

Hexachlorobenzene (HCB)

HCB was particularly used as a fungicide in the 1960s for the treatment of seed grains. The present environmental presence is, however, mainly due to HCB being formed as a by-product in a large variety of combustion processes. HCB is also formed as an unwanted by-product in the production of various other OCs such as pentachlorophenol, chlorinated solvents (e.g., perchloroethylene and carbon tetrachloride) and chlorine containing pesticides (De March et al. 1998). Once in the environment, HCB breaks down very slowly with a half-life of 3-6 years in sediments. HCB is transformed by detoxifying enzymes in higher

organisms to other toxic substances; of which pentachlorophenol is probably the most important (van Raaij et al. 1993a). Concerning its toxicity, HCB like some PCBs elicit dioxin toxicity through the Ah-receptor (van Birgelen 1998) Furthermore, concerning endocrine disruptive potentials, HCB and the metabolite pentachlorophenol reduces plasma thyroid hormone concentrations in laboratory animals (e.g., rodents) by many of the same mechanisms as the PCBs (De March et al. 1998; van Raaij et al. 1993a,b). As described above in glaucous gulls from Svalbard, HCB was found to be one the most prominent OCs to alter thyroid hormone levels (Verreault et al. 2004), suggesting a potential endocrine disruptive effect of this compound in wildlife species.

Mirex

Mirex (1,1a,2,2,3,3a,4,5,5,5a,5b,6-dodecachlorooctahydro-1,3,4-metheno-1H-cyclobutal[*cd*]pentalene) was introduced as an insecticide in 1959, and used to control fire ants, primarily in the USA and Canada until it was banned in 1978 (De March et al. 1998; Jemal and Hugh-Jones 1993; International Agency for Research on Cancer, www.iarc.fr). Mirex is a white, odourless non-flammable crystallised solid, and does not evaporate to any great extent from water or soil, and due to its high lipophilicity it does not dissolve easily in water but adders to particulate matter. Mirex is persistent in the environment with a half-life reported to be up to five to ten years in soil and sediments (De March et al. 1998). Mirex can build up in fish and other organisms that live in contaminated waters, and its presence in the Lake Ontario food web has been well documented (Makarewicz et al. 2003). Information on the toxic effects is limited, but it has been classified as a possible human carcinogen (De March et al.1998).

The grey seal

The grey seal female (*Halichoerus grypus*) reaches sexual maturity at 3-5 years and males at 4-6 years. Grey seals are social and congregate during breeding, moulting and at haul-out sites. The grey seal is polygamous and the male is substantially larger than the female. Reproduction is similar to that of other phocids, with a delayed implantation and post-implantation gestation of approximately nine months, with the female giving birth to one pup annually. Mating occurs in the breeding colony during the end of the lactation period (Bonner 1981). The grey seal is an opportunistic feeder and prey on a variety of species, predominately on fish, with crustaceans included in the diet (Bowen and Harrison 1994; Bowen et al. 1993; Hammond et al. 1994; Mikkelsen et al 2002; Thompson et al. 1996). Like most other phocids, the grey seal undergo extensive seasonal changes in their fat reserves because they fast during breeding and moulting seasons, and gain weight during the intervening seasons (Beck et al. 2000).

The grey seal pups are nursed for approximately 2-3 weeks with milk that is highly nutritional and lipid-rich (up to 60% fat) (Lydersen et al. 1995). During this period the body mass of the mother grey seal is reduced by 65% and as much as 57% of the energy of the mobilised lipids is made available to the pup via the milk (Iverson et al. 1993; Lydersen et al. 1995; Mellish et al. 1999). The pup is born with almost no subcutaneous lipid but assimilates about 75% of the milk derived lipids into its blubber, allowing the body mass of the pup to increase three- to four folds during the short nursing period (Lydersen et al. 1995). The growth of the pup thus depends on the nutritional condition and output of milk energy from the mother seal (Iverson et al. 1993; Mellish et al. 1999). Following weaning, the pup is abandoned by the mother and may stay ashore or on the sea-ice for several days or even weeks, losing on average 0.5 kg of body mass daily during the post-weaning fast before it eventually seeks the water and starts feeding on its own (Bonner 1981).

The grey seal is endemic to the northern Atlantic Ocean, and there are several populations that are separated geographically. These populations are also distinguished by differences in body size and breeding and moulting seasons. Three distinct populations are recognised: the Baltic, the East-Atlantic (e.g., Norwegian and British) and the West-Atlantic (US and Canadian) stocks. A study of geographical distribution of mitochondrial DNA has revealed that there are significant genetic variations between these stocks (Boskovic et al. 1996).

The population size in the Western Atlantic is estimated to constitute more than 190,000 animals, occurring mainly around the Scotian Shelf and the Gulf of St. Lawrence (Lesage and Hammill 2001). This population breeds during January and February, both on sea ice (e.g., Gulf of St. Lawrence, Canada) and on land (e.g., Sable Island, Canada). The East Atlantic population is found along the shores and waters of Iceland, The British Isles, The Netherlands, Norway and the Kola Peninsula of Russia (Bonner 1981; Haug et al. 1994; Reijnders et al. 1995), with the breeding season ranging from September to December depending on the breeding sites. The population in the British waters consists of more 130,000 animals (Matthiopoulos et al. 2004), whereas the Norwegian and Russian populations consist of about 5,000 seals each (Ekker et al. 1995; Haug et al. 1994). The Baltic population, which was thought to be around 100,000 individuals at the beginning of 1900s, was estimated to be only around 2,000 individuals in the 1970s, mainly due to hunting and possible reproductive impairments caused by high exposure to pollutants such as PCBs and DDTs (Harding and Harkonen 1999). This drastic decline has been followed by a recent recovery in the numbers to at least 7,000-8,000 seals counted at the present (Mart and Ivar Jüssi, personal communication).

Distribution and fate of OCs in the marine environment

Due to their physical and chemical properties, organochlorines are globally distributed even to the most remote and pristine regions of the earth (Mackay et al. 1992; Muir et al. 2000). This is particularly the case for the more volatile and/or water soluble compounds (e.g., HCH and low-chlorinated PCB congeners), which are readily transported in the atmosphere as gasses or in aerosols. This enables these compounds to be deposited through precipitation and condensation at locations far away from their application. The lower vapour pressures and water-solubility of more chlorinated OCs (e.g., high-chlorinated PCBs) causes these compounds, to a greater extent, to be partitioned away from air and water into particulate matter. Thus in aquatic environments, the highest concentrations of these particle-bound compounds are typically found in the particulate matter and sediments of lakes, rivers, estuaries, coastal and semi-closed seawaters in close vicinity to industrial and agricultural application and spillage/run-off (Aguilar et al. 2002; Muir et al. 2000).

OCs such as high-chlorinated PCBs, are under most environmental conditions highly persistent against degradation by abiotic and biotic factors. This persistence against degradation of OCs is highly associated to the degree of chlorination of the compound. For instance, although photolysis is a significant chemical degradation process of many organic compounds in the environment, the degradation by sunlight is substantially reduced with the increasing chlorination of the substances (Erickson 1997). Likewise, although organic compounds are biodegraded by a large variety of micro-organisms by both aerobic and anaerobic processes, the biotic degradation seems to be very slow for the high-chlorinated organic compounds (Erickson 1997; Maldonado and Bayona 2002; Wiegel and Wu 2000). Moreover, although OCs may be subject to enzymatic transformation and excretion in higher organisms (Boon et al. 1997), many of the high-chlorinated compounds have extremely long half-life times in most organisms. For example, the half-life of PCB-153 (the most abundant

PCB congener in most biological samples) has been found to be as long as ten years in eels (de Boer et al. 1994).

Lipophilic OCs are predominately accumulated in the marine organisms via food intake, but different to the nutrients in the food which are utilised to cover energetic demands of the organisms, the dietary derived OCs are persistent against degradation and accumulate in the organisms. Thus, provided that the dietary intake of these OCs is greater than the excretory loss, the body burdens of these compounds will increase with the lifespan of the organism (Bernhoft et al. 1997; Bernt et al. 1999; Ross et al. 2000). Persistent and thus bioaccumulative OCs are also subject to the process of biomagnification, which is the increase of contaminants as a function of trophic level in the food chain (Kelly et al. 2004; Ruus et al. 2002), leading to a high body burden in marine apex predators such as fish-eating marine mammals and seabirds (Tanabe 1988). The substantially higher body burdens of OCs typically reported in marine mammals and seabirds as compared to many of their prey species of invertebrates and fish are, however, also explained largely by these species being homeotherms, which means they have a greater energy demand and dietary intake as compared to their predominately poikilothermic prey-species of lower energy demands (Hop et al. 2002).

The main mechanism of detoxification and excretion of OCs involves a two-phased biotransformation and metabolic pathway. Phase I is catalyzed by a large variety of enzymes, including the cytochrome p450 family. One of the main phase I reactions involves an oxidative reaction, where oxygen is introduced into the lipophilic substrate of the OC creating a functional group (-OH) for attachment of larger molecules of endogenous origin by phase II reactions. In this way, a lipophilic OC is transformed into a more polar and water-soluble end product that can be excreted through bile and urine (Iyer and Sinz 1999: Parkinson 1996). Phase II enzymes are generally transferases, and glutathione-S-transferase, UDP-glycosyl transferases, sulfotransferases and methyltransferases are considered the major enzymes (Visser et al. 1994).

Noteworthy, the oxidative step of phase I enzymes may also lead to the formation of reactive intermediates (e.g. OH-PCB metabolites) with mutagenic, carcinogenic or other toxic properties (Shimada et al 1996; Sandau et al. 2000, 2002). Moreover, actions of phase II enzymes might form toxic and persistent metabolites, such as methyl sulfone ($MeSO_2$) PCB and DDE metabolites (Bergman et al. 1994). Dechlorination is also an important reaction in the biotransformation and/or metabolism of OCs. For example, the metabolite DDE is a result of enzymatic dechlorination of the parent DDT by the activity of the enzyme DDT-dehydrochlorinase (Parkinson 1996). Other degradation by-products of DDT such as the toxic metabolites DDD and DDA are formed by a series of reductive dechlorination and oxidative reactions. Both DDE and DDD remain highly lipophilic and therefore persistent and bioaccumulative in most species. Other important toxic OC metabolites are oxychlordane and pentachlorophenol derived from the metabolism of chlordanes and HCB, respectively (De March et al. 1998). The detoxifying apparatus are therefore not only responsible for detoxification, but also for the formation of metabolites more toxic (and in some cases more persistent) than the original or parent compounds.

The detoxifying capacity varies among the different species, with lower capacities typically observed in invertebrates and fish as compared to mammals and birds (Borgå et al. 2004: Boon et al. 1987; Ruus et al. 2002). Low chlorinated OC compounds are generally more readily metabolised and excreted than the higher chlorinated compounds. Also, the substitution pattern of chlorines on the OC compounds affects their resistance against metabolism (Boon et al. 1987, 1997), causing the bioaccumulation and biomagnification potential to vary substantially among the different OCs. Noteworthy, marine mammals and seabirds typically express lower detoxifying capabilities as compared to terrestrial

counterparts, further exemplifying the particular concern of bioaccumulation of OCs in marine mammals (Tanabe et al. 1988). The metabolism of OCs is often enhanced in a concentration-dependent manner by the increased induction of detoxifying enzyme activities with increasing contaminant exposures (Boon et al. 1997). This may cause the more metabolisable OCs to be depleted at a higher rate in highly contaminated populations as compared to lightly contaminated populations. The formation and presence of toxic metabolites might therefore be greater in the more contaminated populations. In addition to excretion and loss of OCs through enzymatic induced metabolism, substantial amounts of the body burden of these compounds may be lost through generational transfer, exemplified by placental and lactational transfer of OCs in mammals. This explains why reproductively active female marine mammals often have lower body burdens of OCs as compared to males and/or non-reproductively active females (Bernhoft et al. 1997; Bernt et al. 1999; Ross et al. 2000).

Tissue partitioning and concentration of OCs

Knowledge about the variability in the partitioning/distribution of OCs in different tissues, in response to different biological circumstances, is important for standardisation and sampling procedures in studies aiming to assess trends in exposure levels and possible toxic effects. Due to their lipophilicity OCs tend to dissolve into the lipids of the organism, causing wet weigh concentrations of these lipophilic compounds to vary among the different tissues, with higher concentrations in lipid-rich compared to lean tissues. Since the blubber represents the bulk amount of lipids in marine mammals, almost all their body burdens of OCs are stored in this tissue (Addison and Stobo 1993). Lipid weight based concentrations of OCs in lipid-rich and lean tissues, however, often yields fairly comparable concentrations, suggesting an equilibrium partitioning of lipophilic compounds between the different body lipid compartments (Bernhoft et al. 1997; Matthews and Dedrick 1984). Importantly, changes in the lipid content or nutritional condition of the organism may severely affect the body lipid concentrations of OCs. Thus, during periods of little or no dietary intake when lipid stores are utilised to cover energetic demands, OCs typically concentrate in the diminishing lipid stores. In contrast, during periods of intensive foraging (preferably when digesting a diet of low contaminant levels), the lipid build-up typically dilutes the concentrations of OCs (Kleivane et al. 2004). This variability in lipid content of the organism may have toxic implications, because higher concentrations in body lipids causes higher concentrations of these compounds in blood and subsequently in target organs of toxic damage (Kleivane et al. 2004; Lydersen et al. 2002).

Equilibrium of OCs between the different body lipids depends on the compounds that can readily and rapidly exchange between the different body compartments. Thus, if storage lipids are utilised and OC concentrations increase in this compartment, similar changes in concentrations are anticipated in the circulatory lipids and lipids elsewhere in the body. Likewise, when milk lipids are produced in the mammary gland, the concentrations of OCs in the milk lipids is anticipated to be comparable to those of lipids elsewhere in the body of the mother seal. However, in lactating grey seals substantially lower concentrations (more than 10 folds) of high-chlorinated PCBs have been reported in the milk as compared to the blubber, whereas low-chlorinated PCBs show fairly comparable concentrations between these two compartments (Green et al. 1996). The lower concentrations of the high-chlorinated compounds in the milk probably relates to a limited ability of such highly hydrophobic compounds to mobilise from the maternal blubber into the aqueous environments of the blood, and subsequently through the aqueous layers of the mammary gland. Such low mobility of OCs may also explain the observations of stratification of these compounds in different concentrations in the blubber layers of marine mammals (Aguilar and Borrell 1991; Debier et

al. 2003; Severisen et al. 2000). This stratification may be because the blubber of marine mammals is not a homogenous tissue, as the outer layers serves the purpose of thermal insulation, whereas the inner layers are the more metabolically active in term of lipid mobilisation and deposition (Koopman et al. 1996). Thus, extensive foraging and lipid build-up may result in lipids derived from the diet diluting the OCs in the inner layers, whereas the concentrations in the more metabolically static outer layers remain fairly unaffected (Severinsen et al. 2000). In contrast, rapid utilisation of the inner blubber lipids may concentrate the OCs in the inner but not the outer layers of the blubber. OC concentrations also increase in the blood following dramatic weight loss (Chevrier et al. 2000; Lydersen et al. 2002), exemplified by captive and wild fasting harp seals (*Phoca groenlandica*) utilising vast amounts of their lipids showing a many time and significant time-dependent increase in blood concentrations of high-lipophilic OCs (e.g., DDTs and most PCBs). Corresponding biopsy concentrations obtained from the metabolically static outer blubber layer of these seals remained, however, fairly unaffected by the weight loss (Kleivane et al. 2004). This discrepancy between the tissues may be of particular importance to the present study since the lactating grey seal utilises vast amounts of her body lipids to cover the energetic demands of the lactation process (Lydersen et al. 1995). Thus, as knowledge of the transfer of the different OCs and how they are partitioning in the maternal tissues in response to the lactation process in phocid seals is poorly understood, this issue will be dealt with in the present thesis (Paper I).

Toxic effects of organochlorines in marine mammals

Toxicological effects of organochlorines and of PCBs in particular, have been observed and/or suspected in several species of marine mammals. A high frequency of complex pathological disorders and reproductive impairments were described in Baltic grey and ringed seals (*Phoca hispida*) during the 1960s and 1970s, when these seals were exposed to particularly high burdens of PCBs and DDTs (Bergman and Olsson 1986; Bergman et al. 1999; Helle et al. 1976a,b; Olsson et al. 1994). These pathological changes now seem to be less frequent, probably because the concentrations of these pollutants have decreased in the Baltic seals over the last two decades (Bergman et al. 1999). The toxic mechanisms linking the pathological disorders reported in the Baltic seals to their body burdens of OCs are not clear. However, new information on toxic effects in laboratory and in free-ranging mammals may elucidate this linkage. For instance, several biochemical parameters including vitamin A and E have been shown to differ in grey- and ringed seals from the Baltic Sea as compared to their less contaminated counterparts in the waters of the Atlantic Ocean (Nyman et al. 2003). Furthermore, PCBs and DDTs have been shown to affect seal reproduction, immune, endocrine (thyroid and sex-steroids) and vitamin A systems in several experimental studies conducted on captive harbour seals (*Phoca vitulina*) fed fish from contaminated waters such as the Baltic Sea and Dutch parts of the Wadden Sea (Brouwer et al. 1989; De Swart et al. 1996; Reijnders 1986). Other linkages to possible endocrine disruptive properties of these compounds include the observation of negative correlations between plasma thyroid hormone concentrations and concentrations of PCBs in Larga- (*Phoca larga*) and ribbon seals (*Phoca fasicata*) from Japanese waters (Chiba et al. 2001). Furthermore, decreased progesterone- and increased testosterone metabolism with increasing liver concentrations of PCBs have been reported in the harbour seal (Troisi and Mason 2000).

The potential toxic effects of OCs have been reported in species of marine mammals other than seals. High body burdens of PCBs have been associated with population declines in the European otter (*Lutra lutra*), including populations from the Baltic Sea (Roos et al. 2001; Sjöasen et al. 1997). Negative correlations between concentrations of plasma vitamin A (retinol) and PCBs have been reported in free-ranging European otters (Murk et al. 1998). In

harbour porpoises (*Phocoena phocoena*) from British waters, high concentrations of PCBs have been associated with increased mortality due to infectious diseases (Jepson et al. 1999). Furthermore, PCBs and DDE has been shown to decrease plasma testosterone levels in Dall's porpoises (*Phocoenoides dalli*) of the North Pacific (Subramanian et al. 1987). In polar bears (*Ursus maritimus*), high concentrations of PCBs have been shown to impair various immunological parameters, and to affect vitamin A, thyroid- , cortisol and sex hormone homeostasis (Braathen et al. 2004; Haave et al. 2003; Lie et al. 2004; Oskam et al. 2004; Skaare et al. 2002).

Given that studies on laboratory animals have shown that the developing and/or newborn mammal might be more vulnerable than the adult with respect to the effects of OCs (Brouwer et al. 1995, 1998a), studies of their effect on newborn seal pups exposed to very high levels of environmental occurring OCs may be particularly relevant. Biomarkers are biochemical, physiological or histopathological indicators of exposure to and/or effects from anthropogenic substances, in the present thesis thyroid hormone and vitamin A levels were chosen as biomarkers (Paper III: IV) as these parameters have been shown to be susceptible to exposure to OCs in a wide range of experimental model-species (Brouwer et al. 1998b; Rolland 2000), and are critical parameters in the development of the neonate mammal (see below).

Thyroid hormones

The thyroid gland is located in the lower part of the neck wrapped around the front of the trachea, and secretes the two thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3) (Fig. 1). The thyroid gland predominately secretes T4, whereas the biological active hormone, T3, is predominately produced in the tissues by outer ring deiodinase enzymes (type I and II deiodinase) that convert T4 to T3. Following the binding to the TH receptor, the THs (predominately T3) exert their action at the nuclear level by regulating the transcription of TH-responsive genes (McNabb 1992). In homeothermic animals THs are mainly involved in increasing the rate of glucose oxidation and additionally increasing the metabolic heat produced and thus maintaining normal body temperature (McNabb 1992). Other important functions of THs are the regulation of growth and differentiation of tissues, including the regulation of cell proliferation processes, cell migration and differentiation of the developing animal (Zoeller et al. 2002). Thyroid hormones are essential for neuron formation, synapse development, formation of myelin, and the migration of neurons to their proper places in the brain (Brown 2003). This development starts during the early stages of gestation, in humans within the first weeks and months peaking in the child's first of year of life. Thus, the developing organism is particularly susceptible to any deficiency of THs (i.e., hypothyroidism) during gestation and the early stages of postnatal life. This is exemplified in children born with low levels of THs who typically express reduced cognitive abilities, difficulties with motor co-ordination, balance, and other psychomotor skills (Brown 2003; McNabb 1992;). Even small changes in TH availability during critical periods of brain development may have harmful results (Colborn 2004). The World Health Organization (WHO), reports that iodine deficiency and subsequent hypothyroidism is the most common cause of preventable mental retardation in the world (Brown 2003).

The activity and secretion of the thyroid gland is regulated by the homeostatic control of the pituitary glycoprotein hormone known as thyroid-stimulating hormone (TSH) (Fig. 1), exemplified when TSH secretion is strongly suppressed by T3 and up-regulated in the absence of T3 (Shupnik et al. 1986) (Fig. 1). TSH stimulates the thyroid gland to capture and store more iodine from the blood and to synthesise, store and release THs (McNabb 1992). Circulatory T4 has been suggested to regulate circulatory levels of T3 because increased plasma T4 concentrations increases the conversion of T4 to T3 in the tissues (Wade et al.

2002). However, a severe depletion of plasma T4 concentrations in PCB exposed rats was not found to effect plasma and tissue concentrations of T3 (Morse et al. 1996), indicating that regulatory mechanisms largely independent of the thyroid gland may be involved in the tissue-homeostasis of T3 (McNabb 1992; Morse et al. 1996).

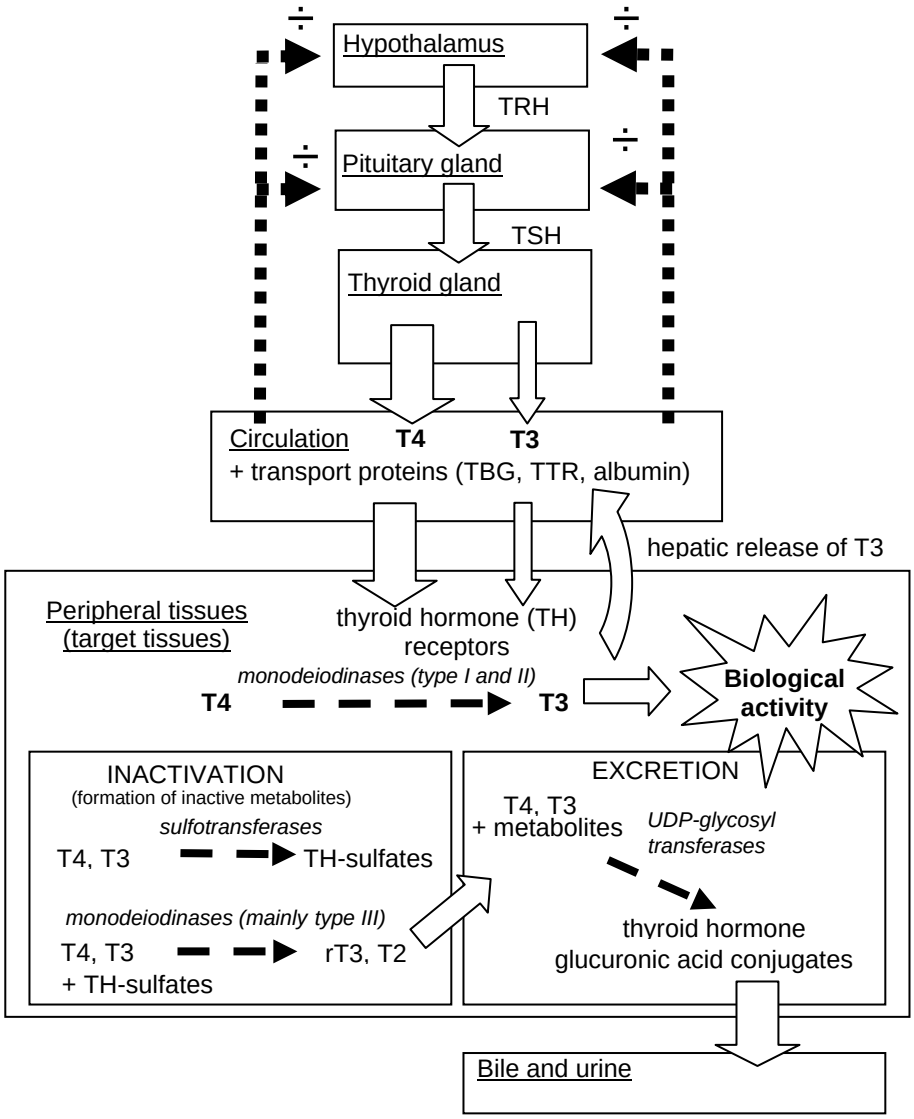

Fig 1. Regulation of thyroid hormones in mammals (see text for detailed explanations).

The regulation of THs also involves various inactivation pathways, including inner ring deiodinase enzymes (type III), which convert T4 to the inactive metabolite reverse T3 (Fig 1). Thus, the bioactivation of T4 is controlled by competing deiodinase pathways; that is, type I and II enzymes that convert T4 to the bioactive form T3, or type III enzymes that converts both T4 and T3 to inactive products such as reverse T3 and T2 (McNabb 1992; Visser 1994). Glucuronidation and sulfation of the THs phenolic hydroxy group are other important mechanisms in the hormone regulation (Fig. 1). TH glucuronides are readily excreted in the bile. Sulfation of THs by sulfotransferases on the other hand facilitates the rapid deiodinase of THs in the liver, causing little of these conjugates to be excreted intact in the bile or appearing in the plasma (Visser 1994). However, high plasma concentrations of TH sulfates are present during the fetal and early stages of postnatal life in mammals, and it

has been suggested that enzymatic desulfation of these conjugate producing T3 may be important in the regulation of TH homeostasis in these early developmental stages in mammals (Chopra et al. 1992; Kester et al. 2002). Since many OCs, and especially the PCBs, have been found to be strong inhibitors of sulfotransferases (Brouwer et al. 1998b), this might be of particular relevance to the present study of TH levels in relation to OCs in neonatal grey seals (Paper III).

Most T4 and T3 in the blood are bound to proteins. In humans, only 0.5% of T4 and 0.3% of T3 is free in plasma. The rest is bound to three transporting proteins: thyroxine-binding globulin (TBG), trans-thyretin (TTR) and albumin. TBG is the least abundant in human plasma, but carries about 75-80% of the THs because TBG has the highest affinity for T4 and T3. Its affinity to T3 is about 1/10 that for T4, causing T3 to be more readily lost to the peripheral tissues than T4. Other mammals (e.g., rodents), reptiles and birds lack TBG, resulting in albumin and TTR as the main TH carriers in these species (Withers 1992). To my knowledge there is no information about which transport proteins are involved in the circulatory transport of THs in seals. Moreover, 'the free hormone concept' suggests that the free TH fractions (i.e., free T4 [FT4] and free T3 [FT3]) determine the availability of hormones for entry into the tissues and thus are the physiologically relevant indicators of TH activity (McNabb 1992).

Concerning THs in seals, seasonal variations in plasma total T4 (TT4) and total T3 (TT3), and free T3 (FT3) but not free T4 (FT4) concentrations have been found in adult harbour seals, as higher concentrations were observed during winter as compared to summer (Oki and Atkinson 2004). This suggests that environmental temperature is one factor that can alter the thyroid activity of seals, and that THs are important for seals in order to efficiently adapt to colder environments. As in many other neonate mammals, newborn seal pups have higher plasma TH concentrations as compared to their mothers, with TSH and T4 peaking in particularly high concentrations during the first 1-3 days following birth. Following this peak in plasma T4 at birth, TH concentrations decline and remain fairly unchanged during the following weeks of the neonate life of phocids (Haulena et al. 1998; Ortiz et al. 2003; Stokkan et al. 1995; Woldstad and Jenssen 1999), with the possible exception of FT3 that has been found to decrease somewhat during the first weeks following birth in harbour seal pups (Haulena et al. 1998). The peak in T4 at birth probably serves the function of initiating thermoregulatory responses in precocial species, possible initiated by the cooling of the newborn and/or cessation of the umbilical cord (McNabb 1992).

Organochlorines and plasma thyroid hormones

Controlled exposure studies of laboratory animals have shown that OCs and particularly PCBs (including their metabolites) may affect TH function through several mechanisms (Brouwer et al. 1998b; Gould et al. 1999; Hood and Klaassen 2000; Iwasaki et al. 2002; Schuur et al. 1998a,b; van Raaij et al. 1993a,b). They can interfere with hepatic type I deiodinase activity, reducing the conversion of T4 to the main active hormone T3; induce increased TH glucuronidation, which causes increased biliary excretion of THs as glucuronic acid conjugates; interfere with sulfotransferases, which are important enzymes in the metabolism of THs; alter plasma concentrations of TSH, which is important to regulate production and release of THs from the thyroid gland; they can directly affect the thyroid gland epithelium structure; or interfere with the binding of THs to the TH receptor, thereby interfering with the gene expression normally induced by the THs. Furthermore, due to structural similarities, T4 and some PCB congeners and/or hydroxylated PCB-metabolites (OH-PCBs) compete for binding sites on trans-thyretin (TTR), and thereby reduces plasma levels of T4 (Brouwer et al. 1998b). Also, methyl sulfone ($MeSO_2$) PCB metabolites,

including those detected in human milk, liver and adipose tissues have been found to reduce plasma TH concentrations in rat studies (Kato et al. 1998).

Although it has been shown that PCBs and other OCs induce alterations in plasma TH concentrations in wildlife, experimental and field studies, these studies have produced somewhat divergent findings (Rolland 2000). For instance, in most experimental animals (e.g., rodents) pronounced decreases in plasma T4 concentrations are the most frequently reported endpoint following PCB exposure, whereas plasma T3 concentrations often show more modest decreases or no alterations (Brouwer et al. 1998b; van der Plas et al. 2001). In contrast, in experimental studies of carnivorous animals such as the mink (*Mustela vison*), increased plasma T4 and decreased T3 concentrations has been reported following PCB exposure (Heaton et al. 1995; Käkelä et al. 1999, 2003; Nieminen et al. 2000). Likewise, in free-ranging otter's plasma T4 concentrations increased with increasing concentrations of dioxin-like PCB congeners (Murk et al. 1998). Furthermore, negative correlations between plasma T3 concentrations and concentrations of PCBs have been reported in seals from Japanese waters, whereas no effect was found on plasma T4 concentrations (Chiba et al. 2001). Moreover, PCBs were found to affect plasma T3 concentrations to a larger degree than T4 in polar bears from Svalbard, Norway (Braathen et al. 2004)

Vitamin A (retinol)

Vitamin A is required for vision, immune function, the maintenance of differentiated epithelia and mucus secretion, and is essential for fetal development and normal growth in mammals (Clagett-Dame and DeLuca 2002). Vitamin A is present in the body tissues in different forms, namely as retinol, retinal, retinoic acid or retinyl esters (Dawson 2000). Dietary vitamin A, predominately retinyl esters, is stored mainly in the liver. Due to their lipophilic characteristics, large quantities of retinyl esters are also stored in the blubber of marine mammals (Schweigert and Stobo 1994; Schweigert et al. 2002). Retinyl esters are hydrolysed to retinol in the liver and transported in the blood bound to retinol binding protein (RBP) (Burri et al. 1993). Retinol is mainly delivered to target tissues via cellular receptors specific to RBP, and dependent on the tissues; circulatory retinol is stored as retinyl esters or transformed to the biological active forms, retinoic acid or retinal. Retinal is essential for vision, whereas retinoic acid is a transcription factor ligand that has important roles in regulating genes involved in cell morphogenesis, differentiation, and proliferation (Dawson 2000).

While hepatic levels of retinyl esters are affected by dietary supplements, retinol levels in the liver and subsequently in the circulation are subjected to mechanisms of homeostasis, and therefore referred to as a 'dietary hormone' (Dawson 2000; Ross and Zolfaghari 2004; Simms and Ross 2000a). Hepatic retinol levels regulate the secretion of retinol bound to RBP into the circulation (Ross and Zolfaghari 2004). Through positive feedback mechanisms excess retinol esterifies into retinyl esters by the actions of the enzyme lecithin: retinol acyltransferase (LRAT), and/or metabolises to more polar forms through oxidation by one of the members of the cytochrome p450 family, CYP26, and subsequently through conjugation to water-soluble retinyl glucuronides found in the bile. There are two isoforms of RBP, one that binds to TTR and a second one that does not bind to TTR, and the relative contributions of these isoforms vary among different species of mammals. The TTR-RBP complex is believed to increase the affinity of retinol to RBP and to decrease glomerular filtration and loss of retinol from the circulation (Burri et al. 1993). However, the role of TTR in retinol homeostasis is somewhat unclear, exemplified by TTR-deficient mice with negligible RBP-TTR-mediated transport of retinol have been found to remain healthy and to maintain normal tissue levels of vitamin A (van Bennekum et al. 2001).

In carnivore mammals, different from most other mammals, a large percentage of the retinol seems to be transported by proteins other than RBP such as various lipoproteins (Burri et al. 1993; Käkelä et al. 2003). For instance in pups of harbour seals, only 20-40% of the retinol was found to be transported bound to TTR-RBP or to RBP (Simms et al. 2000b). Carnivores are also unusual among mammals in that large quantities of vitamin A are also transported in the circulation as retinyl esters (Käkelä et al. 2003). However, although seals are considered as carnivores, retinyl esters have not been found in seal plasma, including plasma obtained from suckling grey seal pups (Schweigert et al. 2002). Furthermore, different from most terrestrial carnivorous mammals where the percentages of retinol in the liver rarely exceed 5-10 % of total vitamin A, the percentages of retinol in the liver of grey seal can be as high as 40% (Schweigert et al. 2002).

Newborn phocid seals have been shown to have low vitamin A stores in their blubber and liver as compared to adult seals, but these stores increase over the course of the lactation period; suggesting the limited placental transfer of vitamin A (Debier et al. 2003). Also, plasma retinol concentrations are low in the neonate grey seal compared to in adult seals, but increase during the suckling period to reach adult levels within 10 days of birth (Debier et al. 2003; Schweigert et al. 2002). Following weaning of the grey seal pup, the reports are somewhat deviating as some report no changes in retinol levels in the weaned grey pups (Schweigert et al. 2002), whereas others report a slight decrease the first few days following weaning after which concentrations stabilise (Debier et al. 2003). Similar increases in plasma retinol over the suckling period have been reported in the harbour seal pup; but although total plasma retinol concentrations increased in the harbour seal pup, the plasma concentrations of retinol bound to its transport proteins (i.e., TTR-RBP and/or RBP) remained fairly constant (Simms and Ross 2000b).

Organochlorines and vitamin A

With regard to the effect of OCs on plasma retinol concentrations, rats exposed to complex mixtures of OCs representing compounds found in Baltic herring such as polychlorinated dioxins and furans, planar and non-planar PCBs, as well as rats exposed to Arochlor 1254 (technical PCB) showed reduced plasma retinol concentrations (van der Plas et al. 2001). The mechanism behind this decrease in plasma retinol concentrations is believed to be due to the formation of OH-PCB metabolites that displace T4 from TTR, leading to the destabilisation of the TTR-RBP complex, and the subsequent decrease of plasma retinol (and T4) (Brouwer and van der Berg 1986; Brouwer et al. 1998b). In contrast, increased plasma concentrations of retinol have been reported in rats exposed to single PCB and PCDD congeners of high Ah-receptor activity, but which are not readily transformed to OH-metabolites (e.g., TCDD, PCB-156 and -169) (van Birgelen et al. 1994a,b; 1995; van der Plas et al. 2001); thus suggesting possible antagonistic effects of the different PCBs. Moreover, PCBs, both dioxin-like and none-dioxin like have been shown to decrease hepatic stores of vitamin A (i.e., retinyl esters) in several species (Brunström et al. 1991; Käkelä et al. 2003; Murk et al. 1998; van der Plas et al. 2001). The mechanisms explaining this loss of hepatic vitamin A stores is unknown.

Although many carnivore mammals differ from the frequently used experimental animals, and from humans with respect to various vitamin A variables, both reduced lipid stores of vitamin A esters and plasma retinol levels have been reported in seals feeding on PCB-contaminated fish from the Baltic Sea and the Dutch Wadden Sea (Brouwer et al. 1989; Swart et al. 1994; Nyman et al. 2003).

AIMS OF THE STUDY

The main objective of the present thesis was to examine differences in uptake and accumulation of OCs (i.e., PCBs, DDTs, chlordanes, HCHs, HCB and Mirex) in grey seal pups from the highly polluted Baltic Sea and in corresponding pups from the less polluted waters of the Atlantic Ocean, and to examine possible biological responses in plasma thyroid hormone and vitamin A levels caused by the exposure to these pollutants. This is to elucidate the possible use of plasma thyroid hormone and retinol levels as biomarkers for exposure and/or effects of OCs in free-ranging grey seal pups. In order to achieve this objective, the following second-order objectives were addressed.

1. The dynamics of maternal transfer of OCs from reproducing female grey seals to their offspring pups were investigated by analyzing for these compounds in some of the matrices that can be obtained non-destructively from mother-pup pairs of grey seals over the course of lactation (i.e., maternal blubber biopsies, maternal blood and milk sample, pup blubber biopsies and blood) (Paper I: IV). This to provide data on the quantitative and qualitative maternal transfer of the different OCs in the grey seal, and on the temporal development in the tissue-partitioning of these compounds among the different matrices during the course of the lactation process.

2. Baseline data of exposure levels and patterns of OCs in the different populations is important for the assessment of these compounds in grey seal pups. To identify geographical variations in OCs exposure and patterns, and to identify grey seal populations of particular concern, blubber biopsy concentrations of a wide range of OCs were examined in grey seal pups of West-Atlantic (Canadian), East-Atlantic (Norwegian) and Baltic (Estonian) origins (Paper II). A second aim of this study was through various principal component analyses (PCAs) to identify metabolisable OCs from persistent OCs in the grey seal. This because potentially metabolisable compounds may be transformed to toxic metabolites that may elicit TH and vitamin A toxicity, suggesting that compounds identified as metabolisable might show the strongest correlations with the investigated biomarkers.

3. The possible effect OCs may have on thyroid hormone status in grey seal pups were investigated by examining levels of plasma thyroid hormones in pups from the polluted Baltic Sea and from the cleaner waters of the open Atlantic Ocean (i.e., Norwegian Sea) (Paper III). In addition, the effects of possible confounding factors such as age following birth, pup body mass and gender on plasma thyroid hormone levels were examined.

4. In a fourth study (Paper IV) the main aim was to investigate the relationship between plasma retinol status and PCB concentrations in blood. This study was conducted on Norwegian grey seal pups sampled over the course of lactation. To consider if there were other important factors that may affect retinol status, the effects of possible confounding factors, such as body mass and age were examined.

MATERIAL AND METHODS

Sampling, ethical aspects and permissions

Details of the handling of the animals can be found in the individual papers and the references therein. Samples of maternal blubber, blood and milk, as well as pup blubber from grey seal mother-pup pairs used in Paper I was collected during January 1995 from specimens captured on the sea ice surrounding Amet Island, Gulf of St. Lawrence, Canada. The blubber and blood samples from the grey seal pups used in paper II and III were sampled from Norwegian Sea specimens captured in Froan Nature Reserve, Norway (October 1997) and from Baltic Sea specimens captured along the Saaremaa Island shorelines, Estonia (March 1998). The blood samples used in Paper IV were obtained from specimens sampled at Froan in October 1993.

All animals were sampled non-destructively and were released following handling and sampling. The Canadian seals were handled in accordance with the principles and guidelines of the Canadian Council of Animal Care. The Norwegian and Estonian seals were handled in accordance with the principles and guidelines of the Norwegian Animal Research Authority. Permissions to conduct the studies were given by Canadian, Norwegian and Estonian authorities.

Chemical quantification of organochlorines

Concentrations of OCs were quantified at the Environmental Toxicology Laboratory, Veterinary School of Veterinary Sciences / National Veterinary Institute, Oslo. Details of the analytical methods can be found in the individual papers and the references therein. In short, the lipids and OCs were extracted with cyclohexane and acetone by ultrasonic disintegration followed by centrifugation. Sulphuric acid was used to remove lipids from the extracts. OC concentrations were determined using a gas chromatograph equipped with a capillary column. The analytical quality of the laboratory is certified through participation in international intercalibration tests, including the four steps of the International Council for Exploration of the Sea/International Oceanographic Commission on PCBs in marine mammals. The laboratory was accredited on April 11, 1996 by the Norwegian Accreditation as a testing laboratory according to the requirements of the Nova Scotia Environmental Network 45001 and the International Organisation for Standardisation/International Electrotechnical Commission Guide 25.

Analysis of plasma TH and vitamin A concentrations

The methods of blood sampling and determination of plasma thyroid hormone and retinol levels can be found in Paper III and IV, respectively, and references therein. In short, plasma concentrations of THs were determined using radioimmunoassay (RIA) and plasma concentrations of retinol were determined using high-performance lipid-chromatography (HPLC).

RESULTS AND DISCUSSION

Lactational transfer and tissue-partitioning of OCs

The present study confirms that the lactational transfer of OCs is very selective in the grey seal (see also Green et al. 1996). While the lactational transfer of HCH, HCB and low-chlorinated PCBs were highly efficient, the transfer of mirex and the high chlorinated PCBs were highly inefficient (Paper I). This low lactational transfer probably relate to inefficient transfer of high-lipophilic and hydrophobic compounds from the maternal blubber into the milk lipids via the circulation and aquatic layers of the mammary gland (Fig. 2a-c). It is, however, noteworthy that HCB, which is relatively lipophilic, was more efficiently transferred to the milk and subsequently to the offspring seal than larger molecules of similar lipophilicity (e.g., DDTs, chlordanes) (Fig. 2a-c), suggesting that other physico-chemical properties, including molecular size or association with plasma proteins, might also affect the lactational transfer rates of OCs in the grey seal female (Paper I).

Blood and milk OC concentrations in the lactating grey seal increased rapidly and substantially during late lactation. This was particularly apparent for the higher chlorinated PCBs and mirex (Paper I), and is consistent with previous studies showing increases in the blood OC concentrations in seals following weight loss (Kleivane et al. 2004; Lydersen et al. 2002). Corresponding blubber biopsy concentrations of the lactating seals showed no or only slight increases in contaminants over the course of lactation (Paper I); suggesting that the increase in blood concentrations probably reflect an increase of OCs confined to the metabolically active inner blubber layers of the lactating mother seal out of reach for the biopsy punch. This assumption is in accordance with findings in a more recent study of grey seal-pup pairs from British waters (Debier et al. 2003). These authors found that PCB concentrations in the inner layers of the mothers blubber increased slightly over the course of the lactation period, whereas the corresponding concentrations in the outer layers remained largely unaffected by the lactation process. Moreover, in that particular study increases in blood and milk PCB concentrations were only observed in some of the mother seals, and confined to seals that showed a corresponding many-fold increase in contaminant concentrations in the their inner blubber layers during late lactation. Also, the blood PCB concentrations of the pups of these mothers increased during late lactation; suggesting that pup blood concentrations may be severely altered during the lactation period (Debier et al. 2003).

In my present study, increases in mother seal blood and milk OC concentrations during late lactation were only apparent in the mother seals that either showed low body masses or were nursing relatively large pups (~50 kg); whereas milk and blood concentrations remained fairly unchanged in larger mothers nursing pups below ~40 kg (see Table 2, Paper I). This suggests that although many of the OCs in the mother grey seals due to their lipophilicity or other physico-chemical properties reluctantly mobilise from the diminishing lipid stores, their mobilisation seems inevitable when the lipid stores of the inner blubber begins to be exhausted; suggesting a rapid and exponential increase in maternal blood (and milk) concentrations during the later phases of lactation. Other compounds (e,g., HCB, HCH and to some lesser extent the low-chlorinated PCBs) on the other hand seem to be mobilised at the same rate as the blubber lipids, and thus do not show the same increase in maternal blood and milk at late lactation. The present observations also suggests that fat females with large lipid stores might provide their pups with a milk of relatively low and stable OC concentrations over most parts of the lactation period, possibly even during the whole lactation period, dependent to which extent the mother seal utilises her lipid stores.

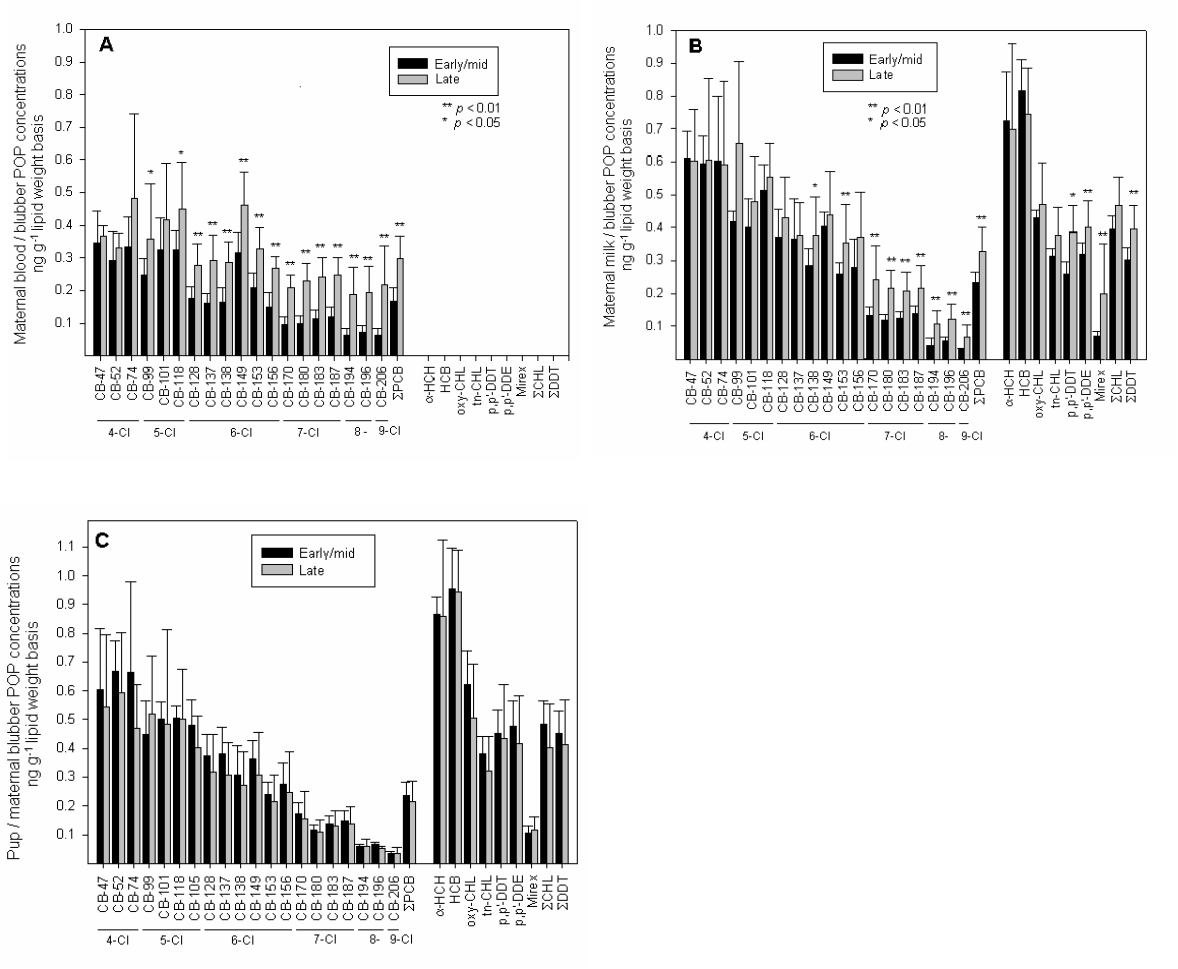

Fig. 2.
The lactation transfer (from mother seal to pup) of OCs appears to be highly selective in the grey seal (*Halichoerus grypus*), and particularly low for the high-chlorinated PCBs and for mirex, suggesting a lower lactational transfer of highly lipophilic compounds. Moreover, the mother seals mobilisation of the most lipophilic compounds into blood and the milk seemed to be greater relative to the less lipophilic compounds at late lactation (Paper I).

During most parts of the lactation period, milk concentrations of OCs were fairly comparable or slightly lower than corresponding concentrations in the blubber biopsies of the pups (Fig 3) (Paper I). However, from approximately Day 10 (i.e., at late lactation) pup blubber concentrations of many OCs, particularly of mirex and of high-chlorinated PCBs, were lower than in the milk the pups were drinking (Fig 3). As the grey seal pup is born without almost any subcutaneous lipids and assimilate as much as 70-75% of milk-derived lipids into its blubber, the comparable concentrations of OCs in pup blubber and milk during early and mid-lactation strongly suggest a highly efficient intestinal uptake and assimilation of milk-associated OCs in the suckling grey seal pup. This observation of a highly efficient and none-selective uptake of milk-derived OCs of grey pups opposes a previous assumption concerning assimilation of OCs in suckling grey seal pups (Addison et al. 1999). These authors stated that the absorption of high-chlorinated PCBs were less efficient compared to low-chlorinated congeners. However, in that particular study milk to pup ratios were only obtained during the later lactating stages, which may explain their conclusion.

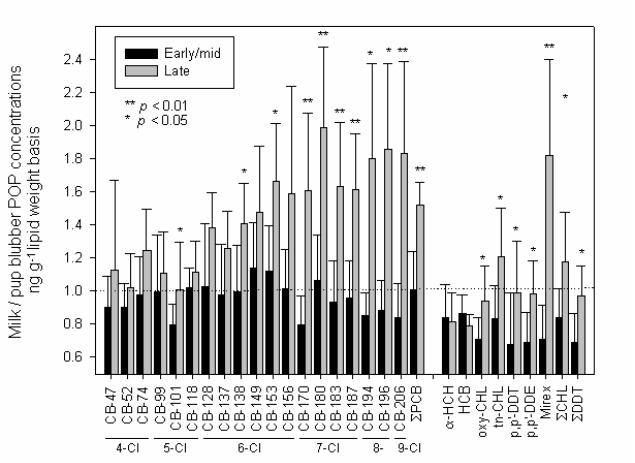

Fig. 3.
During the early and mid-phases of the lactation period, OC concentrations in the grey seal milk were highly comparable to those observed in the pup blubber, suggesting that both milk lipid and milk-associated OCs were efficiently taken up and assimilated into the pups blubber. At late lactation the pups were drinking milk more contaminated than their blubber; this was particularly evident for the high-chlorinated PCBs and for mirex (Paper I).

With respect to selection of matrices to quantify OC exposure of the grey seal pups in trend and effect studies, the present study suggests that concentrations quantified in the blubber biopsies obtained from pups at all stages of the lactation period most likely reflect the concentrations in the milk that pups were drinking at the earlier phases of their suckling period. Milk on the other hand seems to be more efficient to reflect the development of increasing OC concentrations in the mother seal lipid stores over the course of the lactation. Moreover, provided the assumption of efficient absorption of milk-derived lipids in the suckling grey seal pup, it can also be anticipated that pup blood concentrations of these compounds will reflect this increase in milk concentrations at late lactation (see Debier et al. 2003). These temporal changes in concentrations of lipophilic OCs in the different matrices in response to the lactation process raises caution about their use in studies aiming to assess exposure and/or toxic effects of these compounds in grey seal pups. Since blubber biopsy concentrations of most OCs, both in the mother and her pup, seems to be fairly unaffected by the lactation process, I recommend the use these matrices in studies aiming to monitor possible trends (geographical or time trends) in OC exposure within and among grey seal populations (Paper II). These more static blubber biopsy matrices may also be most suitable when assessing toxic effects, particularly if the effects are believed to be the consequence of chronic exposure for prolonged periods of time, including prenatal exposure of the offspring seal. On the other hand, blood is the substance that transports the OCs to the different sensitive tissues and organs, and episodes of elevated blood concentrations in the pup in response to the lactation process may induce and/or intensify toxic damage. This may be particularly important if the toxic effects are assumed to be acute, responding rapidly to rises in concentrations of these compounds in the circulation and target tissues. For instance, increased circulatory concentrations of OCs might enhance their competition with endogenous substances to binding sites on transport plasma proteins (e.g., TTR, RBP).

In the present thesis, pup blubber biopsy concentrations were used to assess possible effects of OCs on plasma TH concentrations in the grey seal pups (Paper III), whereas blood

concentrations were used to assess the possible effect of these compounds on plasma vitamin A concentrations (Paper IV). Thus, the reader should be aware of the respective potential weaknesses and strengths of the use of the different matrices of blubber and blood to measure OC exposure in the grey seal pups in these two studies. The increase in OC concentrations in milk and pup blood at late lactation, however, seem to be highly dependent on the nutritional condition and to which extent the mother seal utilises her lipid stores. The observation of no increase in blood PCB concentrations over the course of the nursing period in Norwegian grey seal pups (Paper IV), and that only a few pups showed increased milk and blood PCB concentrations in the above mentioned study of British grey seal pups (Debier et al. 2003), may indicate the infrequent occurrence of increased milk and blood OC concentrations at late lactation during most circumstances in the lactating grey seal pup.

Geographical differences in OC levels and pattern

The present thesis shows that grey seal pups from the Baltic waters have higher concentrations of many OCs (e.g., PCBs, DDTs and HCHs) as compared to their relatives in Norwegian and Canadian waters (Paper II). PCB concentrations were about 4 times higher in the Baltic pups, whereas DDT concentrations in the Baltic seals were 2 and 4 times higher, respectively, as compared to Canadian and Norwegian pups (Fig. 4). Concentrations of chlordanes did not differ between Baltic and Atlantic seals, whereas HCB were about 2 times higher in Norwegian and Baltic pups as compared to Canadian pups. HCHs and β-HCH in particular, were more than 10 times higher in the Baltic pups as compared to the Atlantic counterparts (Paper II).

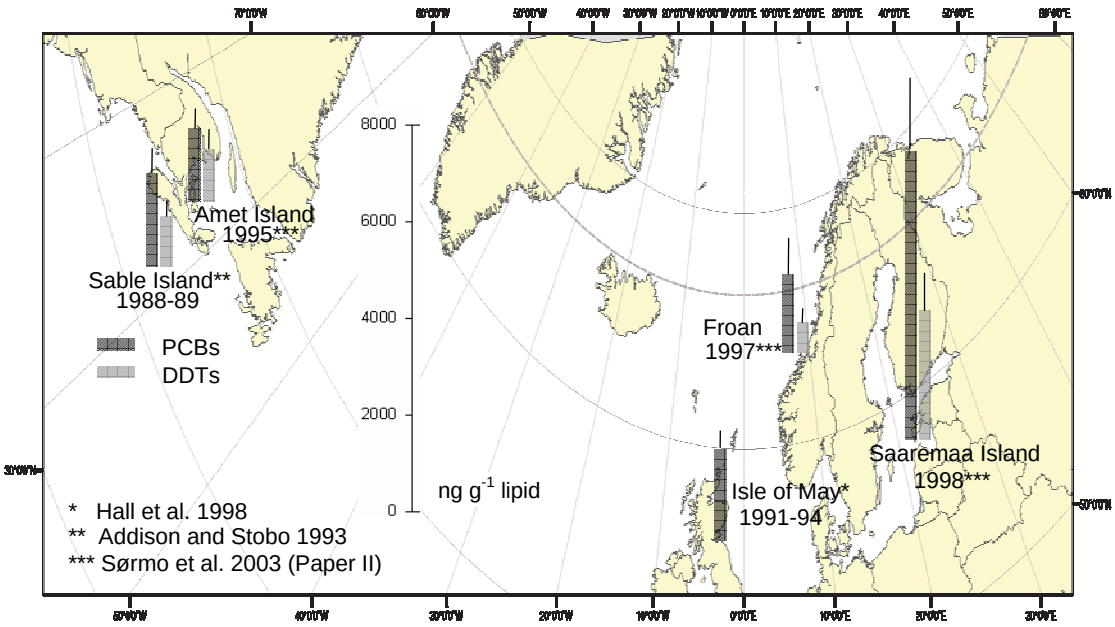

Fig. 4.
Mean ±SD blubber biopsy concentrations of PCBs and DDTs in suckling or newly weaned grey seal pups at different breeding sites (data from Paper II).

The higher OC concentrations in the Baltic grey seal pups relative to their Atlantic counterparts observed herein are consistent with the long record of reports of higher concentrations of OCs of the marine biota of the Baltic Sea relative to corresponding biota from the open waters of the Atlantic Ocean. Baltic herring (*Glupea harengus*) and harbour porpoise have been reported to have 4 and more than 10 times higher PCB and DDT

concentrations, respectively, compared to specimens from the open waters of the north-eastern Atlantic Ocean (Berggren et al. 1999; De Swart et al. 1995). Thus, the difference in DDT concentrations between Baltic and Atlantic grey seals was lower than previously reported for herring and porpoises, probably related to the better capabilities of grey seal to metabolise DDD (see Paper II), which are present in particularly high levels in Baltic fish (Falandysz et al. 1994). Moreover, similar to the grey seals of the present study, β-HCH concentrations were found to be 10 times higher in Baltic compared to Atlantic herring (De Swart et al. 1995). The higher β-HCH concentrations in the Baltic biota probably relates to recent use of technical HCH as an insecticide in the region close to the Baltic Sea (Voldner and Li 1995). The PCB concentrations in the Norwegian and Canadian pups found in the present study were similar to concentrations previously reported in the blubber of grey seal pups from other breeding sites in British and Canadian waters (Addison and Stobo 1993; Addison et al. 1999; Green et al. 1996; Hall et al. 1998), suggesting similar exposure to PCBs in the major populations of grey seals from the both sides of the Atlantic (Fig. 4).

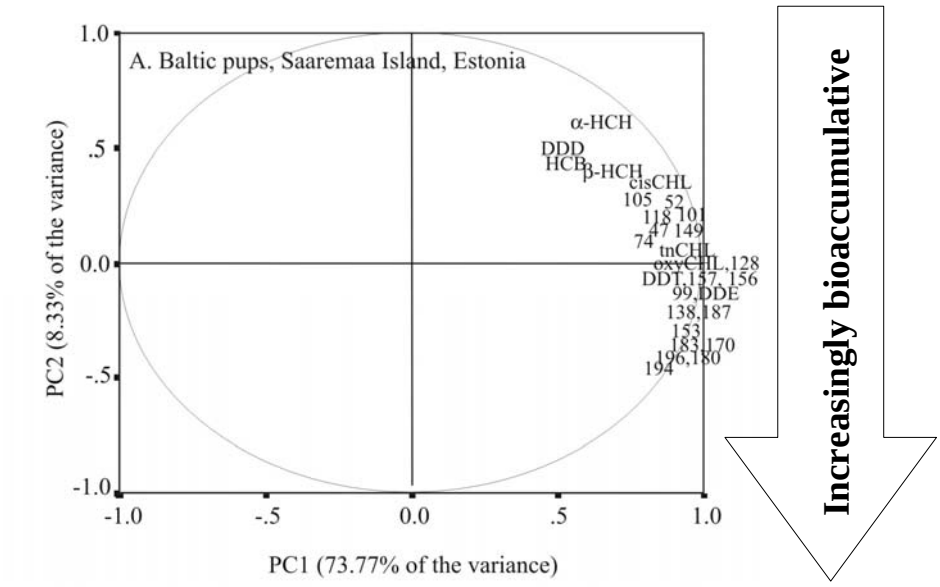

Fig. 5.
Identification of persistent versus 'metabolisable' OCs in the grey seal (*Halichoerus grypus*) by using intercorrelations of OC concentrations in Baltic grey seal pups (Paper II).

The OC patterns and particularly the pattern of PCBs and DDTs differed in the Baltic pups compared to Atlantic counterparts (Paper II). Several factors might account for such differences; but of particular importance is the possibility of concentration-dependent metabolism of OCs. Thus, the more metabolisable compounds are metabolised at higher rates and thereby less bioaccumulative in the more highly-exposed populations (e.g., Baltic) (Boon et al. 1997; De Swart et al. 1995). Studies of intercorrelations of OC concentrations have been suggested as a tool to identify compounds of different bioaccumulative properties (Boon et al. 1997). It can be assumed that compounds of low mother-pup transfer rates and/or high resistance against enzymatic degradation should be identified as bioaccumulative (e.g., persistent); whereas the compounds of high mother-pup transfer rates and/or low resistance against enzymatic degradation should be identified as less bioaccumulative. Consistent with this assumption, in the Baltic group of seals it was shown, in large, that compounds of high mother-pup transfer rates (e.g., HCB, HCH and to a somewhat lesser extent lower chlorinated

PCBs) intercorrelated less with compounds of low mother-pup transfer rates (e.g., high chlorinated PCBs) (Fig 5). However, intercorrelations also deviated among compounds of similar mother-pup transfer rates, suggesting that other factors than mother-pup transfer rates such as enzymatic degradation/transformation affect the bioaccumulative properties of the OCs in the grey seal. For instance, PCB-99 seem to be much more bioaccumulative than other penta-CBs (i.e., PCB-101, -105 and -118) and PCB-187 seem to be less bioaccumulative than other hepta-CBs (i.e., PCB-170, -180, and -183) (Fig. 5). Also the hexa-CBs shows variability in their bioaccumulative potentials, PCB-149 and to lesser extent -128 seems to be less bioaccumulative than PCB-138, -156, -157 and in particular PCB-153. This indicates that PCB-101, -105, -118, -128, -149 and -187 may be 'more or less' metabolisable and available for production of metabolites; for example to the metabolite 4-OH-PCB-107 that is predominately derived from the hydroxylation of both PCB-105 and -118. This metabolite has been reported to be one of the main OH-metabolites in humans and wildlife (Malmberg et al. 2004; Sandau et al. 2002). In blood from Latvian men with a high intake of fat fish from the Baltic, 4-OH-PCB-107 was found to be the most abundant OH-PCB metabolite (Sjödin et al. 2000). Furthermore, 4-OH-PCB-187 derived from the hydroxylation of PCB-187 (and to lesser extent PCB-183) has been reported to be the main OH-metabolites in other human populations (e.g., Canadian Inuit's) (Sandau et al. 2000, 2002). The present intercorrelation analysis also seems to identify the lower bioaccumulative potential of p,p'-DDD and cis-chlordanes in the grey seal as compared to other DDTs and chlordanes quantified in the present study (Fig. 5) (Paper II).

Consistent with the above was the lower proportions PCB-47, -52, -74, -101, -105, -118 and -187 relative to the total PCB load in the higher exposed Baltic seals as compared to the Atlantic seals (Paper II); suggesting a concentration-dependent metabolism of these metabolisable PCBs in grey seals (Fig. 6). The lower proportions are consistent with reports of higher hepatic 7-ethylxyrosurofin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activities in Baltic as compared to Atlantic grey seal females (Nyman et al. 2002), where EROD is particularly involved in the metabolism of coplanar PCBs (e.g., PCB-105 and -118) and PROD in the metabolisms of non-planar PCBs (Safe 1992). Furthermore, previous observations of high concentrations of $MeSO_4$ PCB metabolites derived from the PCB-52 and -101 in Baltic grey seals (Bergman et al. 1994) also support the assumption of metabolisms of metabolisable PCBs in the grey seal. Important is that OH-PCB metabolites readily cross the placenta and accumulate in high concentrations in the blood of human foetuses (Sandau et al. 2002). This raises the concern of the presence of high concentrations of such metabolites in grey seal pups, particularly in pups from the Baltic Sea. Thus, in future, complementary studies efforts should be made to quantify levels and effects of these metabolites in phocid pups.

Other factors may contribute to the differences in the contaminant patterns observed between the Baltic and Atlantic grey seal. For instance, the lower proportions of the less-chlorinated and more volatile OCs (e.g., PCB-47, -52, -74) in the Baltic pups may relate to these compounds being readily distributed globally via the atmosphere, resulting in their higher relative contribution to the total PCB load in biota of pristine locations far away from their release (e.g., the open waters of the Atlantic Ocean) (Muir et al. 2000). However, the similar to lower proportions of the high-chlorinated PCBs (i.e., PCB-170, -180, -183, -187, -194, and -196) in the Baltic seals (Fig. 6) do not fit with the assumption that biota close to the primary source of PCB releases have higher proportions of low-volatile compounds (Mackay et al. 1992; Muir et al. 2000; Sánchez et al. 1993). Several explanations may contribute to this peculiar phenomenon in the present study; including the possible usage and release of other commercial PCB mixtures in the Baltic region. Enhanced biodegradation of the high-chlorinated PCB congeners by anaerobic microorganisms of the anoxic environment of the

eutrophicated Baltic Sea might also be a plausible explanation for this observation. This degradation by the microorganisms is possible through a process where these compounds are biodegraded via chlororespiration by using the PCBs as electron acceptors (Abraham et al. 2002). Likewise, the higher occurrence of DDD in the Baltic pups (Paper II) probably relates to the eutrophication situation of the Baltic Sea, because DDT is believed to be rapidly broken down to its metabolites in eutrophicated waters (Maldonado and Bayona 2002); exemplified with the observation of much higher proportions of DDD and DDE relative to non-metabolised DDT in sediment layers representing the periods of eutrophication of the Baltic Sea (from 1970s and onwards) compared to sediment layers from the 1940-50s (Olsson et al. 2000).

Differences in prey availability and prey preferences between the seal populations could also contribute to the observed differences in the PCB pattern. Thus, Baltic grey seals predominately prey on herring (*Glupea harengus*) which again feed on zooplankton (Sjöberg 1999), whereas Atlantic grey seals predominately prey on a large variety of fish, including piscivorous species of higher trophic levels (Bowen et al. 1993; Bowen and Harrison 1994; Hammond et al. 1994; Mikkelsen et al. 2002). This might be important since the more lipophilic and persistent OC compounds are the most likely to increase with trophic level in the marine food chain (Ruus et al. 2002). Variability in age-structures of the mother seals from the Baltic and Atlantic could also contribute to differences in this pattern. For instance, in a recent study where relatively large numbers of mature grey seal females were shot and aged in Baltic and Canadian waters, the Baltic females were found to be significantly younger than their Atlantic counterparts (Nyman et al. 2000). This might be important since the more bioaccumulative compounds (e.g., high-chlorinated PCBs) are the most likely compounds to increase in concentration with the lifespan of the female seals (see also Bernt et al. 1999).

In summary, higher body burdens of PCBs, DDTs and HCHs were found in Baltic grey seal pups relative to their Atlantic counterparts. The OC patterns differed significantly between Baltic seals and Atlantic grey seals, possibly related to between-population differences in the rate of OC metabolism, proximity to areas of OC use, differences in prey availability or preferences, and/or age-structures of the lactating females. It is also possible that the anoxic conditions caused by the present eutrophication of the Baltic partly explain some of the differences in the OC pattern of Baltic and Atlantic grey seals. However, irrespective of the causes, the variability in the OC and PCB pattern both within and among the populations imply that parameters such as total PCBs and total DDTs represent mixtures of different composition and possible toxic potentials in Baltic and Atlantic grey seal pups. This suggests that efforts should be made to quantify concentrations of individual OC compounds in studies aiming to assess the toxic effects of these compounds in grey seal pups (Paper III; IV).

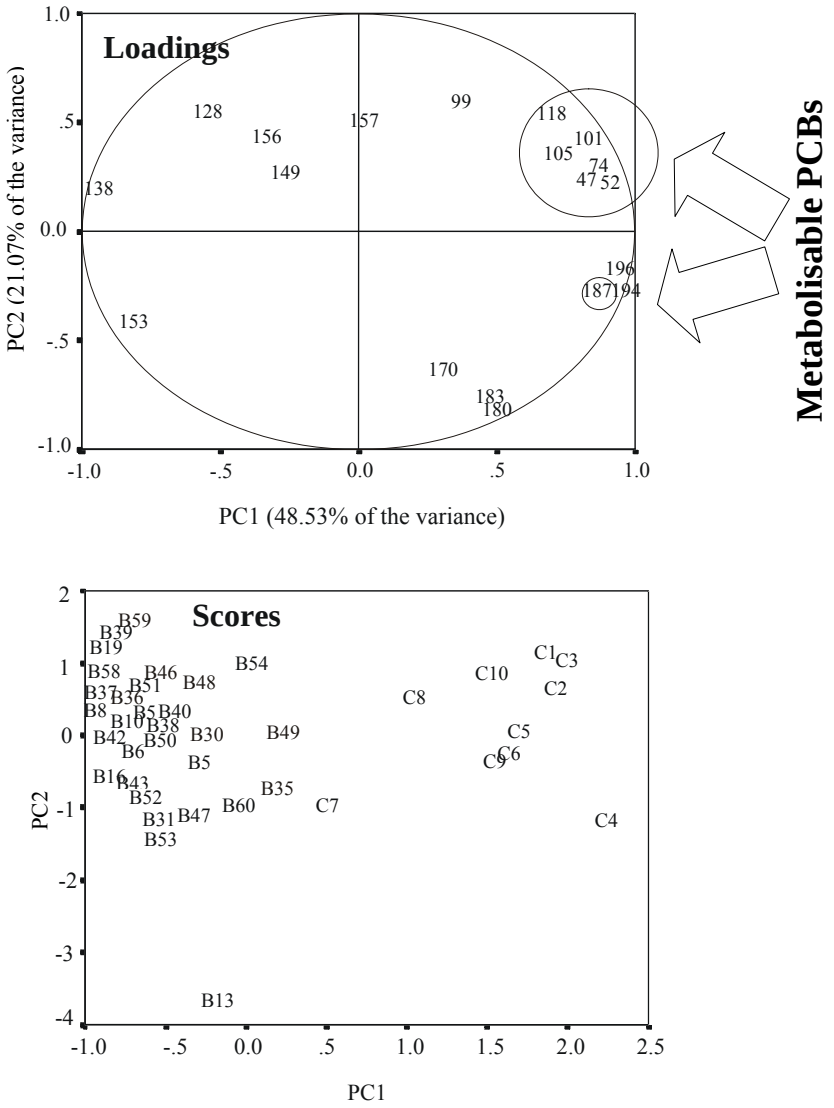

Fig. 6.
The lower relative contribution of 'metabolisable' congeners relative to total PCB in Baltic compared Atlantic grey seals (*Halichoerus grypus*), expressed by principal analysis showing the difference in PCB pattern between Baltic and Atlantic (represented by Canadian) grey seal pups (from Paper II). Baltic pups are labelled B+ pup ID, and Canadian pups are labelled C+ pup ID (Paper II).

Plasma thyroid hormone concentrations

Concentrations of FT4, TT3 and FT3 concentrations in the grey seal pups of the present study (Paper III) were similar to concentrations reported in harbour seal pups of similar age. Plasma TT4 concentrations were on the other hand somewhat lower than reported in harbour seal pups (~45 nmol/L) (Haulena et al. 1998). Similar to the grey seal pups of the present study, FT3 was also the only plasma TH reported to decrease in harbour seal pups in the first weeks following birth (Haulena et al. 1998). To my knowledge the present study is the first that investigates gender differences in plasma TH concentrations in neonate phocids, and thus the first to show that FT3 concentrations were higher in the female pups (Paper III). The observation that both gender and age explained relatively large proportions of the variation in FT3, but that these factors did not account for variation on the other THs, probably relates to FT3 being the physiologically relevant indicator of hormone action (McNabb 1992); as the actions of THs are mediated by the nuclear receptor that has its

highest affinity for T3. The effect of age and gender on plasma TH levels also suggests the importance in accounting for confounding factors when assessing the possible effects of pollutants on plasma TH parameters.

Effects of OCs on plasma thyroid hormone concentrations

Grey seal pups from the Baltic Sea had lower plasma TT3 and FT3 concentrations compared to pups from the open and cleaner waters of the Atlantic Ocean (e.g., Norwegian Sea), whereas there were no differences in plasma TT4 and FT4 concentrations between the two groups (Paper III). Since blubber concentrations of most OCs were higher in the Baltic seals, including compounds that have been reported to disrupt TH functions in experimental studies, it can be argued that the present study indicates that plasma T3 concentrations in neonate phocids might decrease following exposure to high levels of environmentally occurring OCs. Such a statement is, however, challenging in studies involving free-ranging organisms where there are possibilities of a wide range of confounding intrinsic (e.g., genetic) and extrinsic (e.g., environmental) factors that make it difficult to provide clear evidence that populations and sub-populations are affected by pollution (Jenssen 2003). Exemplified in the present study where the factor population (i.e., whether the pups were from Baltic or Norwegian waters) explained variations in plasma T3 concentrations to a greater extent than most OCs did (Paper III). This suggests weak TH disruptive properties of the majority of the OC compounds quantified in the present study. Also important in this context is the observation that despite a 10-fold variation in OC concentrations within the Baltic group (Paper II); there were no correlations between THs and OCs within this group of seals (Paper III). The observation that population was a stronger predictor does not, however, rule out the possibility that toxic compounds other than those quantified (including metabolites), as well as higher overall toxicity due to population-dependent variability in interaction of toxic compounds (e.g., antagonistic, synergistic, etc) could contribute to the lower TT3 and FT3 levels in the Baltic grey seal pups. Noteworthy is that irrespective of whether population was included or not in the statistical analyses, PCB-118 was found to correlate negatively with plasma concentration of FT3 when the pups of both populations were merged in the analyses (Paper III). This suggests that this congener or other compounds that correlate or interact with this congener might be particularly potent to disrupt plasma TH, at least FT3, concentrations in grey seal pups.

Negative correlations between blubber PCBs and plasma T3 concentrations have also be reported in Larga- and ribbon seals from Japanese waters, whereas plasma T4 concentrations were not found to be affected in either species (Chiba et al., 2001). Likewise, negative correlations between serum total PCB and total PCB TEQ concentrations, and plasma TT3 (FT3 not analysed) have recently been reported in free-ranging juvenile Californian sea lions (*Zalophus californianus)*, plasma TT4 was, however, not found to be affected in the sea lions (Debier et al. 2005). In captive harbour seals it was shown that following fasting, that plasma TT3 and not TT4 was lowered in seals previously fed herring from the Baltic Sea compared to control seals fed cleaner herring from the open waters of the Atlantic Ocean (De Swart et al. 1995). Greater effects on T3 to T4 following PCB exposure have also been observed in other carnivorous mammals. PCBs were found to affect plasma T3 concentrations to larger degree than T4 in polar bears from Svalbard, Norway (Braathen et al. 2004). In an experimental study on mink exposed to PCB contaminated carp (*Cyprinus carpio*) from Saginaw Bay, Michigan, plasma TT3 and FT3 concentration decreased with increasing TEQ levels in their food, whereas total and free T4 increased (Heaton et al. 1995). Thus, the effect of OCs on plasma T3 concentrations, and the lack of effect on T4 observed in the present study are in accordance with some previous studies involving carnivorous mammals. This disruption predominately on plasma T3 levels observed in carnivorous mammals

following PCB exposure is in strong contrast to numerous reports of disrupted plasma T4 concentrations in experimental rodent models (see Brouwer et al. 1998b). Some of this difference may relate to that TTR has a major role of TH transport in the rodents, but plays a minor role compared to TBG and albumin in non-rodents (McNabb 1992). Thus, the limited ability of OH-PCBs to compete with T4 for binding sites on TBG and albumin (Cheek et al. 1999) might explain why no difference was observed in plasma TT4 and FT4 concentrations between seals from the heavily polluted Baltic Sea and the somewhat less polluted Atlantic Ocean.

The lower plasma T3 levels in the Baltic seals may be of particular concern since the actions of thyroid hormones are mediated by nuclear TH receptors that have the highest affinity for FT3 (McNabb 1992). Because knowledge of normal baseline thyroid hormone levels in grey seals is sparse, it is not possible to judge whether any of the grey seal of the present study suffered from hypothyroidism. However, the emerging observations of negative relationships between OCs and T3 in carnivore mammals raises concern and suggests that more focus should be put on T3 when addressing the possible effects of OCs on thyroid hormone function in non-rodent models.

The present study only allows speculations about possible mechanistic explanations for the lower T3 levels in the more OC contaminated grey seal pups. However, the present study of grey seal pups and those of other carnivorous mammals suggest that mechanisms involved in the synthesis-, metabolism- and/or excretion of circulatory T3 may be particularly susceptible to disruption actions of environmentally occurring OC pollutants in these species. In most mammalian species, circulatory T3 originates predominately from hepatic type I (mono) deiodinase (MDI-I) activity, and to lesser extent from MDI-I activity in the thyroid gland (McNabb 1992). Thus, inhibition of pollutants on hepatic MDI-I activity may lower plasma T3 concentrations in the more exposed seals. For instance it has been documented that strong inducers of AhR-mediated toxicity (e.g., PCB-77 and TCDD), as well as technical PCB mixtures and several OH-PCBs inhibit hepatic MDI-activity (Brouwer et al. 1998b). In this context it is noteworthy that PCB-118 may be the most potent dioxin-like pollutant quantified in the present study (Alhborg et al. 1994; Safe 1992). Furthermore, PCB-118 is also metabolised and transformed to 4-OH-PCB-107, which has been reported to be one of the most potent OH-PCB metabolites to affect various thyroid hormone parameters (Brouwer et al. 1998b). The toxic potential of such OH-metabolites have, for example, been shown in human infants of mothers of high intakes of marine foodstuffs, where cord blood levels of chlorinated phenolic compounds (sum of pentachlorophenol and OH-PCBs) lowered plasma TT3 and FT4 concentrations in the infant. HCB and the parent PCBs were, however, not found to affect TH levels in that particular study (Sandau et al. 2002). Moreover, in rats exposed to subchronic levels of complex mixtures of environmentally relevant OCs (including Arochlor 1254, TCDD and DDTs) and heavy metals such as lead and cadmium, decreased hepatic MDI-activity and increased TSH levels were found to be the most sensitive endpoints for assessing thyroid toxicity (Wade et al. 2002). However, in that particular study, plasma T3 levels remained unaffected, probably related to an observed increased compensatory MDI-activity in the thyroid gland of the more exposed rats, which probably explains their increased TSH levels (Wade et al. 2002). Alternatively, iodothyronine sulfates, such as T3-sulfate (T3-S) have been suggested as an important source of circulatory T3 during fetal and early stages of postnatal life in mammals (Chopra et al. 1992). Several OH-PCB metabolites (e.g., 4-OH-PCB-107) have been reported to inhibit iodothyronine sulfation. Thus, it is possible that low pools of plasma T3-S caused by inhibition of iodothyronine sulfation may result in the reduced T3 concentrations in the Baltic group of grey seal pups. Thus, in future studies, complementary analyses of other TH variable, such TSH and T3-S, should be included to

bring additional insight into the possible endocrine disruptive effects of these compounds in newborn phocids.

Plasma retinol concentrations

The observed increase in plasma retinol concentrations in the grey seal pups over the course of the lactation period in the present study (Paper IV) is in accordance to previous observations in grey- and harbour seal pups (Debier et al. 2002; Schweigert et al. 2002; Simms and Ross 2000b), and probably reflects the high ingestion of milk-derived retinol by the neonate phocid seal. Consistent with this assumption is that orphaned harbour seal pups have been found to have significantly lower plasma retinol concentrations as compared to well-fed counterparts (Simms and Ross 2000b). The importance of ingestion of milk-derived retinol probably explains the positive correlation between plasma retinol concentrations and body masses of the suckling pups of the present study (Paper IV). The plasma retinol concentrations in the Norwegian pups of the present study were fairly comparable or somewhat lower than reported in British grey seal pups (Debier et al. 2002).

Effects of OCs on plasma vitamin A concentrations

Negative correlations were found between plasma retinol and blood PCB concentrations in grey seal pups of the present study (Paper IV). This is consistent with observations reduced plasma retinol concentrations of captive harbour seals fed PCB contaminated fish from the Baltic Sea and the Dutch parts of the Wadden Sea as compared to control seals fed less contaminated fish (Brouwer et al. 1989; Swart et al. 1994). Likewise, just recently negative correlations were reported between serum PCBs and DDTs concentrations, and plasma retinol levels in free-ranging juvenile Californian sea lions (Debier et al. 2005). Moreover, in free-ranging harbour seal pups in the Washington State and British Columbia (Simms et al. 2000), it was reported that pups from the more PCB polluted sites had lower plasma retinol concentration compared to pups from less polluted sites. However, further analysis of these results revealed that plasma retinol levels in both the high and low contaminated breeding sites were positively correlated to PCBs and PCDD/Fs levels expressed as TEQ values (Simms et al. 2000). This observed difference of negative and positive correlations between plasma retinol and PCB exposure in the present study and in the harbour seal pup study might relate to antagonistic mechanisms. That is, compounds of high dioxin-like toxicity (i.e., high TEQ-values) without being transformed to OH-metabolites to any great extent (e.g. PCB-156) have been found to increase plasma retinol levels in experimental rodent-models, whereas compounds found to produce OH-metabolites seems to reduce plasma retinol concentrations in the same rodent models (van der Plas et al. 2001). Variability in the OC compounds included in studies to assess effects on plasma retinol levels may, thus, yield different effects concerning plasma retinol levels.

Noteworthy in the present study was that PCBs identified to be metabolisable (e.g., PCB-101, -105, -118, -128, -149, -187) in the grey seal (Paper II), were all among the PCB congeners that showed the strongest negative correlations with plasma retinol concentrations in the seal pups (Paper IV). Although, it is not yet possible to predict the relative contribution of the different PCB congeners to each one of the OH-PCBs, several studies indicated that several of the commonly occurring OH-PCB metabolites (e.g., 4-OH-PCB-107, 4-OH-PCB-187) might be formed by some of these PCBs (Guvenius et al. 2003; Malmberg et al. 2004; Sandau et al. 2002). The observed strong negative correlations between retinol and concentrations of the more persistent congeners PCB-153 and -138 in the seal pups might not seem to fit with this assumption of effects caused by OH-PCBs. However, one of the three most abundant OH-PCBs detected in human blood, 4-OH-PCB-146 is believed to be derived

from the hydroxylation of these two congeners (Guvenius et al. 2003; Sandau et al. 2002). Thus, although these congeners are slowly metabolised, their high concentrations result in the high presence of their metabolites. Other interesting OH-metabolites with respect to PCB-153 and -138 are also observations of the metabolites 3-OH-PCB-153 and 3-OH-PCB-138 in human blood (Guvenius et al. 2003). Noteworthy, is that the mono-ortho PCB-156 which has been shown to increase plasma retinol levels in rodents (van der Plas et al. 2001) was one of only few PCBs that did not affect plasma retinol levels in the grey seal pups in the present study (Paper IV).

All the above mentioned OH-metabolites have an OH-group in the *para*- or *meta*-position with two chlorine atoms on the neighbouring carbons, suggesting structural similarity to T4 and thereby having high competitive binding potency to the TTR-RBP-complex (Lans et al. 1994). Thus, it is possible that if there is an equilibration between the concentrations of metabolisable congeners and their metabolites (see also Sandau et al. 2002) that the retinol-depressing effects of PCBs in the neonate grey seal pups may be caused by OH-PCB metabolites that interfere with the formation of protein complexes that transport retinol in the plasma. This is in accordance with previous mechanistic explanations of retinol-depressing effects of PCBs in rodent models (Brouwer 1991; Brouwer and van den Berg 1986). The possible effect of PCBs on the binding of retinol on plasma transport proteins may have significant toxic implications, as the fraction of plasma retinol bound TTR-RBP or RBP have been observed to be relatively constant throughout the nursing period in harbour seal pups (Simms and Ross 2000b), suggesting that, like most mammals, the delivery of retinol to target tissues is highly regulated in phocid pups. An alternative mechanism involves the possibility that PCBs may inhibit hepatic retinyl ester hydrolase, which transforms retinyl esters to retinol (Chen et al. 1992). Thus, in future, complementary studies analysis of the possible depletion of the protein bound plasma fraction of retinol should be investigated to add further insight into the possible retinol disruptive potential of these compounds (including their OH-metabolites) in the neonate grey seal pup.

Conclusions and future perspectives

Negative correlations were found between OC contaminants and plasma levels of thyroid hormone (T3) and retinol (Paper III; IV), however, these relationships are not evidence *per se* of direct cause-effect associations, but indicate that, consistent with findings in other experimental animal models, wildlife and human studies, that OCs and PCBs in particular may interfere with TH and retinol homeostasis of free-ranging grey seal pups. Thus, indicating the possible use of these parameters as biomarkers of exposure and/or possible toxic effects of OC exposure in these animals. The biological importance of these alterations is, however, unknown, but the fact that plasma THs and retinol levels are highly regulated and important for developmental functions in mammals, raises concerns of possible harmful and severe effects of these compounds in the most exposed grey seal pups. This indicates the need to focus on possible functional effects of the apparent OC-induced TH and retinol imbalance in grey seal pups. This may include destructive sampling of the seal pups for investigation of parameters such hepatic monodeiodinase activity, TH-receptor activity (e.g., possible compensatory up-regulation related to lowered T3 levels), thyroid histopathology, hepatic vitamin A stores etc. Non-destructively (achievable by blood samples only) investigations of relationships between OCs and parameters such as plasma levels of TSH and T3-sulfate, and the fraction of retinol bound to TTR-RBP may also possibly bring additional insight into the possible endocrine disruptive effects of these compounds in newborn seals.

The present study also revealed that both biomarkers (i.e., THs and retinol) were highly affected by various confounding factors, such as development and age of the pup (Paper III; IV), emphasising the need to identify and account for these factors and/or

standardise the samples included when analysing for pollutant induced effects. For instance, that gender and population affected plasma T3 levels (Paper III) may suggest the need to investigate populations and genders separately when assessing OC-induced effects on this biomarker in grey seal pups.

Moreover, the issue of exposure was found particularly challenging in the present study, both related to the variability to toxicokinetics (e.g., tissue partitioning, concentration-dependent metabolism) and the great variability in exposure between the populations (Paper I; II). One such challenge is choosing the proper matrices (tissue sample) that best reflect the exposure that may induce and/or cause toxic effects. Dealing with developmental toxicology, exposure levels reflecting prenatal exposure may be particularly important. In the present study blubber biopsy concentrations were judged best to predict background and prenatal exposure of OCs, since both concentrations in the milk and possibly in the blood of pups may change rapidly during the lactation period. However, since the nutritional condition and exposure levels of the pregnant grey seal may change over the gestation period, it can be argued that one anyway can not be certain that the measurements of OCs in the newborn pup mirror the exposure during critical periods or windows in the prenatal development of TH- and retinol functions in the pups. On the other hand, blood is the substrate that transports the OCs organs sensitive to effects, and may be the correct matrice related to acute toxic effects. An additional uncertainty is the possible toxic actions of unknown compounds. The present study clearly demonstrates the greater metabolism of some compounds, presuming the possible formation of toxic metabolites. Noteworthy, was that these compounds identified as metabolisable were also those compounds that often showed the strongest negative correlations with the present investigated biomarkers, calling for focus on the toxicokinetic and effects of metabolites in newborn phocid seals in future studies.

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PAPER I

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Partitioning of persistent organic pollutants in grey seal (*Halichoerus grypus*) mother–pup pairs

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Abstract

Phocid seals have lipid rich milk, which is known to serve as a transfer medium through which persistent organic pollutants (POPs) move from mother to offspring during lactation. However, knowledge on this generational transfer of different POPs and the partitioning of these compounds in maternal and offspring tissues over the course of the lactation are limited. In this study we examined the qualitative and quantitative partitioning of a range of chlorinated POPs in maternal blubber, blood and milk as well as in pup blubber, collected early in the lactation period and late in the lactation period. In the lactating female, the high-chlorinated and hydrophobic compounds were passed less efficiently into the milk than the low-chlorinated compounds and more water-soluble compounds. Significantly, lower maternal blood concentrations than in maternal blubber biopsies suggest a stratification of POP concentrations in the blubber column of lactating female and lower concentrations in the metabolic active inner layers. Over the course of lactation, there was a significant increase in maternal blood and milk concentrations of POPs as opposed to no change in maternal blubber biopsy concentrations. This was most apparent for the hydrophobic and high-chlorinated compounds. The most likely explanation for this is that the metabolic active inner blubber layer, from which the milk lipids are derived from, is in steady state with the circulatory system, while the outer layers are more static and only slowly respond to changes in concentrations elsewhere in the body. The concentrations of the high-chlorinated and hydrophobic compounds were substantially lower in pup blubber than in maternal blubber. This probably relates the combined effect of these compounds stratification in maternal blubber and their slow transfer into the milk. The present study shows that the more hydrophobic and high-chlorinated compounds come to steady state less quickly in the different tissues than the more water-soluble and low-chlorinated compounds in the lactating female and her offspring. This has implications for which matrices to choose when sampling for assessing the toxicological risk of POPs in seals.

Keywords: Organochlorines; Maternal transfer; Blood; Milk; Blubber; Marine mammals; Non-destructive monitoring; Grey seal; *Halichoerus grypus*

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1. Introduction

High concentrations of persistent organic pollutants (POPs) have been reported in marine mammals throughout the world (Tanabe et al., 1983; Hutchinson and Simmonds, 1994; Norstrom and Muir, 1994; Jenssen, 1996). Polychlorinated biphenyls (PCBs), chlordanes (*trans*-nonachlor, oxy-, *cis*- and *trans*-chlordane), DDT (1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene), hexachlorobenzene (HCB), hexachlorohexanes (α and β HCHs) and Mirex are among the chlorinated POPs detected in these animals. Phocid seals have lipid rich milk (Lydersen and Kovacs, 1999) that serves as an efficient transfer medium of POPs from mothers to offspring (Addison and Brodie, 1977, 1987). Suckling phocid pups are thus exposed to large amounts of POPs during an early phase of their life, when they are probably most susceptible to harmful effects of these compounds.

Many studies have addressed the mechanisms involved in POPs transfer during lactation between phocid mothers and pups, particularly in the case of grey seals (*Halichoerus grypus*) (Addison and Brodie, 1977, 1987; Schweigert and Stobo, 1994; Green et al., 1996; Pomeroy et al., 1996; Addison et al., 1999). These studies have shown that the transfer of different POPs from maternal lipid stores to pups appears to occur selectively, implying that some POPs are retained in the mother. However, knowledge on the transfer of different POPs and how they are partitioned in maternal and offspring tissues during the course of the lactation is poorly understood. In this study we examine the qualitative and quantitative partitioning of chlorinated POPs (chlordanes, DDTs, HCB, HCH, Mirex and 22 PCBs) in maternal blubber, blood and milk, and pup blubber, of grey seals from early and late in the lactation period.

2. Material and methods

2.1. Animals and sampling

Samples were collected from 10 grey seal mother–pup pairs at Amet Island (45°47′N, 63°13′W), Gulf of St. Lawrence, Canada during January 1995. The adult females were captured using a sling net, chemically immobilized using Telezol® at a dosage of 0.9–1.0 mg kg^{-1} body mass, and weighed using a 500 kg (±1.0) Dillon dynometer. Their pups were hand-captured and weighed using either a 50 kg (±0.1) or a 100 kg (±0.5) Salter spring scale (depending on their size). Pups were aged based on their pelage stage (Kovacs and Lavigne, 1986) and the mothers were aged by counting growth layers in decalcified, stained incisor tooth sections (Bernt et al., 1996). Mother–pup pairs with newborns were not disturbed due to risk of abandonment.

Blood, blubber and milk samples were collected from the 10 mothers and blubber was collected from their pups. Six pairs were sampled early in the lactation period (pups 5–10 days old), and four pairs were sampled late in lactation (pups 12–15 days old). The blubber samples were collected dorsally using a biopsy punch (8 mm), allowing a sample of approximately 0.7–0.8 g to be taken. Milk samples (~15 ml) were taken approximately 10 min after intramuscular injection of 50 IU of oxytocin. Blood samples (~10 ml) from the adult females were obtained from the epidural vein. Samples were stored in bio-freeze polyethylene vials (−20 °C) until they were analyzed. All experimental procedures were conducted in accordance with the principles and guidelines of the Canadian Council for Animal Care.

2.2. Analysis of POPs

Chemical analyses of chlorinated pesticides and PCBs were performed at the Laboratory for Environmental Toxicology at the Norwegian School of Veterinary Science, Oslo, Norway. The blubber biopsies were crudely homogenized in a Petri dish using repeated cutting with a scalpel. Samples of blubber (~0.8 g), milk (~1.0 g) and whole blood (~8 g) were weighed and internal standards (PCB-29, -112, -207; Promochem GmbH, Wesel, Germany; Ultrascientific, North Kingstown, RI) were added. The samples were extracted twice with cyclohexane and acetone (3:2 proportion) using an ultrasonic homogenizer (4710 Series, Cole Parmer Instruments Co., Chicago, IL), followed by centrifugation. The supernatant was evaporated to approximately 1 ml using a

Zymark® evaporation system (Hopkington, MA). The extracts of blubber and milk were added to cyclohexane to a fixed volume of 10 ml. The extractable lipid content in each blubber and milk sample was calculated from an aliquot (blubber 4 ml, milk 5 ml) of the extract that was evaporated to dryness on a 50 °C sand-bath, and the lipid content was determined gravimetrically. Due to the low lipid content of the blood the extract had to be used for both lipid determination and detection of POPs. In order to prevent the loss of the most volatile POPs during evaporation (i.e. sand-bath), this process was performed under constant observation and the final proportion of the solvent was evaporated carefully at room temperature. The blood lipid content was determined gravimetrically and the dried lipids were dissolved by adding cyclohexane for use in the further analyses of the POPs.

Clean-up (i.e. removal of lipids) of the extracts was performed using ultraclean concentrated sulfuric acid, H_2SO_4 (purity 98.8%, Scanpure, Chemscan AS, Elverum, Norway) according to methods described by Brevik (1978), with some modifications by Bernhoft and Skaare (1994). All sample extracts were automatically injected (Fisons Autosamples 800, Manchester, UK) on a Carlo Erbo, High Resolution Gas Chromatograph (5300 Mega Series, Milan, Italy), equipped with split/splitless injection (1 μl, 1:20) and an electron capture ^{63}Ni detector (Carlo Erbo). The splitless time was 2 min. Hydrogen was used as the carrier gas (flow 2 ml min^{-1}) on a SPB-5 or a SPB-1701 capillary column (both 60 m, 0.25 i.d. and 0.25-μm film; Supelco Inc, Bellefonte, PA). The makeup gas was composed of 5% methane/95% argon (flow 30 ml min^{-1}). The temperature program was as follows: start 90 °C (held for 2 min), 25 °C increase min^{-1} to 180 °C (held for 2 min), 1.5 °C increase min^{-1} to 220 °C (held for 2 min), 3 °C increase min^{-1} to 275 °C (held for 2 min). Chromatographic data were calculated using the software Maestro version 4.2 (Chrompack International, Middelburg, Netherlands). The concentrations of the individual POPs were determined by comparison with the corresponding components in the PCB standards (Promochem GmbH, and Cambridge Isotope Laboratories, Woburn, MA), and in the pesticide standards (CPM, Supelco Inc).

The following POPs were determined: (chlordane related compounds) oxychlordane*, *trans*-chlordane*, *cis*-chlordane*, *trans*-nonachlor* and *cis*-nonachlor*; (DDT-compounds) *p,p′*-DDE, *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDD, *o,p′*-DDD; HCB; (HCH-isomers) α, β and γ-HCH; Mirex; and 32 PCB congeners (IUPAC nos, Ballschmiter and Zell 1980) 28, 31, 52*, 47, 74, 66, 56, 101*, 99, 87, 136, 110, 118*, 151, 149*, 114, 153, 105, 141, 137, 138, 187, 183, 128, 156*, 157*, 180, 170*, 199, 196, 189, 206, 209. Compounds marked with an asterisk were determined using the SPB-1701 column.

2.3. Analytical quality assurance

Quantifications were carried out within the linear range of the detector. Detection limits varied among the chemicals and ranged from 0.5 to 2.0 ng g^{-1} lipid in blubber, 0.2–1.35 ng g^{-1} lipid in milk and 0.002–0.41 ng g^{-1} lipid in whole blood. Quantification limits were set 3 times above the detection limits and non-quantified components were assigned a value of 0. Recovery rates via the gas chromatograph were calculated by adding a known amount of POPs to samples of clean material (i.e. pig blubber, bovine milk and sheep blood). Acceptable recovery rates were set as 80–110%. Analyzing a control sample of seal blubber tested reproducibility.

Analytical quality of the laboratory is certified by the participation in international intercalibration tests, including the four steps of the ICES/IOC/OSPARCOM (International Council for Exploration of the Sea/International Oceanographic Commission/Oslo–Paris Commission) on PCBs in marine mammals (Anonymous, 1995). The laboratory was accredited on 11 April 1996 by Norwegian Accreditation as a testing laboratory according to the requirements of NS-EN45001 and ISO/IEC Guide 25.

2.4. Statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 10 for Windows, SPSS

Table 1
Mean (range) POP concentrations (ng g^{-1} lipid weight basis) in 10 mother–pup grey seal (*Halichoerus grypus*) pairs sampled at Amet Island, Canada during the 1995 breeding season

	Maternal blubber	Maternal blood	Milk	Pup blubber
PCB 47	36 (21–57)[a,b,c]	13 (6–21)	21 (13–33)[a]	22 (14–32)
PCB 52	58 (41–94)[a,b,c]	18 (9–31)	34 (24–47)[a]	36 (26–53)
PCB 74	41 (20–62)[a,b,c]	16 (8–40)	22 (16–33)[a]	21(16–27)
PCB 99	371 (153–669)[a,b,c]	122 (56–293)	172 (95–292)[a]	152 (69–227)
PCB 101	174 (113–249)[a,b,c]	66 (33–171)	74 (44–122)[a]	82 (57–107)
PCB 105	20 (10–34)[b,c]	NA	16 (11–25)[c]	9 (6–10)
PCB 118	74 (44–120)[a,b,c]	29 (17–73)	39 (24–59)[a]	37 (23–47)
PCB 128	72 (35–111)[a,b,c]	16 (8–36)	28 (14–42)[a]	24 (14–32)
PCB 137	34 (18–54)[a,b,c]	8 (4–20)	12 (5–18)[a]	11 (7–14)
PCB 138	1279 (674–2070)[a,b,c]	288 (124–701)	416 (186–742)[a]	351 (204–598)
PCB 149	86 (51–153)[a,b,c]	34 (18–85)[c]	36 (22–56)	28 (19–35)
PCB 153	2058 (1110–3490)[a,b,c]	533 (255–1283)	626 (244–1136)[a]	452 (269–819)
PCB 156	48 (24–92)[a,b,c]	10 (5–21)	15 (7–26)[a]	12 (8–16)
PCB 157	8 (3–19)[b]	ND	4 (2–7)	ND
PCB 170	298 (167–433)[a,b,c]	44 (16–98)	53 (21–90)[a]	48 (27–70)
PCB 180	972 (501–1406)[a,b,c]	151 (54–336)	158 (62–272)[c]	107 (61–141)
PCB 183	246 (136–340)[a,b,c]	43 (16–94)	40 (17–95)	33 (19–44)
PCB 187	387 (210–520)[a,b,c]	70 (28–151)	67 (29–117)	54 (32–86)
PCB 194	231 (128–347)[a,b,c]	25 (10–49)[b,c]	17 (7–31)	14 (8–19)
PCB 196	165 (90–232)[a,b,c]	19 (7–37)[b,c]	14 (6–24)	11 (6–14)
PCB 206	111 (67–147)[a,b,c]	13 (5–24)[b,c]	5 (2–8)	4 (2–5)
PCB 209	43 (25–60)	ND	ND	ND
∑PCB	6813 (3715–10 178)[a,b,c]	1537 (705–3566)	1876 (896–3087)[a]	1505 (944–2141)
p,p′-DDE	2152 (1263–4195)[b,c]	NA	779 (337–1754)	931 (579–1879)[b]
p,p′-DDT	357 (200–576)[b,c]	NA	116 (44–181)	126 (74–208)
p,p′-DDD	44 (9–90)[b,c]	NA	18 (10–25)	11 (3–17)
∑DDT	2553 (1501–4615)[b,c]	NA	905 (390–1918)	1072 (656–2027)[b]
Oxychlordane	240 (146–421)[b,c]	NA	106 (61–192)	130 (91–226)
Trans-nonachlor	319 (204–478)[b,c]	NA	110 (66–159)	110 (70–150)
Cis-chlordane	12 (9–16)[c]	NA	NA	7 (5–9)
∑CHL	574 (375–916)[b,c]	NA	244 (143–382)	247 (175–383)
Mirex	78 (39–112)[b,c]	NA	9 (3–15)	8 (5–11)
HCB	21 (4–69)[b]	NA	17 (3–67)	21 (4–76)
α-HCH	39 (24–73)[b,c]	NA	28 (14–68)	33 (12–58)[b]

ND, not detected; NA, not applied.
[a] Significantly higher conc. than corresponding conc. in maternal blood (paired *t*-test, $P<0.05$).
[b] Significantly higher conc. than corresponding conc. in maternal milk (paired *t*-test, $P<0.05$).
[c] Significantly higher conc. than corresponding conc. in pup blubber (paired *t*-test, $P<0.05$).

Inc., Chicago, IL). The results are presented as means and ranges. HCB and α-HCH concentrations were non-normally distributed (Shapiro–Wilcoxon test), and therefore $\log_{10}$ transformed to achieve a normal distribution prior to statistical analysis. The differences in maternal blubber, blood, and milk and pup blubber POP concentrations were tested using the paired Student *t*-tests. Changes in POP concentrations, and partitioning of POPs between the different tissues as lactation progressed were investigated by comparing early and late lactation stages samples using the Mann–Whitney *U*-test. The level of significance was defined as $P<0.05$.

3. Results and discussion

Concentrations of nearly all compounds were significantly lower in maternal blood, milk and offspring blubber as compared to maternal blubber

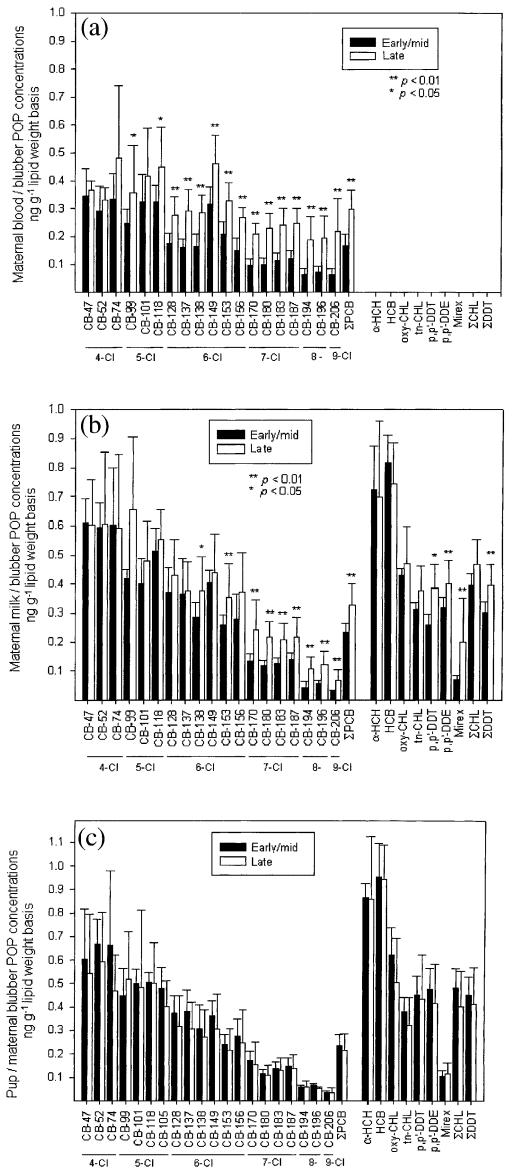

Fig. 1. Maternal blood (a); milk (b) and pup blubber (c) POP concentrations relative to corresponding concentrations (ng g^{-1} lipid weight basis) in maternal blubber in grey seal (*Halichoerus grypus*) mother–pup pairs. Early lactation ($N=6$) is 5–10 days after parturition, late-lactation ($N=4$) is 12–15 days after parturition. Values are presented as means and error bars are 95% CI. Mann–Whitney *U*-test is used to test statistical significance in difference in the partitioning of POPs between early and late-lactation.

(Table 1; Fig. 1a–c). The differences were particularly apparent for the high-chlorinated PCB congeners (7–9 Cl atoms) and for Mirex (Fig. 1a–c). The concentrations of these compounds in milk and offspring blubber were less than 10% of that in maternal blubber. Concentrations of DDTs and chlordanes in milk and offspring blubber were approximately half of the concentration in maternal blubber (Fig. 1b and c). However, concentrations of HCB and α-HCH in milk and offspring blubber was similar to or only slightly lower than in maternal blubber (Fig. 1b and c).

Concentrations of HCB and the pesticide related compounds were not determined in the blood samples due to the small sample volumes. Therefore the interaction of the pesticide compounds between the maternal blood, blubber and milk compartments could not be examined.

When considering the transport of POPs from the maternal blubber to the milk, the results indicate that transport efficiency in the passage of the compounds from blubber to milk depends on the $\log K_{ow}$ values of the compounds. In milk there were high concentrations of the less hydrophobic compounds (HCH, $\log K_{ow} \approx 4$) and low concentrations of the more hydrophobic compounds (DDTs, $\log K_{ow} \approx 6$; *trans*-nonachlor, $\log K_{ow} \approx 6.5$; Mirex, $\log K_{ow} \approx 7$; PCBs, $\log K_{ow}$ ranging from 5 for 4-Cl PCBs to 8 for 10-Cl PCBs; Isnard and Lambert, 1988; Mackay et al., 1992; Simpson et al., 1995). This suggests that the compounds with high $\log K_{ow}$ pass less efficiently from blubber into milk as compared to compounds of low $\log K_{ow}$ (Fig. 1b). Thus, if the transfer between one lipid pool (e.g. the blubber) and another (e.g. the milk) involves diffusion through a largely aqueous microlayer (e.g. the mammary gland), the transfer rate of the compounds are inversely related to their water solubility. Since the milk of the grey seal is rapidly produced there will not be time for the hydrophobic and 'slow moving' POPs to reach steady state milk concentrations with the other lipid pools before the milk is secreted. It is therefore possible that in species with lower milk production rate, a larger proportion of hydrophobic POPs will be able to pass into the milk from maternal lipid stores. Furthermore, it should be noted that HCB,

which has a $\log K_{ow} \approx 6$, seems to be more efficiently transferred to the milk compared to larger molecules that have similar $\log K_{ow}$ values, such as DDTs and penta/hexa PCBs (Fig. 1b). This indicates that other physico-chemical properties of the compounds, including molecular size, might effect the transport of POPs from maternal blubber in milk, as well. Other properties of the compounds such as binding to specific plasma proteins may also be involved.

The maternal whole blood/blubber ratios were also low for all PCB congeners but did not show the same high degree of selectivity related to $\log K_{ow}$ values of the compounds as for the milk/blubber ratios (Fig. 1a and b). In a previous study of grey seal females at early lactation (i.e. 3–4 days post-parturition), Addison and Brodie (1987) reported whole blood/blubber PCB (and DDT) ratios of 0.5, which were comparable to twofold higher than the corresponding ratios reported in the present study (Fig. 1a). Several physical and biological factors may explain the finding that the concentrations in blood were so much lower as compared to blubber concentrations in the lactating grey seal female. This difference between POP concentrations in blubber and blood, may relate to the possible stratification of POPs in the blubber column of marine mammals (Severinsen et al., 2000). The blubber layer of marine mammals is not a homogenous tissue in terms of fatty acid composition. Short-chained fatty acids with relatively low melting points are normally enriched in the outer layer towards the skin and the relative cold environment, while more long-chained fatty acids are enriched in the inner layer (Hart 1987; Aguilar and Borrell 1991; Fredheim et al., 1995; Koopman et al., 1996). This stratification suggests that the inner blubber layer is more metabolically active in terms of mobilization and deposition (Koopman et al., 1996) implying that most of the seasonal variation in blubber thickness is due to variation of the inner parts of the blubber. A stratification of fatty acids in blubber as described above has been documented for grey seals (Fredheim et al., 1995). The cycle of lipid use and deposition in the inner layer is expected to result in lower POP concentrations in inner than outer layers because the newly deposited lipid from the diet dilutes the concentrations in the inner blubber (Severinsen et al., 2000). Thus, the low maternal whole blood/blubber contaminant ratios might reflect the lower concentrations of the more vascularized and more metabolic active and possible 'cleaner' inner layers as compared to the possible more contaminated outer layers, where the biopsies were collected from.

Also, although it was not analyzed, lipid composition in grey seal blubber and blood may be different. The lipids of blubber consists of mainly non-polar triglycerids, while the lipids of blood are more complex consisting of non-polar triglycerids, fatty acid esters of glycerol and cholesterol, and more polar lipids like phospholipids (Ryan and Mills, 1997). To what extent the more polar lipids are extracted from the seal blood when using the method applied in the present study (i.e. cyclohexane and acetone) is unknown. If the more polar lipids are extracted, the blood lipids would be more polar compared to the non-polar blubber triglycerids. Thus the lower concentrations, particularly of the non-polar and high-chlorinated PCB congeners, in blood compared to blubber (Fig. 1b) may indicate that physico-chemical properties of the individual congeners and the lipid composition of the tissues could partly explain the pattern of congener distribution between the maternal blubber and blood samples.

The possible stratification of POPs in the blubber layers may also account for why the concentrations of several POPs in maternal blubber biopsies did not change significantly over the course of lactation, as opposed to the observed increase in concentrations in maternal blood and milk lipids (Table 2). Although it has been reported that grey seal females may feed to some extent during the lactation period, they generally do fast (Lydersen et al., 1994); losing on average more than 5-kg body mass daily (Lydersen et al., 1995). The blubber sampled from the seals in this study was collected with biopsy punches that penetrated only the outer 1–2 cm of the blubber layer. Thus, blubber collected from the lactating females came from a tissue layer that is relatively unaffected by the dramatic mobilization that occurs in the inner layer during lactation. This resulted in the finding of similar concentrations of several POPs in the

Table 2
Age (mother; years, pup; stage), sex, body mass (BM), and lipid content and residue concentrations (ng g^{-1} lipid) of three types of sample in 10 grey seal (*Halichoerus grypus*) mother–pup pairs sampled at Amet Island, Canada, January 1995

Seal ID	Age	Sex	BM (kg)	Lipid content (%)			ΣPCB			ΣDDT		ΣCHL		HCB		α-HCH		Mirex	
				Blubber	Blood	Milk	Blubber	Blood	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk
Early/Mid lactation																			
3092																			
Mother	20	F	164	37.8	0.51	62.1	5085	705	1077	1746	551	459	163	13.5	10.3	32.2	20.6	80.3	5.2
Pup	3	F	42	59.5	–	–	944	–	–	656	–	175	–	10.8	–	26.5	–	5.9	–
3099																			
Mother	17	F	162	45.6	0.79	61.5	9129	1636	2322	2831	959	768	339	10.4	9.3	73.5	68.2	112	10.6
Pup	3	M	40.5	47.3	–	–	1734	–	–	971	–	304	–	10.5	–	58.2	–	10.5	–
3090																			
Mother	16	F	184	55.5	0.61	62.3	3715	821	1012	1501	482	375	155	62.2	61.4	42.3	31.5	52.9	3.6
Pup	3	M	40.5	59.5	–	–	954	–	–	740	–	196	–	69.9	–	39.4	–	5.4	–
3095																			
Mother	21	F	173	57.9	0.72	56.7	6489	780	1272	2039	634	403	160	15.9	12.4	48.0	39.6	86.2	5.2
Pup	3E	F	32.5	57.3	–	–	1402	–	–	911	–	195	–	13.5	–	42.8	–	8.4	–
3309																			
Mother	22	F	183	41	0.49	66.4	5935	806	1466	2267	732	389	164	11.6	8.9	33.8	22.9	91.3	6.4
Pup	3E	F	34.5	37.4	–	–	1646	–	–	1153	–	219	–	10.9	–	31.3	–	11.9	–
3074																			
Mother	7	F	175	56.2	0.50	68.7	4387	810	896	1533	390	419	144	69.0	51.3	28.3	14.8	48.1	3.3
Pup	3E	F	31.5	52.6	–	–	1267	–	–	819	–	228	–	76.4	–	23.4	–	6.4	–
Late-lactation																			
2203																			
Mother	–	F	133	36.5	0.69	85.4	10 178	3566	3071	3074	1411	916	348	10.2	6.4	40.1	18.9	105	15.3
Pup	3L	F	50	63.2	–	–	1935	–	–	1346	–	303	–	8.6	–	28.6	–	6.8	–
2203																			
Mother	–	F	148	43.8	0.50	65.6	7194	2084	2024	3025	1081	694	311	7.3	5.7	38.3	26.9	108	10.3
Pup	3L	M	54	58.5	–	–	1282	–	–	1002	–	215	–	6.8	–	29.3	–	5.5	–
833																			
Mother	22	F	171	55.1	0.71	57.1	6453	1696	2538	1999	897	513	277	5.5	4.5	24.2	20.8	39.4	13.4
Pup	3L	M	51.5	57.1	–	–	1763	–	–	1088	–	246	–	5.8	–	26.5	–	7.4	–

Table 2 *(Continued)*

Seal ID	Age	Sex	BM (kg)	Lipid content (%)			ΣPCB			ΣDDT		ΣCHL		HCB		α-HCH		Mirex	
				Blubber	Blood	Milk	Blubber	Blood	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk	Blubber	Milk
801																			
Mother	8	F	140	49.8	0.62	62.6	9567	2474	3087	4614	1918	777	382	3.7	2.8	31.7	23.9	59.2	12.9
Pup	3L	F	42	60.8	–	–	2141	–	–	2027	–	383	–	3.4	–	27.4	–	9.4	–
Mean-early/mid-lactation)																			
Mother	17	–	174	48.9	0.60	62.9	5790	926	1341	1986	608	469	187	30.4	25.6	43	33	79	5.7
Pup	–	–	37	52.3	–	–	1323	–	–	875	–	220	–	32	–	34	–	8.0	–
Mean-late lactation)																			
Mother	–	–	148	46.3	0.63	67.7	8348	2455[a]	2685[b]	3403	1327[b]	725[b]	329[b]	6.7[c]	4.9[c]	34[c]	23	78	13[b]
Pup	–	–	49.5	59.9	–	–	1780	–	–	1366[b]	–	287	–	6.2	–	28	–	7.3	–

[a] Significantly higher than early lactation ($P<0.01$).
[b] Significantly higher than early lactation ($P<0.05$).
[c] Significantly lower than early lactation ($P<0.05$) (Mann–Whitney *U*-test).

blubber biopsies of the lactating females when comparing early with late stage of lactation (Table 2), implying that the outer layers are more static and will only slowly respond to changes in POP concentrations elsewhere in the body.

Concerning the increased POP concentrations in maternal blood and milk over the course of lactation, this is most likely related to the above-discussed inefficiency in the transfer of several POPs into the milk lipids. Thus, compounds that are mobilized from the inner layers and only to limited extent passed to the milk, will inevitable be retained within the lactating female in increasingly higher concentrations as lactation progresses. This increase is expected to occur in the more vascularized tissues (e.g. muscle tissues, internal organs and the mammary glands). This explains why the PCBs, DDTs, chlordanes and Mirex did, and α-HCH and HCB did not, increase in maternal blood and milk lipids over the course of lactation (Table 2). Their increase in the body core of the seal may explain the higher blood/blubber and milk/blubber ratios of the high-chlorinated PCBs and Mirex at late lactation as compared to early lactation in the mother seals (Fig. 1a and b). Furthermore, the mobilization of a possible more contaminated outer layer at late lactation may also, at least partly, account for the elevated POP concentration in maternal blood and milk at this stage of lactation.

Early in the lactation period POP concentrations in the milk were similar or slightly lower than concentrations in the pup blubber (i.e. the milk/pup blubber ratio ~ 1.0, Fig. 2). During late lactation the POP concentrations were higher in the milk than in the pup blubber (i.e. milk/pup blubber ratio > 1.0). This was particularly apparent for the high-chlorinated PCBs and for Mirex (Fig. 2). It should be noted that the high POPs concentrations in the milk during the late lactation period, coincided with the elevated maternal blood concentrations of POPs (Table 2).

During the early phase of lactation, the uptake of the POPs from milk to the blubber of the pups is highly efficient. The slightly higher concentrations of some POPs in the pup blubber compared to the milk (i.e. milk/pup blubber ratio < 1.0), is probably because the pups utilise some of the

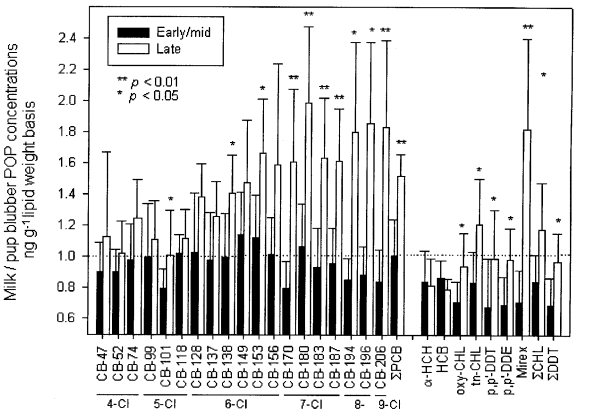

Fig. 2. Milk concentrations of POPs relative to corresponding concentrations (ng g^{-1} lipid) in pup blubber in grey seal (*Halichoerus grypus*) mother–pup pairs. Early lactation ($N=6$) is 5–10 days after parturition, late-lactation ($N=4$) is 12–15 days after parturition. Values are presented as means and error bars are 95% CI. Mann–Whitney *U*-test is used to test statistical significance in difference in the partitioning of POPs between early- and late-lactation.

received milk lipids to cover their energetic requirements. Since the POPs are not metabolised this will result in a higher concentration of POPs in the pups' remaining lipid reserves. Grey seal pups are born with almost no subcutaneous blubber, so the samples collected early in lactation represent newly deposited blubber synthesised from relatively 'clean' milk. As lactation progresses pups deposit more blubber from the increasingly contaminated milk. However, this deposition occurs in the inner blubber layer of the pup, and does not affect the outer 'older' layer where our samples were collected. This can explain why, for instance, ΣPCB and Mirex in pup blubber did not increase between the lactation stages even though it increased significantly in the milk the pups were drinking (Table 2).

In conclusion, for the more water-soluble and/or less chlorinated compounds equilibrium between the different tissues is reached more quickly than for the less water-soluble and/or more chlorinated compounds. This has implications of which matrices to use when assessing the toxicological risk of POPs in the lactating female and her offspring. For instance, the female and proba-

bly her offspring will generally have elevated concentrations of several POPs in their circulation and possible target organs during the late stages of lactation, whereas the POP concentrations in outer part of their blubber will remain relatively stable during the nursing period. It is also noteworthy that the elevated maternal blood concentrations at late lactation coincide in time with their ovulation and mating.

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Negative relationship between PCBs and plasma retinol in low-contaminated free-ranging gray seal pups (*Halichoerus grypus*)

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Abstract

Polychlorinated biphenyls (PCBs) have been shown to affect retinol (vitamin A) homeostasis in adult as well as neonatal seals. The aim of the present study was to examine the relationships between plasma (PL)-retinol status and PCB concentrations in different blood compartments (blood cells (BCs), PL, and whole blood (WB)) of free-ranging neonatal gray seals (*Halichoerus grypus*) and to identify which PCB congeners may be responsible for the PL-retinol-depressing effects of PCBs. PL-retinol concentrations correlated positively with body mass and negatively with ΣPCB (lipid-weight basis, lw) in WB and ΣPCBlw in BCs. ΣPCBlw in WB was the parameter that best described the variation in PL-retinol concentration ($r^2 = 0.455$, $n = 20$, $P = 0.0007$). Furthermore, PL-retinol concentrations correlated with nine of the 15 detected PCB congeners. It is possible that there is an equilibration between the concentrations of metabolizable congeners and their metabolites and that the retinol-depressing effect of PCBs in neonatal gray seals is caused by PCB-OH metabolites that interfere with the formation of PL transport complexes that transport retinol in PL and increase renal excretion of retinol. This suggestion is in accordance with previous mechanistic explanations of the retinol-depressing effect of PCB in rodents.

Keywords: Pinnipeds; Pollution; Persistent organic pollutants; Marine mammals; Arctic pollution

1. Introduction

Polychlorinated biphenyls (PCBs) are man-made chemicals that, due to their persistent and hydrophobic properties, accumulate in organisms and are often biomagnified with increasing trophic level in food webs (Tanabe and Tatsukawa, 1992). Pinnipeds are top predators in many coastal ecosystems and therefore have high body burdens of persistent organic pollutants (Reijnders, 1986; Jenssen, 1996). Retinol deficiency is associated with a diversity of anomalies and defects, such as alterations in both cell-mediated and humoral immunity, leading to decreased resistance to infections (Semba et al., 1992, 1993), growth retardation (Bermudez et al., 1993), and effects on the nervous system (Combs, 1992). Because PCBs are lipophilic, they are transferred from female mammals to their offspring during lactation (Addison and Brodie, 1977; Sormo et al., 2003), and this group of environmental pollutants has been shown to affect several biochemical and physiological factors that regulate the normal development of neonates (Birnbaum, 1994). Because the milk of pinnipeds is particularly rich in lipids (Baker, 1990), the neonatal period of pinniped life appears to be particularly susceptible to PCB exposure and to the harmful effects of this group of contaminants. We have previously found reduced plasma (PL)-retinol concentrations in neonatal gray seals (*Halichoerus grypus*) with increasing blood–cell concentrations of PCBs (Jenssen et al., 1995). Likewise, reduced PL-retinol concentrations have been reported in polar bears (*Ursus maritimus*) with high burdens of PCBs (Bernhoft et al., 1997), in harbor seals (*Phoca vitulina*) fed with PCB-contaminated fish (Brouwer et al., 1989; deSwart et al., 1996), in free-ranging harbor seal pups (Simms et al., 2000), and in experimental studies on many other mammalian species (Brouwer, 1991).

The aim of the present study was to elucidate the effects of PCBs on PL-retinol status in free-ranging

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neonatal gray seals. Thus, relationships between PL-retinol and PCB concentrations in different blood compartments (i.e., blood cells (BCs), PL, and whole blood (WB)) were examined. Furthermore, we aimed at elucidating whether the retinol-depressing effect is related to the concentrations of particular PCB congeners.

2. Materials and methods

Blood samples were collected from 35 gray seal pups in the Froan Nature Reserve (64° 10′ N, 09° 20′ E) on the coast of central Norway. Care and treatment of the animals during blood sampling was conducted in accordance with established national guidelines. The blood samples were collected at the base of one of the hind flippers, from the rich vascular network in the metatarsal region just above the origin of the interdigital webbing in the planar surface (Geraci and Lounsbury, 1993) using vacutainers and a 22 G blood-collecting needle. To protect vitamin A against UV light, the samples were immediately wrapped in aluminum foil, and placed in ice water (0°C) in a coolant box. Following blood sampling, the sex of the pups was determined. The body mass (BM) was determined to the nearest 0.5 kg using a calibrated spring weight (Salter, mod. 235 6S, England), and based on morphological criteria the pups were categorized into one of four age classes (class I: 2.4 ± 4.4 days; class II: 4.8 ± 3.1 days; class III: 12.1 ± 2.9 days; class IV: 16.0 ± 3.0 days) (Kovacs and Lavigne, 1986). For individual recognition upon later encounters, the pups were tagged with numbered PVC tags in the web of one of the hind flippers. The blood samples were transported to a field laboratory and centrifuged (1200 r.p.m., 15 min) within 8 h after sampling. The PL and the BCs from each pup were transferred to separate polyethylene vials and frozen (−18°C). From 20 of the pups, the volume of blood sampled was sufficient to allow a sample of WB to be transferred to cryovials and frozen. All vials were wrapped in aluminum foil immediately after handling.

2.1. PCB analysis

Concentrations of 21 PCB congeners were determined in BC samples from all 35 pups and in WB samples from 20 of the pups. Concentrations of the PCB congeners in PL were calculated in 20 of the pups from the difference in WB and BCs. A short summary of the methods (Bernhoft and Skaare, 1994) is given below.

The lipid fraction was extracted from homogenized WB and BC samples using cyclohexane, distilled water, and acetone. Lipid content in the samples was determined by weighing after the solvents were evaporated. Analyses of the PCB congeners were conducted using a high-resolution gas chromatograph (HRGC 5300 Mega Series; Carlo Erba instrumentation, Milano, Italy) equipped with an electron capture detector (Carlo Erba 63Ni-ECD) and a capillary column (fused silica SPB-5, 60 m, 0.25 mm i.d., 0.25 μm film thickness, Supelco Inc.). The chromatographic data were transferred to a connected computer (Olivetti PC M290) equipped with Maxima 820 chromatography workstation (Millipore waters, Milford, MA, USA). The following 21 PCB congeners were quantified in the samples according to retention times and peak heights (referred to by the IUPAC numbers (Ballschmiter and Zell, 1980)): -28, -52, -74, -99, -101, -105, -114, -118, -128, -138, -141, -153, -156, -157, -170, -180, -183, -187, -194, -206, and -209. The concentrations of ΣPCB as well as the single PCB congeners are presented both on wet-weight basis (ww) and on lipid-weight basis (lw).

2.2. Quality assurance of PCB analysis

Percent recovery was calculated for each series of 12 samples by adding a known amount of a PCB standard (PCB-22; 100 ng/mL) to duplicate samples of unexposed pig fat and including them in the sample series. The PCB standard (obtained from Cambridge Isotope Laboratories, Woburn, MA, USA) contained the 22 previously mentioned PCB congeners. The amount of pig fat was adjusted to approximate the lipid content of the seal blood samples (0.03–0.06 g). The mean recoveries were 100% in the blood samples and 120% in the WB samples. Thus, the PCB concentrations in the WB samples were adjusted with 20%, while the PCB concentrations in the BC samples were not corrected for recovery rates. Unfortunately, the small sample amounts rendered duplicate analysis impossible. Quantification was carried out within the linear range of the detector, and the detection limits of the different PCB congeners varied between 5–15 ng/g lipid. Prior to preparation tetrachloronaphtalene (TCN; 10 ng/mL) was added to each sample as an internal standard. Reproducibility was continuously tested by adding a duplicate control sample (seal blubber from a seal with a known contamination level) to each series of 12 samples. The laboratory (Environmental Toxicology Laboratory, Norwegian School of Veterinary Science, Oslo, Norway) has participated in several intercalibration tests for organochlorine measurements over the past decade. These tests, organized by WHO/UNEP (The World Health Organization/United Nations Environmental Program) and ICES/IOC/OSPARCOM (International Council for Exploration of the Sea/International Oceanographic Commission/Oslo-Paris Commission) have confirmed good competence in PCB determination in marine material for this laboratory compared to other participating laboratories (Bernhoft and Skaare, 1994).

2.3. Retinol analysis

Retinol concentrations were determined in blood PL from all 35 pups using high-performance liquid chromatography (HPLC) with fluorescence detection (Shearer, 1986). Proteins were denaturated using ethanol. Because in seal PL a majority of retinol is in unesterified form, no saponification or analysis of retinol esters was performed, and retinol was extracted with *n*-hexane. The extraction procedure was repeated twice. Following evaporation to dryness, the residue was redissolved in the mobile phase (99.9% HPLC-methanol). An aliquot of 20 μL was injected automatically (Pharmacia LKB autosampler 2157) into the HPLC column (LKB-Bromma 2155 HPLC-column oven). The flow rate of the mobile phase (99.9% methanol) was set to 1 mL/min. Retinol (vitamin A1) was quantified with a fluorescence detector (Pharmacia LKB Fluorescence Detector 2144) by peak integration in relation to standards of retinol (Sigma R-7632) in ethanol. All PL levels of retinol are presented on a ww basis.

2.4. Quality assurance of retinol analysis

Due to the photoreactivity of retinol, all sample handling and experimental steps were carried out in subdued light. All determinations were made in duplicate, and the sensitivity of the assay was improved so that the 200-μg/L PL samples could be assayed with less than a 15% coefficient of variation (CV). In the few cases where the CV exceeded this, new duplicates were made and analyzed. Reproducibility was continuously tested by adding a duplicate control sample to each series of five samples. The control samples consisted of PL from one seal pup from which particularly much blood had been collected. The reproducibility was somewhat variable, but, nevertheless, considered acceptable (mean = 172.2 μg/L, standard deviation (SD) = 50, range = 111.1–294.4 μg/L, $n = 19$). The calibration graph of peak ratios of all-*trans*-retinol was linear over the range 50–1300 μg/L ($r^2 = 0.993$, $P < 0.0001$; simple regression analysis).

2.5. Statistics

The results were examined for normality distribution (Shapiro–Wilcoxon test). The only variables that were normally distributed were BM and PL-retinol concentration. Variables that did not conform to normality were log transformed ($\log[x+1]$), whereby normality distribution was obtained. Parametric tests were thus used for statistical analysis. Paired or unpaired student *t* test was used to compare data sets. When appropriate, ANOVA followed by Dunn/Bonferroni post hoc tests were applied. Relationships between variables were tested using correlation matrixes, and standard Bonferroni correction was applied when appropriate (Rice, 1989). Forward stepwise regression analysis was performed to test which of the independent variables best explained the variation in the dependent variable. In data sets that were normally distributed, Values are presented as means ± SD. Values are presented as geometric means (GM), median (M), and standard deviation (SD). When appropriate, coefficient of determination (r^2) or other coefficients, number of samples (n) and level of significance (P) are also given. A value of $P < 0.05$ was defined as significant. Significance levels following Bonferroni correction are given in the text or tables. Computing was performed on a Macintosh computer equipped with statistical software (Statview 4.02 and SuperANOVA, Abacus Concepts Inc., Berkeley, CA, USA).

3. Results

The BM of the pups differed between the age classes (Table 1, ANOVA, $F = 17.11$, $P < 0.0001$). There were significant differences in BM between pups in age classes I and II, I and III, I and IV, and II and III. There were no differences in BM between the age classes II and IV and III and IV. Thus, the BM of the pups increased with age until the pups reached age class III, where it stabilized. The PL-retinol concentration also varied between the age classes (Table 2, ANOVA, $F = 7.014$, $P = 0.0010$). The retinol concentration was significantly lower in age class I than in the age classes III and IV and also lower in age class II than in III. There were no differences in the retinol concentration between the age groups I and II, II and IV, and III and IV. Thus, the retinol-PL concentration increased with age until age class III, where it stabilized.

There was no differences between the age classes with respect to any of the other examined variables, i.e.,

Table 1
Mean body mass (BM, kg), ±SD, 95% confidence interval (CI), and minimal (Min) and maximal (Max) body mass in different age classes of gray seal pups from Froan, Norway

Age class	Sample size	Mean BM (kg)	SD	95% CI	Min	Max
I	6	19.2[a]	1.4	1.1	17.5	21.0
II	9	32.3[b]	4.3	2.8	26.5	39.0
III	10	43.7[c]	7.8	4.9	26.0	55.0
IV	10	40.0[b,c]	9.7	6.0	21.5	53.0

ANOVA, Dunn/Bonferroni post hoc test: I vs II, $P = 0.0006$; I vs III, $P < 0.0001$; I vs IV, $P < 0.0001$; II vs III, $P = 0.0024$; II vs IV, $P = 0.0.0329$; III vs IV, 0.2929.
Significance level following Bonferroni correction, $P = 0.01$.
The means with no common letter differ significantly. Each pup was placed into one of the age classes based on morphological criteria (Kovacs and Lavigne, 1986).

Table 2
Mean PL-retinol concentration (μg/mL), ±SD, 95% confidence interval (CI), and minimal (Min) and maximal (Max) body mass in different age classes of gray seal pups from Froan, Norway

Age class	Sample size	Mean retinol	SD	95% CI	Min	Max
I	6	300.5[a]	84.3	67.5	189	389
II	9	418.6[a,b]	265.2	173.3	144	861
III	10	800.5[c]	241.5	149.7	478	1244
IV	10	784.3[b,c]	362.6	214.3	161	1422

ANOVA, Dunn/Bonferroni post hoc test: I vs II, $P = 0.384$; I vs III, $P = 0.0007$; I vs IV, $P = 0.0010$; II vs III, $P = 0.0075$; II vs IV, $P = 0.0101$; III vs IV, $P = 0.9042$.
Significance level following Bonferroni correction, $P = 0.01$.
The means with no common letter differ significantly. Each pup was placed into one of the age classes based on morphological criteria (Kovacs and Lavigne, 1986, see Methods for explanation).

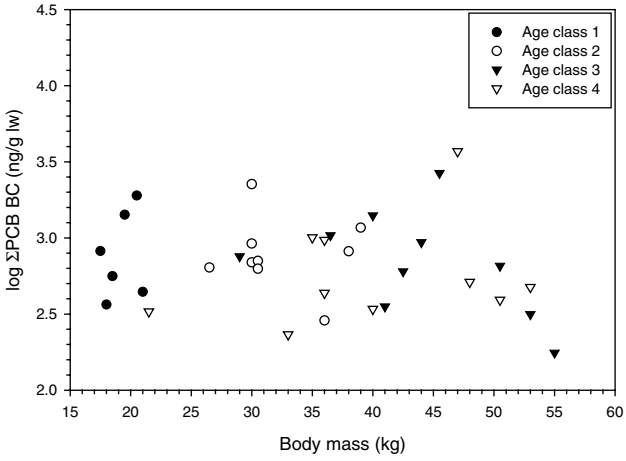

Fig. 1. There was no relationship between log ΣPCBlw (ng/g) in BC and BM (kg) in neonatal gray seals (*H. grypus*) from Froan, Norway.

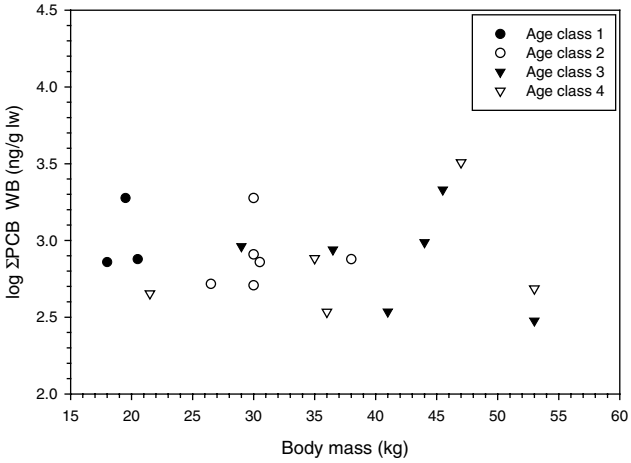

Fig. 2. There was no relationship between log ΣPCBlw (ng/g) in WB and BM (kg) in neonatal gray seals (*H. grypus*) from Froan, Norway.

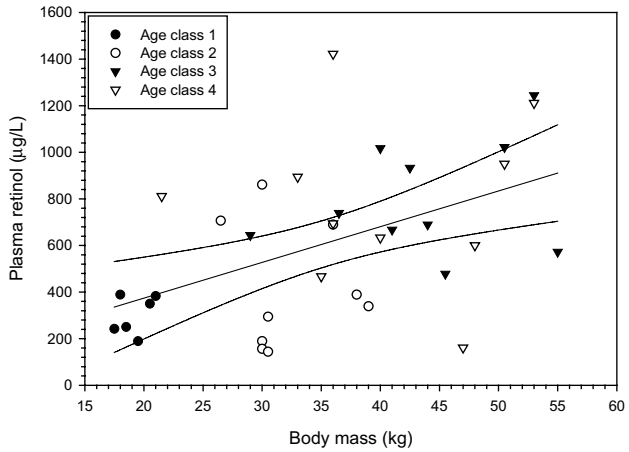

Fig. 3. The PL-retinol concentration (mg/L) increased as a function of increasing BM (kg) in gray seals (*H. grypus*) pups.

extractable lipid content (in BC, WB, or PL), ΣPCBww, or ΣPCBlw (in BC, WB, or PL) (ANOVA, $P > 0.05$). The concentrations of ΣPCBlw in BC and WB as a function of the BM of the pups are shown in Figs. 1 and 2, respectively. The only variable that correlated with the BM of the pups was the PL-retinol concentration (Fig. 3, $r^2 = 0.249$, $P = 0.0266$). Thus, because all other variables were independent of age class and BM, values are listed for the sample population in Tables 3,4 and 5.

The extractable lipid content in PL was significantly higher than in WB, but not in BC. Lipid content in WB was significantly higher than in BC (Table 3). ΣPCBww in PL was significantly higher than in both BC and WB, whereas ΣPCBww in WB was significantly higher than in BC (Table 4). There were no differences in ΣPCB1w between the three blood compartments.

There was no relationship between PL retinol and the lipid content in the three different blood compartments (WB, $r = -0.124$, $P = 0.6079$; BC, $r = -0.190$, $P = 0.4272$; PL, $r = -0.201$, $P = 0.3940$; level of significance, $P = 0.025$). Multiple correlation matrix showed that retinol-PL level correlated with five of the PCB variables, ΣPCBlw and ΣPCBww in WB, ΣPCBlw in BC, and ΣPCBlw and ΣPCBww in PL (Table 6). Because the PL retinol concentration also correlated with BM, forward stepwise multiple regression was conducted to find which of these independent variables best described the variation in retinol. The results showed that the independent variables ΣPCBlw in WB and BM described 62% of the variation in the PL-retinol concentration ($r^2 = 0.624$, $n = 20$, $P < 0.0001$). ΣPCBlw in WB was the single independent parameter that described the variation in PL retinol best (Fig. 4, $r^2 = 0.455$, $n = 20$, $P = 0.0007$).

Of the 21 PCB congeners analyzed, 15 congeners (PCB-101, -99, -149. -118, -153, -105, -138, -187, -128, -156, -180, -170, -194, -206, and -209) were found in detectable concentrations in all samples. To examine if

Table 3
Extractable lipid content (%) in WB, BCs, and PL of gray seal pups from Froan, Norway. Lipid content in the PL is estimated from differences in corresponding WB and BC samples

Variable	*n*	GM	*M*	SD	Min	Max
WB extract lipid	20	0.75	0.76[a]	0.23	0.36	1.31
BC extract lipid	35	0.36	0.38[b]	0.15	0.21	0.82
PL extract lipid	20	1.13	1.21[b]	0.43	0.43	2.06

WB vs BC (unpaired *t* test), $t = 4.068$, d.f. = 53, $P < 0.0001$; WB vs PL (paired *t* test), $t = 11.63$, d.f. = 19, $P < 0.0001$; BC vs PL (unpaired *t* test), $t = 1.519$, d.f. = 53, $P = 0.135$.
Significance level following Bonferroni correction, $P = 0.025$.
Data are presented as geometric mean values (GM), mean (M), standard deviation (SD) minimum value (Min) and maximum value (Max). The means with no common letter differ significantly ($P < 0.025$).

Table 4
Concentration of ΣPCBww (ng/g) in WB, BCs, and PL of gray seal pups from Froan, Norway. The concentration in PL is estimated from differences in corresponding WB and BC samples

Variable	*n*	GM	*M*	SD	Min	Max
WB PCBww	20	6.03	7.74[a]	6.90	2.35	28.03
BC PCBww	35	2.44	3.26[b]	3.11	0.65	14.91
PL PCBww	20	8.51	11.42[c]	10.21	3.06	41.16

WB vs BC (unpaired *t* test), $t = 4.158$, d.f. = 53, $P < 0.0001$; WB vs PL (paired *t* test), $t = -14.068$, d.f. = 19, $P < 0.0001$; BC vs PL (unpaired *t* test), $t = 6.00$, d.f. = 53, $P = < 0.001$.
Significance level following Bonferroni correction, $P = 0.025$.
Data are presented as geometric mean values (GM), mean (M), standard deviation (SD), minimum value (Min) and maximum value (Max). The means with no common letter differs significantly ($P < 0.025$).

Table 5
Concentration of ΣPCBlw (ng/g) in WB, BCs, and PL of gray seal pups from Froan, Norway. The concentration in the PL is estimated from differences in corresponding WB and BC samples

Variable	*n*	GM	*M*	SD	Min	Max
WB PCBlw	20	799	977[a]	717	299	3218
BC PCBlw	35	684	882[ab]	745	175	3702
PL PCBlw	20	689	846[b]	608	181	2858

WB vs BC (unpaired *t* test), $t = 8.817$, d.f. = 53, $P = 0.4173$; WB vs PL (paired *t* test), $t = 1.719$, d.f. = 19, $P = 0.102$; BC vs PL (unpaired *t* test), $t = 0.445$, d.f. = 53, $P = 0.6584$.
Significance level following Bonferroni correction, $P = 0.025$.
Data are presented as geometric mean values (GM), mean (M), standard deviation (SD), minimum value (Min) and maximum value (Max). The means with no common letter differ significantly ($P < 0.025$).

the variation in retinol could be subscribed to one or more of the PCB congeners, relationships between retinol and each of the 15 detected PCB congeners were analyzed. Multiple correlation matrix showed that in WB there were significant relationships between retinol concentration and nine of the PCB congeners (Table 7: PCB-149, -128, -99, -118, -138, -101, -153, -187, -105).

Table 6
Correlation coefficients and levels of significance in the relationships between ΣPCB variables and PL-retinol concentration in gray seal pups from Froan, Norway

Variable	*n*	Correlation coefficient	Level of significance
WBPCBlw	20	−0.696	0.0004*
BCPCBlw	34	−0.670	0.0008*
PLPCBlw	20	−0.624	0.0030*
WBPCBww	20	−0.612	0.0034*
BCPCBww	34	−0.400	0.0107
PLPCBww	20	−0.631	0.0032*

*Significant following Bonferroni correction ($P < 0.01$).

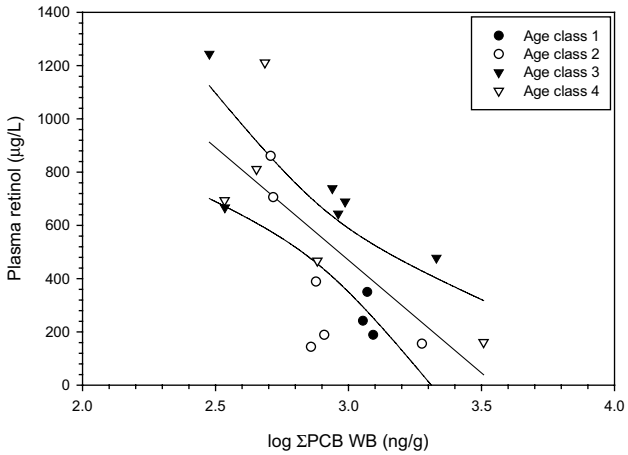

Fig. 4. The PL-retinol concentration (mg/L) decreased as a function of increasing log ΣPCBlw (ng/g) concentration in WB of gray seals (*H. grypus*) pups. The results also indicate that there were negative correlations between log ΣPCBlw (ng/g) and retinol concentration within each age class (see text for further description).

4. Discussion

There were significant negative correlations between the PL-retinol concentration and the ΣPCB concentrations in all blood fractions (WBlw, WBww, BClw, PLlw, and PLww). Forward stepwise regression analysis showed that of the independent variables that correlated with retinol, ΣPCBlw in WB was the single independent variable that affected the variation in PL-retinol concentration most, explaining 46% of the variation in PL retinol (Fig. 4, $r^2 = 0.455$). The negative correlation between PL-retinol concentration and PCB concentrations in blood documented in this study is in accordance with previous reports in many other mammal species, such as harbor seals (Brouwer et al., 1989), polar bears (Bernhoft et al., 1997), and rodents (Byrne et al., 1987;

Table 7
Correlation coefficients and levels of significance in correlations between PCB congeners in WB samples (lw) and PL-retinol concentration in gray seal pups from Froan, Norway

Congener number	Correlation coefficient	*P* value
101	−0.722	0.0002*
99	−0.730	0.0001*
110	−0.035	0.8839
149	−0.833	<0.0001*
118	−0.725	0.0002*
153	−0.702	0.0003*
105	−0.635	0.0020*
138	−0.722	0.0002*
187	−0.648	0.0014*
128	−0.737	<0.0001*
156	−0.222	0.3514
180	−0.528	0.0155
170	−0.598	0.0044
194	−0.304	0.1949
206	−0.269	0.2552
209	−0.087	0.7202

*Significant following Bonferroni correction ($P<0.0033$).

Chen et al., 1992). It should be noted, however, that it has been reported that chronic exposure to some PCB congeners (PCB-126, -169, -156) has resulted in increased PL-retinol levels in rodents (van Birgelen et al., 1994a, b).

In a similar study on harbor seal pups in Washington State and British Columbia (Simms et al., 2000), it was reported that pups from sites polluted by PCBs, PCDDs, and PCDFs had lower PL-retinol levels than pups from less contaminated sites. However, further analysis of these results revealed that PL-retinol levels were positively correlated with contaminant level (PCBs, PCDDs, PCDFs) in blubber and that there were confounding effects of nutritional status (Simms and Ross, 2000a). Thus, the lower retinol levels in the pups from the polluted sites most likely reflected their weaned status (Simms et al., 2000). In the present study, there were negative relationships between ΣPCBlw in WB and PL retinol in all four age classes (Fig. 4). Even though the relationships within each age class were not significant, mainly due to the low sample sizes within each group, the results strongly indicate that PCBs influence PL levels of retinol in gray seal pups regardless of their nursing status. Because fluctuations in contaminants between the blood and blubber compartments probably are highly influenced by the nutritional status of the pups, the positive relationship between PL-retinol concentration and contaminant level reported in harbor seal pups from western USA and Canada may be related to the fact that contaminant levels in these particular animals were measured in blubber samples (Simms et al., 2000). In the present study, a stepwise multiple regression model revealed that ΣPCB in WB and BM described 62% of the retinol variation. Thus, it is apparent that BM as well as nutritional status (Simms et al., 2000) has confounding effects on the PL-retinol levels, and we support the conclusion reached by Simms and Ross (2000b) that confounding factors must be characterized before retinoids can be used as an effective indicator of adverse health effects related to elevated levels of environmental contaminants.

The low retinol concentrations in the newborns are consistent with previous reports of low liver-retinol concentrations in neonatal gray, hooded (*Cystophora cristata*), harp (*P. groenlandica*), and harbor seals (Rodahl and Davies, 1949; Schweigert et al., 1987; Simms and Ross, 2000a). The increase in PL-retinol concentration in older pups is in accordance with results from a similar study on harbor seal pups from western USA and Canada (Simms and Ross, 2000a) and is due to transfer of retinol from the mother to the pup via the milk (Schweigert and Stobo, 1994). After weaning, the retinol is at first released efficiently from short-time storage in the liver, and the release from adipose tissues during lipid metabolism is slow (Schweigert and Stobo, 1994). Thus, PL-retinol levels remain relatively stable in newly weaned pups. The PL-retinol concentrations in pups from Froan were similar to those reported in adult Dutch harbor seals, which were fed low-level PCB-contaminated fish from the north Atlantic (380–580 μg/L) (Brouwer et al., 1989), and in healthy free-ranging harbor seal pups from western USA and Canada (144 ± 13.9 μg/L in newborns and 431 ± 35.8 μg/L in weaners (Simms and Ross, 2000a)). The present retinol concentrations were, however, somewhat higher than serum concentrations previously reported in suckling gray seal pups and adult males from Sable Island, Canada, which were 272 ± 159 μg/L ($n=5$) and 260 ± 56.7 μg/L ($n=12$), respectively (Schweigert et al., 1987). Since PCB burdens in gray seal pups at Froan and Sable Island do not appear to differ (Addison and Stobo, 1993; Jenssen, 1996), the difference in retinol levels between the populations is probably not caused by the retinol-depressing properties of PCBs. The difference, however, can be due to the use of different analytical methods and/or to different handling of the samples.

Organochlorines seem to affect retinol homeostasis through several mechanisms. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, non-ortho and mono-ortho PCB congeners appear to cause reductions in the retinol content of the liver as well as in other organs (Håkansson and Hanberg, 1989; Brunström et al., 1991; Murk et al., 1998), which may lead to increases in PL-retinol levels (Håkansson and Ahlborg, 1985). These effects could be mediated through binding of pollutants to the cystolic Ah receptor. However, herein there was a negative relationship between pollution load and PL-retinol concentration.

Many PCB congeners are metabolized in the cytochrome *P*450 system to form hydroxymetabolites of

PCBs (PCB-OHs). It has been shown that some PCB-OHs have structural similarities with thyroxin (T4), which allows them to bind to binding sites for T4 on transport proteins, such as transthyrethin (TTR). In the PL, retinol is transported bound to a retinol-binding protein (RBP), which is bound in a complex to TTR. The PL-retinol-depressing effect of PCBs is a result of these metabolites binding to TTR and causing a conformational change in TTR so that the RBP–TTR complex is not formed, and PL-retinol concentration is reduced due to glomerular filtration (Brouwer and van den Berg, 1986a, b; Brouwer et al., 1990; Brouwer, 1991).

In the present study the retinol concentration in the PL correlated significantly with nine of the 15 quantified PCB congeners (Table 7, PCB-149, -128, -99, -118, -138, -101, -153, -187, -105). The PCB congeners that did not correlate with PL retinol are characterized by being relatively highly chlorinated (>7 Cl atoms) and persistent against metabolic degradation. Of the congeners that did correlate with PL retinol, six (PCB-128, -99, -118, -138, -101, -105) are congeners that have vicinal H atoms and thus potentially can be metabolized to PCB-OHs. It is possible that there is an equilibration between the concentrations of the above-mentioned congeners and their metabolites and that the retinol-depressing effect of PCBs in the gray seal pups may be linked to binding of PCB-OH metabolites to the binding sites for T4 on TTR. As previously mentioned, this will hinder the formation of the TTR–RBP complex resulting in increased renal excretion of the retinol–RBP complex (Brouwer and van den Berg, 1986a, b; Brouwer et al., 1990; Brouwer, 1991). It should be noted, however, that the studies on PCB-OHs and their interactions with the TTR–RBP compelex are rat studies. In phocids, the importance of TTR compared to other thyroid-hormone-carrying proteins is unknown. For example, PCB-OHs do not inhibit the binding of thyroid hormones to other major transport proteins such as thyroxin-binding globuline, which is the most important thyroid hormone transport protein in humans and non-rodents (Lans et al., 1994; Cheek et al., 1999; Meerts et al., 2002). This may indicate that PCB-OHs have a direct effect on RPB in seal pups.

A third possible mechanism involves the possibility that PCBs may inhibit hepatic retinyl ester hydrolase, which transforms retinol esters into retinol that is released into circulation. The mechanisms for this inhibition seem to be unknown (Chen et al., 1992). A fourth mechanism that could cause depressed PL-retinol concentrations involves the direct histological and structural damaging effects of PCBs on the liver.

The PCB concentrations in the gray seal pups were relatively low as compared to other studies where reduced PL-retinol concentrations have been linked to PCB exposure. For instance, in free-ranging adult polar bears PL-retinol concentrations seem to be affected at PCB concentrations in BCs of 5000–7000 ng/g lw (Bernhoft et al., 1997), which is nearly 10 times higher than the concentrations reported herein. Even though in a different way, retinoid dynamics also have been reported to be affected by low levels of contaminants in free-ranging harbor seals pups (Simms and Ross, 2000a). This could indicate that seal pups, and perhaps neonatal mammals in general, are especially sensitive to retinoid-related effects of PCB exposure.

5. Conclusions

ΣPCBlw in whole blood (WB) described 46% of the variation in PL-retinol concentration in gray seal pups. Six of the congeners that were identified to correlate best to PL-retinol levels can potentially be metabolized to PCB-OHs. This suggests that the negative relationship between polychlorinated biphenyls (PCBs) in blood and PL-retinol were related to interference between PCB metabolites and PL-transport proteins for retinol. The fact that the PCB burdens were relatively low in the seal pups indicate that seal pups, and perhaps neonatal mammals in general, are especially sensitive to retinoid-related effects of PCB exposure. However, even though ΣPCBlw in WB was the single independent variable that best described the variation in PL retinol, the study also demonstrated that BM had confounding effects. Thus, in order to apply retinoids as a biomarker of exposure or effects to elevated levels of environmental contaminants, confounding factors should be identified, especially in developing animals.

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