Control of Toxic Chemicals in Puget Sound Phase 3:

Toxic Contaminants in Harbor Seal (*Phoca vitulina*) Pups from Puget Sound

Washington Department of
FISH and WILDLIFE

Toxic Contaminants in Harbor Seal (*Phoca vitulina*) Pups from Puget Sound

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Acronyms, Abbreviations and Units

Acronyms and abbreviations used frequently in this report are listed below, those used infrequently are excluded.

AhR	Aryl hydrocarbon receptor
DDT	Dicholorodiphenyltrichloroethane
ERα	Estrogen receptor alpha
FT3	Free thyroxine
FT4	Free triiodothyronine
GR	Glucocorticoid receptor
Hg	Mercury
Hsp 70	Heat shock protein 70
OC	Organochlorine
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
POP	Persistent organic pollutant
PPARγ	Peroxisome proliferator-activated receptor gamma
TH	Thyroid hormone
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta
TT3	Total triiodothyronine
TT4	Total thyroxine
Vit D	Vitamin D receptor

Units of Measurement

dw	Dry weight
g	Gram
lw	Lipid weight
mL	Milliliters
mm	Millimiters
µg/g	Micrograms per gram
‰	Permille

Abstract

Harbor seals are non-migratory, occupy a high position in the Puget Sound food web, and consume a variety of prey species, making them useful indicators of marine ecosystem health. A history of industrialization and a vulnerable receiving environment has resulted in the contamination of Puget Sound with a multitude of contaminants, including chemicals deemed to be persistent, bioaccumulative and toxic. These include the polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polychlorinated dibenzo-*p*-dioxins (PCDDs or dioxins), polychlorinated dibenzofurans (PCDFs or furans), and the organochlorine (OC) pesticides, as well as the organic form of the metal mercury (Hg). Regulations and mitigation measures rely on up-to-date measurements in indicator species, and an understanding of what, if any, health impacts are due to any of the contaminants of concern. To this end, we live-captured 24 harbor seal pups in 2009 from four sites in Puget Sound designated in Ecology's box model as Hood Canal (south), Whidbey Basin, Main Basin, and South Sound (east), and collected blood, fur and skin/blubber biopsy samples. Stable isotopes, contaminant concentrations, thyroid hormones, vitamin A, and 11 genomics endpoints were measured in these animals, providing insight into feeding ecology, contaminant trends over space, and effects on their health. Results indicate that Hood Canal (south) seal pups are least contaminated, with Main Basin seal pups having up to four times higher PCB, PBDE, OC pesticide and Hg concentrations. While a small sample size precluded a full evaluation of health effects, we were able to determine that vitamin A, estrogen receptor-alpha, heat shock protein-70, and peroxisome proliferator-activated receptor in Puget Sound harbor seal pups were influenced to varying degrees by contaminant levels. Among the chemical classes measured, the PCBs continue to dominate in terms of both concentration and health risk to harbor seals. While current PCB concentrations continue to present a health risk to harbor seals, the lower levels relative to previous reports suggest an improving PCB trend in Puget Sound's aquatic biota.

Introduction

Persistent and fat-soluble environmental contaminants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins / dibenzofurans (PCDDs/Fs), organochlorine pesticides, and polybrominated diphenyl ethers (PBDEs) are widely distributed in the marine environment and accumulate in the aquatic food chain. Similarly, mercury (Hg), in the form of methylmercury (MeHg) also has the ability to bioaccumulate. Fish and wildlife are exposed to high concentrations of these compounds in many parts of the industrialized world. Despite the implementation of regulations on the production and use of several of these chemicals, there has been little change reported in the concentrations of many of these chemicals in biota in Europe and North America since the mid-1980s (Calambokidis *et al.* 1999; Olsson *et al.* 2000). This likely reflects a combination of continued leakage from storage sites, cycling among environmental compartments such as sediment, air, water, and long-range atmospheric transport from distance sources, where they may still be in use (e.g. developing nations, Asia) (Noël *et al.* 2009; Aguilar and Borrell 2005).

PCBs were used as electrical transformer and capacitor fluids, flame retardants, hydraulic lubricants, sealants, and paints because of their heat resistance and good insulating capacities. They were banned in the 1970s in most industrialized countries resulting in a decrease in their environmental concentrations (Muir *et al.* 1999). PCDD/Fs have no commercial or domestic uses. They are formed as by-products during different industrial and thermal processes such as emissions from metallurgical industries, municipal incinerators, pulp and paper mills, and the manufacture of chlorinated chemicals. Similarly to PCBs, regulations on PCDD/Fs emissions resulted in a decrease in their environmental concentrations. DDT is one of the most widely reported organochlorine pesticides found in marine mammals. It was used to control insects on agriculture crops as well as insects that were vectors of human diseases such as malaria and typhus. The progressive reduction of agricultural uses of DDT in the developed world led to its almost total withdrawal by the mid 1980s. In contrast to those highly regulated products, PBDE levels, chemical still widely used as flame retardants, are increasing rapidly in a variety of biota (Elliott *et al.* 2005; Lebeuf *et al.* 2004). All three commercial formulations (Penta-, Octa-, and Deca-BDE) are now banned in Europe and Canada. While Penta- and Octa-BDE were removed from the US market at the end of 2004, Deca-BDE remains largely in use although some States, including Washington, have moved to regulate this product. Recent studies have reported a possible decline of PBDE concentrations in polar bears (*Ursus maritimus*) from western Hudson Bay and as well as in ringed seals (*Pusa hispida)* from the western Canadian Arctic suggesting a potential effect of restrictions and bans of Penta- and Octa-BDE (McKinney *et al*. 2010; de Wit *et al.* 2010). In addition to those country specific regulations, several persistent organic pollutants (including PCBs, PCDDs, PCDFs), pesticides such as DDT, and recently tetra-, penta-, hexa- and hepta-BDEs are listed under the Stockholm Convention, a global treaty that requires parties to take measure to eliminate or reduce the release of these contaminants in the environment. In Asia, there are no current regulations on the use of the three PBDE mixtures (http://bsef.com/).

As opposed to those man-made chemicals or by-products, Hg is emitted from both natural (~60% of the total Hg atmospheric emissions) and anthropogenic sources. Electric power generation facilities using coal are the number one source of anthropogenic Hg contributing more than 50% of the total anthropogenic emissions. In Canada, most European countries, and Japan, there are

regulations to limit mercury emissions from coal fired power plants. No efficient regulations are currently in place in the United States, China, India, Russia and other countries from the former Soviet Union. Emissions of anthropogenic Hg have been increasing since the industrial revolution. Asia is currently the major emitter of Hg and its contribution is expected to become more significant due to anticipated increases in emissions related to their fast economic development, particularly in China (Pacyna *et al.* 2010).

Over the past 20 years, researchers from the Puget Sound Assessment and Monitoring Program (PSAMP), including researchers from the Washington Department of Fish and Wildlife (WDFW), have monitored and assessed a wide range of bioaccumulative and other Persistent Organic Pollutants (POPs) in a number of species. They studied important ecological guilds, including a variety of fish and marine mammals i.e. harbor seals, that are considered to be sentinel species in Puget Sound. These efforts have provided a picture of the geographic extent of ecosystem contamination by POPs, the magnitude of contamination, and temporal trends in these patterns. In addition, monitoring and assessment studies have raised questions regarding the pathways by which POPs from terrestrial sources find their way into the Puget Sound food web, and why Puget Sound's pelagic food web exhibits an unusually high exposure to some POPs (O'Neill and West 2009; West *et al.* 2008).

Long-term PSAMP and WDFW studies support the hypothesis that benthic (bottom-dwelling) species reflect contaminant conditions in sediments. However, assessments of pelagic (open water) species, such as Pacific herring (*Clupea pallasi*), suggest that the pelagic food web is more directly linked to POPs that occur in Puget Sound waters and pelagic biota (rather than sediments). Pacific herring hold unusually high tissue burdens of bioaccumulative POPs (e.g., PCBs), an observation that is not typically predicted from sediment-as-source models. In addition, other research indicates that PCBs and PBDEs have biomagnified in Puget Sound harbor seals (*Phoca vitulina*) and killer whales (*Orcinus orca*) to levels that have impaired their health including reproductive impairment, immunotoxicity, endocrine disruption, and developmental malformations (Hickie *et al.* 2007).

Department of Ecology Phase 1 and Phase 2 toxics loading and modelling studies found that stormwater and aerial deposition represent the primary conveyance mechanisms for PCBs, PBDEs, and polycyclic aromatic hydrocarbon congeners (PAHs) from terrestrial sources into Puget Sound. These toxicants represent three important POP classes to which Puget Sound biota are exposed in high enough doses to impair their health. Several of these POPs bioaccumulate through the pelagic food web to high-level predators such as salmon, harbor seals, killer whales, seabirds, and humans. However, the pathways of contaminant flow from their abiotic sources to these predators are unclear, making it difficult to prioritize management actions aimed at reducing loading of toxicants, remediating contaminated habitats, or reducing exposure of biota to toxicants. To better protect this biota, we must evaluate: a) where (geographically) POPs enter the pelagic food web from stormwater and the atmosphere; b) the pathways of toxic contaminants within the pelagic food web; and c) the sources of POPs to species at the highest trophic levels (marine mammals, seabirds, and humans).

During the summer of 2009, WDFW scientists conducted sampling surveys of plankton, representative pelagic fish, and harbor seals to document contaminant loading in order to evaluate the extent, as well as the magnitude, of POP exposure in these Puget Sound organisms. These

species or guilds are meant to provide: a) a broad scale evaluation of POP exposure at the lowest trophic levels – i.e., plankton, the putative point of entry of POPs into the food web; b) a better understanding of the role of residency in Puget Sound as a risk factor for POP exposure in pelagic predators; and c) an expanded geographic coverage of exposure and health effects of POPs on harbor seal pups. This report contains data strictly on contaminant levels and health effects on Puget Sound harbor seal pups.

Among marine mammals, the harbor seal has emerged as the primary study species for contaminant science including contaminant-related health effect studies. As top predators, harbor seals serve as an informal sentinel of marine ecosystem contamination by integrating contaminant information from the food chain upon which they depend. Harbor seals are a relatively long-lived species and consume a variety of fish. They do not migrate, so they provide a more 'local' signal of contamination than would, for example, resident killer whales that have seasonal movements and can swim hundreds of miles in a day. Harbor seals are a relatively small marine mammal where capture methods and sampling techniques (e.g. blood sampling, blubber biopsy) have been developed and successfully applied in research programs.

Methods

The Quality Assurance Project Plan was published in December 2009 to describe the objectives of the "Persistent Organic Pollutants in Three Guilds of Pelagic Marine Species from Puget Sound" study. In that document can be found the procedures to be followed to achieve those objectives (West *et al.* 2009). In the present report, we update and further detail the methods employed for field sample collection, laboratory analyses, and data analysis specific to the harbor seal component of the project.

Field Sample Collection

A total of 24 harbor seal pups were live-captured at four different regions in Puget Sound, WA which have been designated in Ecology's box model as Hood Canal (south), Whidbey Basin, Main Basin, and South Sound (east) (Figure 1). The primary method of harbor seal pup capture was using salmon landing nets or by deploying tangle nets from boats. We only sampled harbor seal pups greater than 20 kg in weight. We recorded length and girth measurements for each animal. A plastic identification tag was placed on each hind flipper prior to release (Table 1).

Blood samples were drawn in designated plasma or serum vacuum collection tubes from the extra-dural sinus. These samples were stored on ice for approximately 4 hours before centrifugation and decanting of serum or plasma. Serum and plasma samples were frozen on liquid nitrogen and stored at -80 °C until analysis. Blood collected did not exceed 1 mL per kg for each animal.

Blubber and skin biopsies were taken using sterile 3.5 and 8.0 mm biopsy punches. Blubber core (from skin to muscle) samples were collected from the left side of the animal in the pelvic region. The biopsy site was prepared by shaving the hair with an electric razor, rinsing with isopropyl alcohol, scrubbing with Betadine, and rinsing a second time with isopropyl alcohol. The 8.0-mm biopsy was wrapped in foil, placed into a 5 mL cryovial, and transferred immediately into a dewar filled with liquid nitrogen. This sample was used for vitamin A and POP analysis. The 3.5-mm biopsy was rinsed with buffered saline solution to remove blood, and placed immediately in pre-labeled tubes containing RNAlater solution. These genomic samples were kept in a cooler for 24 hours and then placed in the freezer at -20 °C until analysis. Wounds resulting from the biopsy punch were filled with antiseptic cream and left open to allow drainage. Time of capture and biopsy sampling were recorded.

Hair was collected for stable isotope and mercury analysis in 1.5 mL cryovials and stored dry at room temperature.

Iced or frozen harbor seal samples were shipped via FedEx to the Institute of Ocean Sciences (IOS), Department of Fisheries and Oceans Canada. Samples were packed in styrofoam coolers and sealed with enough wet ice or dry ice to keep a consistent temperature during transit. Samples were tracked via tracking number and communication with Canadian Customs and contacts at IOS.

Harbor seal research activities were conducted under Marine Mammal Protection Act Research Permit 782-1702-05.

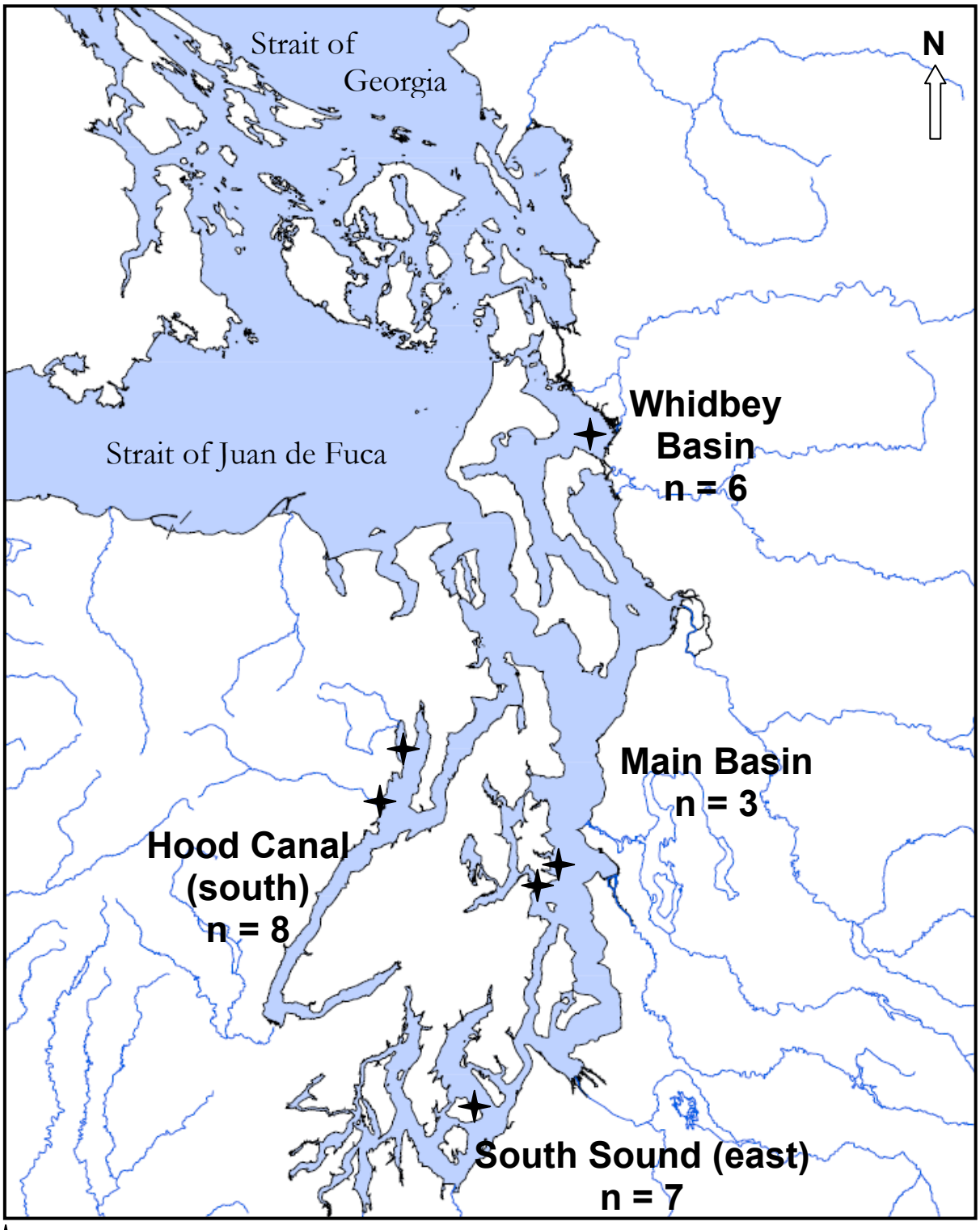


Figure 1: Locations of harbor seal pup live-captures in Puget Sound.

Table 1: Biological information for the 24 live-captured harbor seal pups.

Tag #	Basin[a]	Location	Sex	Weight (kg)	Length (cm)	Girth (cm)	% lipid
Y1586	Hood Canal (south)	Dosewallips River	F	24	91	82.5	91.7
Y1587	Hood Canal (south)	Dosewallips River	F	20	88	71	99.7
Y1588	Hood Canal (south)	Dosewallips River	F	24.5	89	77	97.0
Y1589	Hood Canal (south)	Quilcene Bay	F	20	88	72.5	96.6
Y1590	Hood Canal (south)	Quilcene Bay	F	22	90	72.5	98.7
B1845	Hood Canal (south)	Quilcene Bay	M	21	89.5	73	97.9
Y1591	Hood Canal (south)	Quilcene Bay	F	25	93	82	98.7
Y1592	Hood Canal (south)	Quilcene Bay	F	23	88	72	100.0
B1817	Whidbey Basin	Skagit Bay	M	20	84	68.5	101.2
B1818	Whidbey Basin	Skagit Bay	M	22	85	76.8	99.8
B1819	Whidbey Basin	Skagit Bay	M	21.5	89	71	98.2
Y1561	Whidbey Basin	Skagit Bay	F	22	84	73	98.8
Y1562	Whidbey Basin	Skagit Bay	F	20	78	75	96.5
Y1563	Whidbey Basin	Skagit Bay	F	24	93	76	97.6
Y1564	Main Basin	Orchard Rocks	F	22	91	74.5	75.3
Y1565	Main Basin	Orchard Rocks	F	20.5	90	75	70.4
B1820	Main Basin	Blakely Rocks	M	22	90	74.6	99.4
B1823	South Sound (east)	Gertrude Island	M	23.5	86.5	78	98.2
B1825	South Sound (east)	Gertrude Island	M	20	87	73	97.5
B1836	South Sound (east)	Gertrude Island	M	20.2	93.5	70.5	94.9
B1837	South Sound (east)	Gertrude Island	M	19.8	92	68.5	94.4
Y1585	South Sound (east)	Gertrude Island	F	22	92	70.5	94.8
Y1604	South Sound (east)	Gertrude Island	F	23	101	71	94.6
B1849	South Sound (east)	Gertrude Island	M	22.5	95	74	92

[a] Basin names are based on the map from Herrera, 2010.

Laboratory Analyses

Stable Isotope Analyses

Delta ^{15}Nitrogen and delta ^{13}Carbon were determined in fur samples. Fur (0.1 g) was rinsed in 2:1 chloroform:methanol, then freeze-dried for 48 to 72 hours. Bulk stable carbon and nitrogen isotope ratio (^{15}N:^{14}N and ^{13}C:^{12}C) measurements were carried out at the University of Winnipeg, Manitoba, with equipment and standards described elsewhere (Loseto *et al.* 2008). Isotopic composition was expressed in ‰ notation as the proportional deviation in parts per thousand (‰) of the isotope ratio in a sample from that of a standard:

$$‰X = (R_{sample}/R_{standard} - 1) \times (1{,}000)$$

where X is ^{15}N or ^{13}C, and R_{sample} and $R_{standard}$ are the ratios of ^{15}N:^{14}N or ^{13}C:^{12}C for the sample and standard (Hobson *et al.* 1997). The isotope mass spectrometer was calibrated with reference material in each batch.

PCB, PBDE, PCDD, PCDF, and OC Pesticide Analyses

Each frozen (-80°C) 8 mm tissue biopsy was cut vertically, and the upper skin layer (approximately 2mm) was removed. A portion of each blubber sample (100 mg to 300 mg wet weight) was used for measuring POPs. All congeners of PCBs, as well as specific congeners of PCDDs and PCDFs, were analyzed at the Fisheries and Oceans Canada LEACA (Laboratory of Excellence in Aquatic Chemical Analysis, Institute of Ocean Sciences, Sidney, British Columbia). The 24 seal pup blubber biopsies were organized into three batches of 8 samples. One of the larger samples in each batch was split in order to obtain a replicate sample for quality assurance (QA) purposes. Other QA samples, including a Standard Reference Material (NIST 1945 whale blubber SRM) and two Procedural Blanks containing pure lipid (triolein) to imitate the behaviour of real extracts, were also included in each batch. The procedural blanks, along with weighed amounts of each seal biopsy, replicate and SRM sample, were spiked with a mixture of surrogate internal standards containing ten ^{13}C-labeled PBDEs and thirty ^{13}C-labeled PCBs, ten ^{13}C-labeled PBDEs, and nine ^{13}C-labeled PCDFs and PCDDs obtained from Cambridge Isotope Laboratories (Andover, MA), to enable precise and accurate quantification using the isotope dilution method. Blubber samples were ground with anhydrous sodium sulphate. Using dichloromethane/hexane (1:1 ratio), the samples were extracted from a glass column. A third of the extract was removed and spiked with eleven ^{13}C-labeled organochlorine pesticides and purified separately from the extracts used for PCB, PBDE, PCDD/F analysis. The extracts were evaporated to dryness and weighed. Total lipid concentrations were determined gravimetrically. The residues were resuspended in dichloromethane/hexane (1:1), and analyzed using high resolution gas chromatography and high resolution mass spectrometry (HRGC-HRMS). Details of the chromatography and mass spectrometry conditions, the criteria used for chemical identification and quantification, the quality assurance and quality control practices can be found in Ikonomou *et al*. (2001), report being currently updated to include additional standards and purification procedures that are part of the current method.

Mercury Analyses

Total Hg was measured in fur samples collected from each harbor seal pup. To remove any external contamination, all hair samples were rinsed in series with acetone, de-ionized water, and acetone and left to dry at room temperature. They were then stored in a dessicator until analysis. About 1 mg of hair was analyzed for total mercury using a Zeeman atomic absorption spectrometer RA-915+ coupled with a PYRO-915 attachment (Lumex, St. Petersburg, Russia). The detection limit was 0.002 μg/g dry weight. Details on the methods and instrumentation can be found elsewhere (Sholupov *et al.* 2004).

Two standards were used: a sediment standard NIST 2709 (National Institute of Standards and Technology, Gaithersburg, West Virginia), and a human hair standard NIES 13 (National Institute for Environmental Studies, Ibaraki, Japan). One NIST 2709 and one NIES 13 standard was run every six samples to ensure that there was no deviation from the calibration curve. Seal hair samples were run in triplicates. In every case, variations between triplicates were below 10% which is considered within the instrument / scale / user average precision therefore the average for the three replicates was used and are expressed on a dry weight basis (dw).

Health Indicators

- Thyroid hormone analyses

The concentrations of total thyroxine (TT4), free thyroxine (FT4), total triiodothyronine (TT3), and free triiodothyronine (FT3) were measured in harbor seal pup serum using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer recommended protocol (Calbiotech, California). Frozen (undiluted) serum sample aliquots were thawed on wet ice, and four TH measurements of each sample were obtained within 6 hours in order to avoid repeated freeze-thaw cycles, which can lower the quality of samples. Serum samples were incubated with horseradish peroxidase- (HRP-) labeled hormones in anti-TH antibody coated polystyrene microtiter plates. HRP-labeled hormone and native hormones competitively bind to the antibodies on the wells. After washing off the unbound hormones, the amount of enzyme-labeled hormones was measured by adding substrate: a mixture of 3,3’,5,5’- tetramethylbenzidine (TMB), which changes color by reacting with the HRP. The color intensity of seal serum samples and TH standards will be measured at 450 nm on a MRX microplate reader (Dynatech Laboratories Inc. in Chantilly, Virginia). For each ELISA, reactions were prepared in triplicate, and the sample data was subsequently averaged and compared to the standard curve in order to obtain representative TH concentration values.

Inter-assay variation was evaluated in two ways. The first method employed the regular inclusion of a reference pooled seal serum sample, whereby results were accepted for an assay only when standard results are ± 20% of expected values. In the second method, total hormone measurements (TT3 and TT4) was validated using the manufacturer’s reference standard (Thyroid Cal-ver™ reagent; Casco Neal, Portland, Maine), and results were accepted for an assay only when concentrations were within ± 5% of the expected values.
No purified harbor seal thyroid hormones are commercially available. With this in mind, we validated the thyroid hormone assays for harbor seals by conducting analyses of serial dilutions within a fixed sample volume, and using incremental spikes of seal serum with Thyroid Cal-ver™ reagent. Responses of serial dilutions of seal serum and standard additions of seal serum

with the reference standard both produced linear results. More detailed methods can be found in Tabuchi *et al.* (2006).

- Vitamin A analyses

Skin and blubber biopsy samples required hydrolysis to extract the retinoids quantitatively due to the small sample size and rough texture. In this method, as established by Vahlquist *et al* (1990), all retinol-esters are hydrolysed into all-trans retinol. Several compounds then appear in a common "total retinol" HPLC peak, improving the detection limit of the assay. Also, there is no need for further homogenization. Briefly, samples were saponified in ethanolic KOH solution (10 mL ethanol and 1.6 g KOH per gram sample) in the presence of 0.1% butylated hydroxytoluene to prevent oxidative degradation and TMMP-OH (all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol) as an internal standard. Tubes were flushed with nitrogen and sealed before a 30-minute incubation in a water bath of approximately 80°C. Processed tissue samples were immediately cooled afterwards, and an equal amount of HPLC grade water was added to stop the reaction. Retinol was extracted twice by adding n-hexane (2 mL) and shaking for 3 minutes. The organic layer was evaporated, dissolved in methanol:dichloromethane (9:1), and immediately used for HPLC-analysis. All work was performed under yellow light to prevent vitamin A degradation.

- Gene expression analyses

Because a possible stratification within blubber biopsies could influence our results, the 3.5 mm blubber/skin biopsy was divided into inner blubber, outer blubber, and skin.

Tissue Homogenization

Tissues were homogenized in TRIzol reagent (Invitrogen Canada Inc., Toronto, Ontario) using a Retsch MM301 mixer mill as described by Veldhoen and Helbing (2001). Each blubber sample was homogenized in a 1.5-mL microcentrifuge tube with the addition of 700 µL TRIzol and a 3-mm diameter tungsten-carbide bead. Blubber samples were homogenized 2 times using 5-minute intervals, at a frequency of 20 Hz; skin samples were homogenized 3 times using the same intervals and frequency. For any given sample, an additional 3 minutes of mixing was performed if un-homogenized tissue remained. All samples were cooled on ice between homogenization intervals.

Isolation of Total RNA

Total RNA was isolated from the tissue homogenates in TRIzol reagent as described by the manufacturer. After phase separation by centrifugation at 12000 x g for 10 min at 4°C, 1 mL glycogen (Roche Diagnostics, Laval, Quebec) was added in a clean 1.5 mL micro-centrifuge tube to each retained aqueous phase from blubber homogenates. Chloroform (140 µL) was added to separate the solution into an organic and an aqueous RNA-containing phase. Tubes were vortexed for 15 seconds, incubated for 2 min at room temperature, and centrifuged for 15 min at 4 °C to obtain phase separation. The aqueous phase containing RNA was transferred into a new tube, and RNA was precipitated with the addition of isopropanol (350 µL), a 10-min incubation at room temperature, and centrifugation at 12000 x g for 10 min at 4 °C. The RNA precipitate appeared as a translucent gel-like pellet at the bottom of the Eppendorf tube. The supernatant was then discarded, and the total RNA pellet was washed with 700 µL of 75% ethanol (made with diethyl pyrocarbonate-treated distilled, deionized H20 (DEPC-dH20)). After a 5-min

centrifugation at 7500 x g at 4 °C, total RNA was re-suspended in 23 µL of DEPC-dH20, incubated in a 55 °C water bath for 10 min, and stored at -80 °C.

cDNA Translation

Spectrophotometry was used to determine RNA concentration, and 1 µg of total RNA was used in the preparation of cDNA. Total cDNA was produced using Superscript II RNase H-reverse transcriptase, as described by the manufacturer (Invitrogen, Canada). The final cDNA solutions were diluted 40-fold using DEPC-dH2O prior to quantification.

Real-Time Quantitative Polymerase Chain Reaction (PCR) Assay

Based on the literature, eight genes were selected for their potential to provide evidence of environmental exposure to contaminants in seals and specific primers were designed. These included markers for: (1) endocrine disruption: thyroid hormone receptors alpha and beta (TRα and TRβ), estrogen receptor alpha (ERα), vitamin D receptor (Vit D); (2) organic contaminant exposure: aryl hydrocarbon receptor (AhR); and (3) metal / oxidative / general stress: heat shock protein 70 (hsp 70), glucocorticoid receptor (GR), Peroxisome proliferator-activated receptor gamma (PPARγ). Three additional genes were selected as normalizers: ribosomal protein L8 (L8); cytoplasmique beta-actin (β actin), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2).

Primers were designed and assessed for their ability to amplify a single specific DNA amplicon using a three-tier quality control process; details on the procedure can be found in Veldhoen *et al* (2009). Quantitative DNA amplification reactions (15 µl) were performed on a Realplex4 Eppendorf system (Eppendorf in Westbury, New York) as described previously (Crump *et al*. 2002) using gene-specific primers. The thermocycle program for most gene targets included an initial enzyme activation step at 95 °C (9 min) followed by 40 cycles of 95 °C denaturation (15 sec), 60 °C annealing (30 sec), and 72 °C elongation (45 sec). Quadruplicate reactions were performed for each sample, and data was averaged and normalized to the expression of the gene encoding the ribosomal protein L8 using the comparative (ΔΔCT) method (Livak and Schmittgen 2001). This transcript passed the Bestkeeper method of normalizer determination (Pfaffl *et al.* 2004), and the expression of this gene was invariant in blubber and skin tissue in this study. The other two normalizer genes (βactin and GAPDH) failed to represent an invariant gene expression target. The data was further normalized to the individual showing average contaminant concentrations.

Table 2: Quality control results (blubber and skin) for the 11 DNA primers.

Gene	Amplicon Size (bp)	Pass / Fail	
		Blubber	Skin
Ribosomal protein L8 (L8)	126	Pass	Pass
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	493	Pass	Fail
Cytoplasmic beta actin (βactin)	236	Pass	Pass
Estrogen receptor alpha (Erα)	213	Pass	Pass
Aryl hydrocarbon receptor (AhR)	308	Fail	Pass
heat shock protein 70 (hsp 70)	392	Pass	Pass
Peroxisome proliferator-activated receptor gamma (PPARγ)	398	Pass	Pass
Glucocorticoid receptor alpha (GR)	139	Pass	Pass
Vitamin D receptor (Vit D)	294	Fail	Pass
Thyroid hormone receptor alpha (TRα)	231	Pass	Pass
Thyroid hormone receptor beta (TRβ)	425	Fail	[a]

[a]- Passes the quality control but not highly efficient in replication. Results must be interpreted cautiously.

Data Analysis

All the organic contaminant concentrations were expressed on a lipid weight basis (lw) and were recovery-corrected using the stable isotope dilution method based on ^{13}C-labelled PCB, PBDE, PCDD, PCDF and OC pesticide spikes (Ikonomou *et al.* 2001) (the recovery ranges were a mean of 64% for PCBs (range 20 – 116%), 63% for PBDEs (26 – 102%), 77% for PCDDs (54 – 93%), 82% for PCDFs (62 – 99%), and 52% for OC pesticides (10 – 99%)).

Contaminant concentrations in the procedural blanks were low (< 10%) compared to concentrations reported in the harbor seal pup blubber samples except for BDE-209 where concentrations in the blank were on average 50% of the concentrations observed in the samples. For this reason, and in order to be consistent, it was decided to blank correct BDE-209 concentrations as well as all the other contaminant concentrations. Corrections were applied on a

batch basis using the average of the two blanks for each congener. It can be noted that, besides BDE-209 levels, the blank correction had minor effects on the final contaminant concentrations.

Many congeners were not detected. When congeners were undetected, substitutions were applied according to the following rule: (1) when congeners were detected in less than 70% of the samples, concentrations of 0 were substituted, and (2) when congeners were detected in more than 70% of the samples, a detection limit value substitution was applied. This method was used in several other studies (Cullon *et al.* 2005; Ross *et al.* 2004).

Total toxic equivalents (TEQ) to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were calculated for all dioxin-like PCBs measured (mono-ortho PCBs 105, 114, 118, 123, 156, 157, 167, and 189; non-ortho PCBs 77, 81, 126, and 169) and 2,3,7,8 Cl-substituted PCDDs (n = 7) and PCDFs (n = 10) using the international toxic equivalency factors (TEF) (Van den Berg *et al.* 2006).

Statistical Analysis

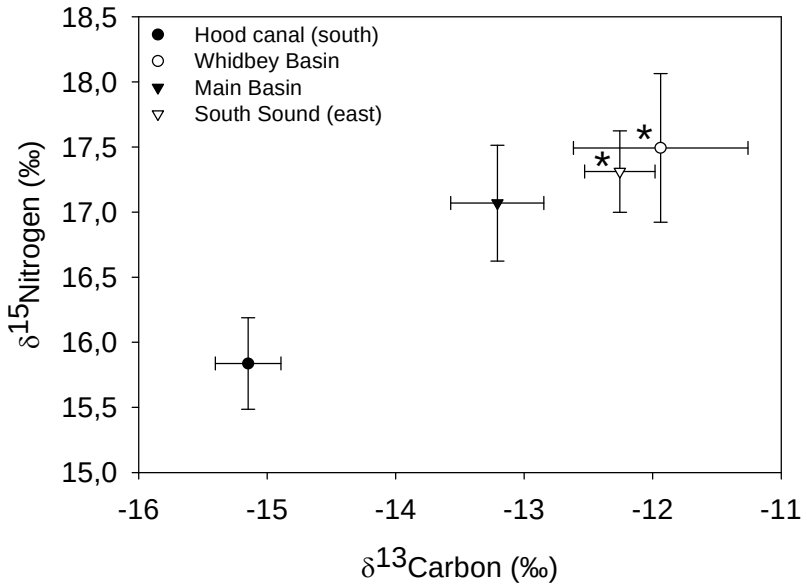
All statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). Spatial differences in contaminant levels and patterns were assessed using a one-way ANOVA followed by a post-hoc Dunnett's test. Seal pups from Hood Canal (south) were relatively less contaminated and were used as a reference group. The criterion for significance was $\alpha = 0.05$. Normality and constant variance were assessed and data were transformed if those test resulted in $\alpha < 0.05$. Relationships between variables were tested using correlation coefficients. The Pearson method was used when the data was normally distributed; otherwise the nonparametric Kendall's tau-b method was used.

Results

Samples from a total of 24 harbor seal pups (Table 1) were collected at four different regions in Puget Sound, WA (from Hood Canal (south) at Quilcene Bay (n=6) and Dosewallips River (n=2); Whidbey Basin in Skagit Bay (n=6); Main Basin at Orchard Rocks (n=2) and Blakely Rocks (n=1), and South Sound (east) at Gertrude Island (n=7) (Figure 1)). Pups were on average 4 to 6 weeks old and weighed between 20 and 25 kg. There were no significant differences in harbor seal pup weights among sites ($p = 0.687$). In addition, there were no significant correlations between contaminant concentrations and weight or length.

Stable Isotopes

$\delta^{15}N$ values ranged from 14.08 to 18.81 ‰ and $\delta^{13}C$ values ranged from -16.04 to -10.23 ‰ in harbor seal pup fur.



Significant differences from the reference site (i.e. Hood Canal (south)) were assessed (* = $p < 0.05$).

Figure 2: ^{15}N and ^{13}C values in the fur of harbor seal pups from four sites in Puget Sound.

The isotopic signature was different among the four sites ($p = 0.028$ and $p = 0.000$ for $\delta^{15}N$ and $\delta^{13}C$, respectively). Harbor seal pups from Hood Canal (south) had lower $\delta^{15}N$ values (15.84 ± 0.35 ‰) than harbor seal pups from Whidbey Basin and South Sound (east) (17.49 ± 0.57 and 17.31± 0.31 ‰, respectively). The same pattern was observed for $\delta^{13}C$ (-15.15 ± 0.26, -11.94 ± 0.68, and -12.26 ± 0.27 ‰ for Hood Canal (south), Whidbey Basin, and South Sound (east), respectively) (Figure 2). $\delta^{15}N$ and $\delta^{13}C$ were not correlated with any of the contaminants.

PCBs

PCB levels in harbor seal pups ranged from 1.0 to 9.4 µg/g lipid weight (lw). There were significant differences among the four sites ($p = 0.001$). Harbor seal pups from the Main Basin were the most PCB contaminated (6.34 ± 1.53 µg/g lw). Their levels were similar to South Sound (east) (4.02 ± 0.73 µg/g lw), but higher than Whidbey Basin and Hood Canal (south) (2.59 ± 0.63 and 1.57 ± 0.21 µg/g lw, respectively) (Figure 3a).

At the four sites, the PCB patterns were dominated by hexa-CBs (49.72 ± 1.47 % on average) followed by penta-CBs (24.58 ± 1.93%) and hepta-CBs (16.01 ± 1.08%). Those three homologue groups contributed to 90.30 ± 0.30 % of the total PCBs on average. Focusing on those 3 dominant homologue groups, there were differences among sites ($p = 0.000$). The contributions of penta-CBs in harbor seal pups from the Main Basin and South Sound (east) were lower than in those from Whidbey Basin and Hood Canal (south) whereas it was the opposite pattern for hepta-CBs. In addition, seal pups from Hood Canal (south) had a lower contribution of hexa-CBs than the 3 other sites (Figure 4a). CB-99, 101, 138, 153, 180, and 187 were the six dominant congeners at the four sites (Table 3).

PBDEs

PBDE levels in harbor seal pups ranged from 0.14 to 1.28 µg/g lw. PBDE concentrations were different among the four sites ($p = 0.002$). Harbor seal pups from Whidbey Basin, Main Basin and South Sound (east) had similar PBDE levels (0.48 ± 0.10 µg/g lw, 0.82 ± 0.14 µg/g lw and 0.66 ± 0.13 µg/g lw, respectively). Those concentrations were higher than in Hood Canal (south) pups (0.25 ± 0.05 µg/g) (Figure 3b).

At the four sites, the PBDE pattern was dominated by tetra-BDEs (78.72 ± 0.60% on average) and penta-BDEs (16.41 ± 0.60%). Those two homologue groups contributed to more than 95% of the total PBDEs. The pattern was similar at all the sites. BDE-47, 49, 99, 100, 153, and 209 were the six dominant congeners at the four sites (Table 3).

Dioxins and Furans

Overall, dioxin and furan concentrations were very low with many congeners being undetected. Of 75 possible dioxin congeners, only up to five congeners were detected. The total PCDD concentrations in harbor seal pups ranged from below the average detection limit (6.8 ± 2.3 pg/g) to 15.02 pg/g lw. There were differences among the four sites ($p = 0.005$). Harbor seal pups from South Sound (east) had significantly higher concentrations (12.37 ± 1.87 pg/g lw) than the three other sites (6.10 ± 2.26, 7.64 ± 1.97, and 6.01 ± 0.96 pg/g lw for Whidbey Basin, Main Basin, and Hood Canal (south), respectively) (Figure 3c). The homologue group patterns were similar among the four sites with hexa-CDDs being dominant (44.23 ± 5.70% on average) followed by penta-CDDs (25.01 ± 5.40% on average) (Figure 4c).

Of 135 possible furan congeners, only up to three congeners were detected in harbor seal pups and the levels ranged from below the average detection limit (3.4 ± 1.4 pg/g) to 5.06 pg/g lw. There were no differences among the four sites ($p = 0.569$) (Figure 3d). 2,3,4,7,8 PeCDF,

1,2,3,4,7,8 HpCDF, and OCDF were the only congeners detected. No clear trend could be concluded as the number of analytes detected was very limited.

OC Pesticides

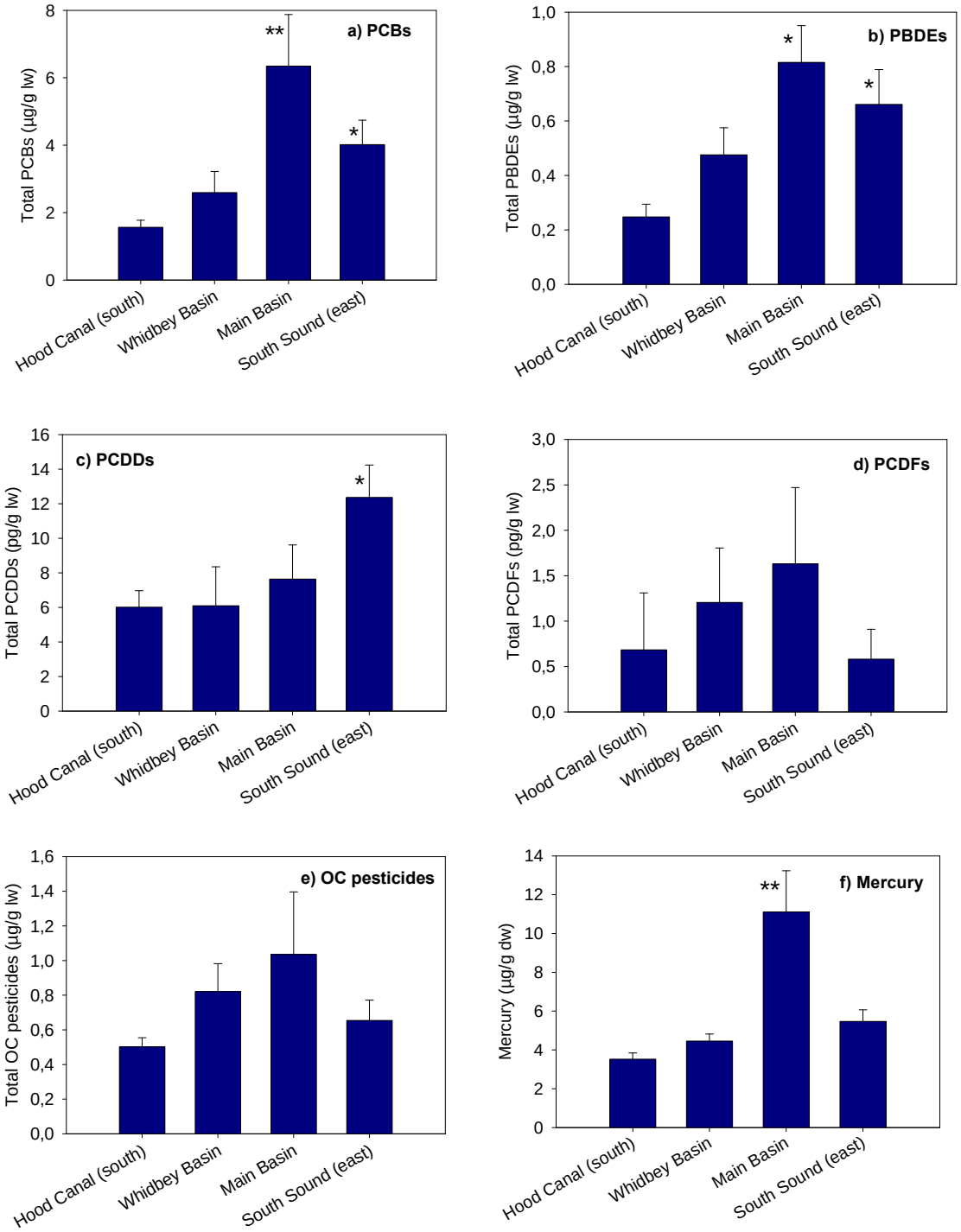
Total OC pesticides levels ranged from 0.36 to 1.74 µg/g lw. The concentrations and patterns were similar at the four sites ($p = 0.147$ and $p = 0.256$, respectively) (Figure 3e). Endosulfan sulphate, aldrin heptachlor and methoxychlor were not detected in any of the blubber samples. ΣDDTs were highly dominant contributing up to 92% of the total OC pesticides followed by Σchlordanes (Figure 4d). The most abundant DDT metabolite was p,p'-DDE accounting for 94.96 ± 0.24 % on average at the four sites followed by p,p'-DDD > o,p'-DDE > o,p'-DDD. The p,p'-DDE/ΣDDTs ratio was similar at all the sites and averaged 0.95 ± 0.01.

Toxic Equivalents (TEQ)

Consistent with the PCB concentration pattern, harbor seal pups from the Main Basin had the highest TEQ values (12.32 ± 2.98 pg/g lw; $p = 0.024$). Pups from Whidbey Basin, Hood Canal (south), and South Sound (east) had similar TEQs (6.86 ± 1.92, 4.09 ± 0.64, and 8.66 ± 1.27 pg/g lw, respectively). The pattern was similar between the four sites ($p = 0.510$); PCBs contributed to up to 89% of the total TEQ and mono-ortho PCBs contributed to most of the ΣPCB TEQ. CB-118, 126, and 105 were the dominant contributors to total TEQ (Table 4).

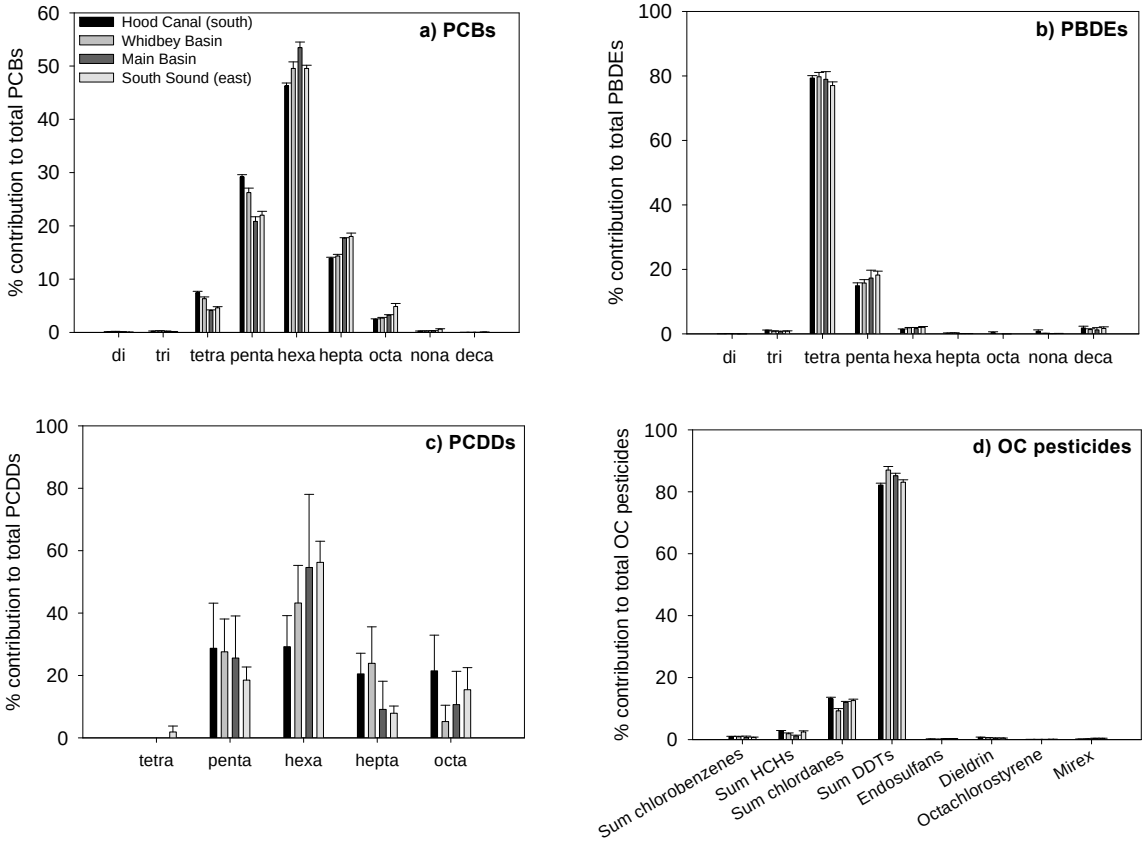
Mercury

Mercury levels in harbor seal pups ranged from 2.83 to 15.23 µg/g dw. Mercury concentrations were different among the four sites ($p = 0.000$). Harbor seal pups from the Main Basin had significantly higher concentrations (11.11 ± 2.12 µg/g) than those reported at the three other sites (4.46 ± 0.36, 3.52 ± 0.32, and 5.47 ± 0.60 µg/g for Whidbey Basin, Hood Canal (south), and South Sound (east), respectively) (Figure 3f).



- Average ± standard error are presented
- Significant differences from the reference site (i.e. Hood Canal (south)) were assessed (* = p <0.05; ** = p< 0.01).

Figure 3: Spatial variations of PCB, PBDE, PCDD, PCDF, OC pesticide, and mercury concentrations.



- The PCDF homologue group pattern is not shown as only a few congeners were detected.

Figure 4: PCB, PBDE, and PCDD homologue group patterns, and OC pesticide pattern.

Table 3: Top six PCB and PBDE congeners in harbor seal pups from four different sites.

	Hood Canal (south)		Whidbey Basin		Main Basin		South Sound (east)	
PCBs (µg/g)	153	0.28 ± 0.03	153	0.54 ± 0.15	153	1.47 ± 0.38	153	0.84 ± 0.16
	138	0.20 ± 0.03	138	0.37 ± 0.09	138	1.04 ± 0.30	138	0.55 ± 0.10
	101	0.12 ± 0.02	99	0.19 ± 0.05	99	0.47 ± 0.16	99	0.26 ± 0.05
	99	0.11 ± 0.01	101	0.18 ± 0.04	187	0.32 ± 0.07	101	0.22 ± 0.04
	187	0.07 ± 0.01	187	0.11 ± 0.03	180	0.31 ± 0.06	187	0.22 ± 0.04
	118	0.06 ± 0.01	180	0.10 ± 0.02	101	0.31 ± 0.04	180	0.19 ± 0.03
∑ top 6		0.84 ± 0.02		1.52 ± 0.06		3.92 ± 0.17		2.23 ± 0.07
% of ∑PCBs		*53.95 ± 0.59*		*57.57 ± 1.61*		*61.85 ± 1.25*		*56.68 ± 0.40*
PBDEs (ng/g)	47	188.4 ± 33.96	47	375.3 ± 78.4	47	628.2 ± 104.1	47	496.6 ± 96.31
	99	20.63 ± 5.99	99	50.97 ± 11.98	99	93.73 ± 38.79	99	85.88 ± 20.32
	100	17.34 ± 3.91	100	22.90 ± 4.99	100	47.47 ± 6.82	100	37.41 ± 6.84
	49	5.71 ± 1.58	153	7.36 ± 1.82	49	13.07 ± 1.50	153	10.39 ± 2.09
	209	4.20 ± 0.99	209	5.12 ± 1.11	153	10.93 ± 3.91	49	10.18 ± 2.23
	153	2.38 ± 0.55	49	4.27 ± 1.08	209	8.19 ± 3.85	209	7.52 ± 1.50
∑ top 6		238.7 ± 7.83		465.9 ± 16.56		801.6 ± 26.50		647.9 ± 21.55
% of ∑PBDEs		*96.20 ± 0.72*		*97.84 ± 0.14*		*98.27 ± 0.21*		*97.98 ± 0.13*

Table 4: Contribution of the non-ortho PCBs, mono-ortho PCBs, PCDD, and PCDF to the total toxic equivalents (TEQ) (calculated using the 2005 TEFs).

	PCB congeners	Mean TEQ ± st error (pg/g lw)			
		Hood Canal (south)	**Whidbey Basin**	**Main Basin**	**South Sound (east)**
Non-ortho PCBs	77	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	81	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	126	0.00 ± 0.00	0.92 ± 0.92	3.06 ± 3.06	0.00 ± 0.00
	169	0.00 ± 0.00	0.19 ± 0.19	0.00 ± 0.00	0.00 ± 0.00
	∑ non-ortho PCBs	0.00 ± 0.00	1.11 ± 0.91	3.06 ± 3.06	0.00 ± 0.00
	% of ∑PCB TEQ	*0.00 ± 0.00%*	*9.86 ± 6.70%*	*17.39 ± 17.39%*	*0.03 ± 0.03%*
Mono-ortho PCBs	105	0.59 ± 0.08	0.77 ± 0.14	1.53 ± 0.03	1.13 ± 0.19
	114	0.03 ± 0.01	0.05 ± 0.01	0.12 ± 0.02	0.08 ± 0.01
	118	1.92 ± 0.24	2.34 ± 0.43	4.68 ± 0.23	3.45 ± 0.50
	123	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
	156	0.22 ± 0.03	0.39 ± 0.09	1.16 ± 0.22	0.67 ± 0.12
	157	0.06 ± 0.01	0.10 ± 0.02	0.27 ± 0.06	0.17 ± 0.03
	167	0.03 ± 0.00	0.04 ± 0.01	0.08 ± 0.00	0.06 ± 0.01
	189	0.01 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	0.05 ± 0.01
	∑ mono-ortho PCBs	2.87 ± 0.35	3.74 ± 0.71	7.92 ± 0.34	5.65 ± 0.86
	% of ∑PCB TEQ	*100.00 ± 0.00%*	*90.14 ± 6.70%*	*82.61 ± 17.39%*	*99.97 ± 0.03%*
Sum PCB TEQ		2.87 ± 0.35	4.85 ± 1.52	10.98 ± 3.31	5.65 ± 0.86
		76.43 ± 7.95%	*73.86 ± 6.85%*	*88.81 ± 11.19%*	*68.54 ± 6.08%*
Sum PCDD TEQ		1.17 ± 0.51	1.96 ± 0.70	1.35 ± 1.00	3.01 ± 0.63
		4.09 ± 0.64%	*6.86 ± 1.92%*	*12.34 ± 2.98%*	*8.66 ± 1.27%*
Sum PCDF TEQ		0.04 ± 0.02	0.05 ± 0.05	0.01 ± 0.01	0.00 ± 0.00
		1.17 ± 0.51%	*1.96 ± 0.70%*	*1.35 ± 1.35%*	*3.01 ± 0.63%*
Total TEQ		**4.09 ± 0.64**	**6.86 ± 1.92**	**12.34 ± 2.98**	**8.66 ± 1.27**

Health Indicators

Thyroid Hormones

The concentrations of different thyroid hormone (TH) forms were measured in the serum of harbor seal pups (Table 5). There were no significant differences in the levels of TT4, TT3, free T4, and free T3 among sites ($p = 0.097$, $p = 0.518$, $p = 0.079$, and $p = 0.249$, respectively). There was a correlation between TT4 and free T4 ($r^2 = 0.24$; $p = 0.015$) as well as between TT3 and free T3 ($r^2 = 0.73$; $p = 0.000$). There were negative correlations between TT3, FT3 and total PCDDs ($r = -0.482$, $p = 0.022$ and $r = -0.566$, $p = 0.004$, respectively). They were the only significant relationships observed between any TH forms and contaminant concentrations (Table 6). There was no effect of body mass on the different forms of TH. Similarly, there was no difference between males and females.

Vitamin A

Vitamin A concentrations were measured in the blubber samples. The levels ranged from 1.70 to 56.3 μg/g blubber. There were differences among sites ($p = 0.001$) with harbor seal pups from Hood Canal (south) (32.24 ± 4.19 μg/g) having higher levels of vitamin A than pups from Whidbey Basin and South Sound (east) (7.82 ± 1.60 and 13.62 ± 2.60 μg/g, respectively) (Table 5). Vitamin A levels were decreasing with ΣPBDEs and Hg ($p = 0.043$ and $p = 0.042$, respectively). We could also notice nearly decreasing trends with ΣPCBs and ΣOC pesticides ($p = 0.054$ and $p = 0.056$, respectively). There was no effect of body mass on vitamin A level ($p = 0.458$) and no differences between males and females ($p = 0.740$). Vitamin A levels in harbor seal pup blubber were not correlated with thyroid hormone levels.

Table 5: Total T4, free T4, total T3, and free T3 levels in serum and vitamin A levels in blubber.

	Hood canal (south)	Whidbey Basin	Main Basin	South Sound (east)
TT4 (µg/dL)	5.01 ± 0.43	6.97 ± 0.66	5.45 ± 0.43	5.63 ± 0.57
	(3.00 - 7.22)	*(5.29 - 9.54)*	*(4.66 - 6.12)*	*(3.94 - 7.70)*
FT4 (ng/dL)	1.34 ± 0.11	1.71 ± 0.17	1.64 ± 0.02	1.26 ± 0.12
	(0.99 - 1.96)	*(1.35 - 2.29)*	*(1.60 - 1.67)*	*(0.87 - 1.66)*
TT3 (µg/dL)	1.17 ± 0.09	1.45 ± 0.23	1.21 ± 0.15	1.31 ± 0.07
	(0.78 - 1.39)	*(0.80 - 1.96)*	*(1.02 - 1.51)*	*(0.91 - 1.48)*
FT3 (ng/dL)	0.38 ± 0.03	0.48 ± 0.06	0.43 ± 0.05	0.36 ± 0.04
	(0.26 - 0.45)	*(0.31 - 0.59)*	*(0.34 - 0.51)*	*(0.19 - 0.53)*
Vit A (µg/g)	32.24 ± 4.19	7.82 ± 1.60	17.40 ± 15.73	13.62 ± 2.60
	(20.40 – 56.32)	*(3.80 – 13.04)*	*(1.67 – 33.13)*	*(6.96 – 26.19)*

- Mean ± standard error
- Ranges are presented in brackets

Table 6: Correlation coefficients between contaminant concentrations and thyroid hormone levels (serum) as well as vitamin A levels (blubber).

	TT4	FT4	TT3	FT3	Vit A
ΣPCBs	0.105	0.062	0.204	0.119	-0.392
p-value	*0.472*	*0.773*	*0.164*	*0.581*	*0.054*
ΣTEQ	0.076	-0.104	0.051	-0.094	-0.217
p-value	*0.602*	*0.629*	*0.728*	*0.662*	*0.146*
ΣPBDEs	0.088	-0.027	0.133	0.034	**-0.426***
p-value	*0.551*	*0.899*	*0.537*	*0.874*	*0.043*
ΣPCDDs	-0.105	-0.394	**-0.482***	**-0.566****	0.023
p-value	*0.472*	*0.056*	***0.017***	***0.004***	*0.918*
ΣPCDFs	-0.047	0.419	-0.372	**-0.327***	0.151
p-value	*0.765*	*0.199*	*0.073*	***0.041***	*0.491*
ΣOC pesticides	0.182	0.187	0.249	0.263	-0.404
p-value	*0.214*	*0.381*	*0.241*	*0.215*	*0.056*
Hg	-0.011	0.136	0.131	0.041	**-0.304***
p-value	*0.941*	*0.525*	*0.371*	*0.849*	*0.042*

- * $p < 0.05$; ** $p < 0.01$

Gene Expression

A total of 11 harbor seal specific primers were designed and their specificity and sensitivity were assessed using the three-tier quality control process (Veldhoen et al., 2009). Eight genes (L8, GAPDH, βactin, ERα, hsp 70, PPARγ, GR, and TRα) passed the quality control test in blubber and nine passed it in skin (L8, βactin, ERα, AhR, hsp 70, PPARγ, GR, Vit D, and TRα) (Table 2). Gene expression was investigated separately in inner blubber, outer blubber, and skin. Correlation coefficients with contaminant concentrations are presented in table 7, 8, and 9. Biological variables such as body weight, length, and girth did not have any influence on gene expression in inner blubber, outer blubber, and skin (not shown) and there were no differences between males and females for any of the tested genes.

In inner blubber, the expressions of TRα and GR were not correlated to any of the contaminant concentrations. TRα did not appear to be associated with the levels of circulating hormones. ERα and hsp 70 expressions were strongly correlated with Hg concentrations ($r = 0.721$, $p = 0.005$; and $r = 0.733$, $p = 0.002$, respectively). PPAR γ expression was strongly correlated with ΣPCBs, ΣOC pesticides, and Hg ($r = 0.566$, $p = 0.035$; $r = 0.600$, $p = 0.023$; and $r = 0.633$, $p = 0.015$, respectively).

Less significant results were reported for outer blubber. TRα and GR expression were the only one to be significantly correlated with Hg ($r = 0.367$, $p = 0.048$; and $r = 0.486$, $p = 0.012$, respectively).

In skin, contaminants did not seem to influence the expression of any of the genes investigated. We can notice that, even though the primer for TRα passed the three-tier quality control process, the expression of this gene appeared to be too low in skin so the data could not be used. The same was true for TRβ.

Table 7: Correlation coefficients between contaminant concentrations and gene expression in inner blubber.

	ΣPCBs	ΣTEQ	ΣPBDEs	ΣPCDDs	ΣPCDFs	ΣOC pesticides	Hg
β actin	-0.273	-0.333	-0.198	-0.303	0.080	0.015	0.061
p value	*0.217*	*0.131*	*0.372*	*0.170*	*0.742*	*0.945*	*0.784*
GAPDH	-0.055	0.011	-0.078	0.055	0.014	0.133	0.055
p value	*0.784*	*0.956*	*0.701*	*0.784*	*0.949*	*0.511*	*0.784*
TRα	0.294	0.188	0.230	-0.062	-0.164	0.267	0.276
p value	*0.308*	*0.519*	*0.429*	*0.834*	*0.576*	*0.356*	*0.340*
TRβ							
p value							
ERα	0.278	0.176	0.145	-0.391	0.100	0.181	**0.721****
p value	*0.358*	*0.565*	*0.636*	*0.186*	*0.744*	*0.553*	***0.005***
Vit D							
p value							
AhR							
p value							
hsp 70	0.505	0.256	0.504	-0.473	-0.016	0.548	**0.773****
p value	*0.066*	*0.399*	*0.066*	*0.103*	*0.960*	*0.052*	***0.002***
GR	0.440	0.297	0.353	-0.011	-0.155	0.381	0.405
p value	*0.115*	*0.303*	*0.216*	*0.970*	*0.596*	*0.180*	*0.151*
PPARγ	**0.566***	0.440	0.409	-0.147	-0.016	**0.600***	**0.633***
p value	***0.035***	*0.115*	*0.146*	*0.616*	*0.956*	***0.023***	***0.015***

- * $p < 0.05$; ** $p < 0.01$
- Grey areas are for genes that did not pass the QA/QC so no data available

Table 8: Correlation coefficients between contaminant concentrations and gene expression in outer blubber.

	ΣPCBs	ΣTEQ	ΣPBDEs	ΣPCDDs	ΣPCDFs	ΣOC pesticides	Hg
β actin	0.121	0.091	0.076	0.091	0.055	0.182	0.061
p value	*0.583*	*0.681*	*0.731*	*0.681*	*0.817*	*0.411*	*0.784*
GAPDH	-0.013	0.090	0.000	0.013	0.075	0.234	-0.013
p value	*0.951*	*0.669*	*1.000*	*0.951*	*0.738*	*0.270*	*0.951*
TRα	0.024	0.083	0.209	0.195	0.240	0.075	**0.367***
p value	*0.930*	*0.653*	*0.437*	*0.469*	*0.371*	*0.784*	***0.048***
TRβ							
p value							
ERα	0.209	0.333	0.341	0.151	0.094	0.118	0.228
p value	*0.455*	*0.083*	*0.214*	*0.592*	*0.739*	*0.674*	*0.414*
Vit D							
p value							
AhR							
p value							
hsp 70	0.152	-0.200	0.226	-0.094	0.225	0.121	0.270
p value	*0.589*	*0.299*	*0.417*	*0.740*	*0.419*	*0.666*	*0.330*
GR	0.082	0.181	0.225	0.210	0.063	0.019	**0.486***
p value	*0.771*	*0.347*	*0.420*	*0.452*	*0.823*	*0.946*	***0.012***
PPARγ	-0.228	0.029	-0.025	-0.178	0.121	-0.043	0.055
p value	*0.414*	*0.882*	*0.929*	*0.526*	*0.667*	*0.880*	*0.846*

- * $p < 0.05$; ** $p < 0.01$
- Grey areas are for genes that did not pass the QA/QC so no data available

Table 9: Correlation coefficients between contaminant concentrations and gene expression in skin.

	ΣPCBs	ΣTEQ	ΣPBDEs	ΣPCDDs	ΣPCDFs	ΣOC pesticides	Hg
β actin	0.030	-0.182	0.062	-0.061	0.202	-0.123	0.061
p value	*0.891*	*0.411*	*0.783*	*0.784*	*0.397*	*0.582*	*0.784*
GAPDH							
p value							
TRα							
p value							
TRβ							
p value							
ERα	0.061	0.091	0.123	-0.091	0.312	0.308	-0.152
p value	*0.784*	*0.681*	*0.582*	*0.681*	*0.190*	*0.168*	*0.493*
Vit D	0.121	0.030	-0.031	0.277	-0.018	-0.369	-0.091
p value	*0.583*	*0.891*	*0.890*	*0.383*	*0.939*	*0.098*	*0.681*
AhR	0.063	-0.030	0.092	0.152	0.092	-0.215	0.294
p value	*0.786*	*0.891*	*0.679*	*0.493*	*0.700*	*0.335*	*0.354*
Hsp 70	-0.152	-0.182	-0.185	-0.182	0.495	-0.215	0.061
p value	*0.493*	*0.411*	*0.408*	*0.411*	*0.067*	*0.335*	*0.784*
GR	-0.107	0.015	-0.047	-0.198	-0.259	0.326	-0.321
p value	*0.630*	*0.945*	*0.836*	*0.372*	*0.280*	*0.147*	*0.149*
PPARγ	-0.382	-0.127	-0.426	0.127	0.384	-0.389	-0.200
p value	*0.102*	*0.586*	*0.072*	*0.586*	*0.124*	*0.100*	*0.392*

- * $p < 0.05$; ** $p < 0.01$
- Grey areas are for genes that did not pass the QA/QC so no data available

Discussion

During the summer of 2009, biopsy samples from free-ranging harbor seal pups were collected in Puget Sound, WA from areas designated in Ecology's box model as Hood Canal (south), Whidbey Basin, Main Basin and South Sound (east), in order to measure the concentrations of contaminants of concern (PCBs, PBDEs, PCDDs, PCDFs, OC pesticides, and mercury), and to assess their possible effects on health.

Organic contaminant and mercury concentrations are known to be affected by biological variations such as age, sex, or body condition in marine mammals. A continuous increase of POP concentration with age is usually observed in immature individuals of both sexes and in adult males. In adult females, concentrations start to decrease when they reach sexual maturity because of the offload of fat-soluble contaminants to their young during gestation and nursing (Borrell *et al.* 1995; Ylitalo *et al.* 2001; Ross *et al.* 2000). Hg concentrations have also been reported to increase with age in various species of pinnipeds (Ikemoto *et al.* 2004; Brookens *et al.* 2007). Similarly, hormone levels such as thyroid hormones and vitamin A are subject to variations associated with age, nutritional status and / or physiological state (Rolland 2000). In this study, we limited our sample collections to seal pups of comparable age, weight, and body condition, in order to reduce confounding factors and improve our spatial contaminant assessment and the evaluation of contaminant-health interactions. Comparisons to the literature must be made with caution, with due consideration to these aforementioned factors (e.g. Table 10).

Stable Isotopes

Diet being the main source of contaminants in marine mammals, it is an important parameter to take into account when analysing spatial variation in POP and Hg concentrations in harbor seals. Based on relative isotopic fractionation, $\delta^{15}N$ can be used to describe trophic level and $\delta^{13}C$ values can help identify the food web carbon sources (Kelly 2000). Harbor seal pups from Hood Canal (south) had a different stable isotope signature, with lower $\delta^{15}N$ and $\delta^{13}C$ values compared to other sites (Figure 3). The difference in $\delta^{15}N$ values observed in Hood Canal (south) harbor seal pups was however within 3‰ % suggesting that, even though their diet might be different, they still feed at a similar trophic level (Cabana and Rasmussen 1994). From a previous report, diet information was only available for harbor seals from Hood Canal and the South Sound. Harbor seals usually forage within approximately 10 km from their haulout sites so their diet reflects local prey species. While harbor seals from Hood Canal are thought to feed primarily on hake, herring, adult salmon, shiner surfperch, and cephalopod species, those from the South Sound mainly feed on flatfish, midshipman and shiner surfperch species, these latter species being associated with sandy substrates (Lance and Jeffries 2009). An earlier study reported Pacific hake as the principal prey item recovered from scat of harbor seals from Hood Canal and Pacific Staghorn sculpin for the seals from southern Puget Sound (Calambokidis *et al.* 1978). The lower $\delta^{13}C$ values reported here in harbor seal pups from Hood Canal (south) may reflect a more pelagic signal (Hobson *et al.* 1993) in accordance with a diet comprised of mostly pelagic prey species.

POPs and Hg are known to biomagnify in aquatic food webs. Several studies have reported positive relationships between both POPs and methylmercury, and $\delta^{15}N$ (Das *et al.* 2003; Jarman

et al. 1996). In this study, harbor seal pups from Hood Canal (south) had lower $\delta^{15}N$ values, suggesting foraging at a slightly lower trophic level than seal pups from the other areas where we sampled (Figure 2). Hake is an important prey item for harbor seals, and otolith analyses suggest that the hake age structure in Hood Canal is skewed towards younger age classes as opposed to older hakes in other parts of Puget Sound. In addition, $\delta^{15}N$ has been found to increase with hake size (West J., WDFW, personal communication) which could help explain the lower $\delta^{15}N$ values reported in our harbor seal pups from Hood Canal (south). Even though we did not find any correlations between contaminant concentrations and stable isotope ratios in the present study, the low $\delta^{15}N$ reported in harbor seal pups from Hood Canal (south) were associated with the lowest concentrations of PCBs, PBDEs, PCDDs, OC pesticides, and mercury.

PCBs

Among the four sites, harbor seal pups sampled from the Main Basin appeared to indicate a "hot spot" for PCB contamination while those sampled from Hood Canal (south) suggested an area of lowest contamination in Puget Sound. While variations in feeding ecology (as above) must be reconciled with this interpretation, our results are consistent with proximity of the city of Seattle and contaminant reports from other matrices, such as sediment, water, and other biota. Elliot Bay reportedly had one of the greatest unit area loading rates for PCBs, and surrounding residential areas contributed the greatest amount of PCB loading to Puget Sound. In contrast, the Hood Canal study areas had among the smallest unit area loading rates for PCBs (Table 4 and B1, (Herrera Environmental Consultants, 2010)).

PCB levels in harbor seal pups from Puget Sound were in the same range as those reported for harbor seal pups from the southern California coast (Blasius and Goodmanlowe 2008), an order of magnitude lower than those observed in harbor seal pups from the northwestern Atlantic (Shaw *et al.* 2005), and an order of magnitude higher than in harbor seal pups from the northern gulf of Alaska (Wang *et al.* 2007) (Table 10).

Temporal POP data are only available for harbor seal pups from the South Sound and revealed that the present PCB levels in harbor seal pups collected in the South Sound were, on average, four times lower than the levels reported in pups collected in 1996-1997 from the same area (Ross *et al.* 2004). Following their ban in the 1970s, decreasing PCB concentrations have been reported in various marine mammal species such as harbor seals from British Columbia, Canada, and Washington State (Calambokidis *et al.* 1999), ringed seals from the Canadian Arctic (Braune *et al.* 2005), northern fur seals (*Callorhinus ursinus*) from the Pacific coast of Japan (Kajiwara *et al.* 2004), and grey seals (*Halichoerus grypus*) from the Baltic Sea (Nyman *et al.* 2002).

Hexa-CBs, penta-CBs, and hepta-CBs were the dominant homologue groups in our harbor seals pups. The lighter pattern observed in harbor seal pups from Hood Canal (south) suggests that these pups are either further away from major PCB sources (Ross *et al*., 2004), or that feeding differences underlie these observations. Similar to our results, PCB-153 and PCB-138 were the dominant congeners in ringed seals from Svalbard (Wolkers *et al.* 1998); harbor seals from the gulf of St Lawrence (Hobbs *et al.* 2002), and Baikal seals (*Pusa sibirica*). This is likely due to the fact that PCB-153 was a major component of the most heavily used PCB technical mixture, and,

as it has no vicinal hydrogen and is Cl-substituted at both para positions, is resistant to metabolism (Boon and Eijgenraam 1988).

PBDEs

Similar to our observations for PCBs, harbor seal pups sampled from the Main Basin appeared to reflect a PBDE "hotspot". Seal pups from Hood Canal (south) had the lowest PBDE concentrations. In a study of Dungeness crab (*Metacarcinus magister*), Ikonomou *et al.* (2006) concluded that the highest PBDE concentrations are mainly found at major harbors and adjacent to population centers followed by industrial / pulp mill towns (Johannessen *et al.* 2008; Ross *et al.* 2008).
PBDE levels in harbor seal pups from Puget Sound were seven times lower than those reported in harbor seal pups from the northwest Atlantic (Shaw *et al.* 2008), three times lower than in harbor seals from California (She *et al.* 2002), and about three times higher than in ringed seals from Greenland (Letcher *et al.* 2009) (Table 10).

Although preliminary, our findings suggest that PBDEs have been increasing in South Sound harbor seals since the early 1980s (Ross *et al*. in prep). Previous studies reporting concentrations doubling every two years in harbor seals from the San Francisco Bay area (She *et al*., 2002), or every six years on average in ringed seals from the Canadian Arctic (Ikonomou *et al.* 2002) and is likely reflecting the increasing regulations of these chemicals in North America in recent years.

Our findings that tetra-BDEs and, to a lesser extent, penta-BDEs, dominate harbor seal pup PBDE profiles in consistent with widespread observations. BDEs 47, 99, 100, and 153 were also reported as the dominant congeners in California sea lions (*Zalophus californianu*) (Stapleton *et al.* 2006), harbor seals from the San Francisco Bay area (She *et al*., 2002), and grey seals from the North Sea (Kalantzi *et al.* 2005). This pattern has been defined as a typical marine mammal blubber congener profile (Ikonomou *et al*., 2002). It can be related to an environmental contamination by the Penta-BDE commercial mixture in which these congeners are dominant. However, in this commercial mixture, the amount of BDE-99 exceeds BDE-47, suggesting that this mixture is not stable in the environment and/or biota, with BDE-47 being the most persistent and bioaccumulative. BDE-47 could also be generated by the debromination of other congeners. Metabolisation of BDE-99 into BDE-47 has been demonstrated in common carp (Stapleton *et al.* 2004) and suggested for California sea lions (Stapleton *et al*., 2005).

BDE-209 was one of the top 6 PBDE congeners detected in our harbor seal pups contributing to 1.57 ± 0.23% of total PBDEs, on average. Whether or not this particular congener bioaccumulates and represents a threat for aquatic top predators is unclear. Because of its high degree of bromine substitution and therefore high molecular weight, BDE-209 has a high affinity to particles in air, water, and sediments making it less bioavailable and less likely to cross biological membranes (Rahman *et al.* 2001). However, relatively high levels of BDE-209 have been reported in eggs of peregrine falcons (*Falco peregrinus*) breeding in Sweden (Lindberg *et al.* 2004), Vancouver Island marmots (*Marmota vancouverensis*) (Lichota *et al.* 2004), and interior grizzly bears (*Ursus arctos horribilis*) from British Columbia, Canada (Christensen *et al.* 2005), showing that even the higher brominated congeners have the potential to bioaccumulate in certain food webs. In addition, a study on captive juvenile grey seals demonstrated that deca-

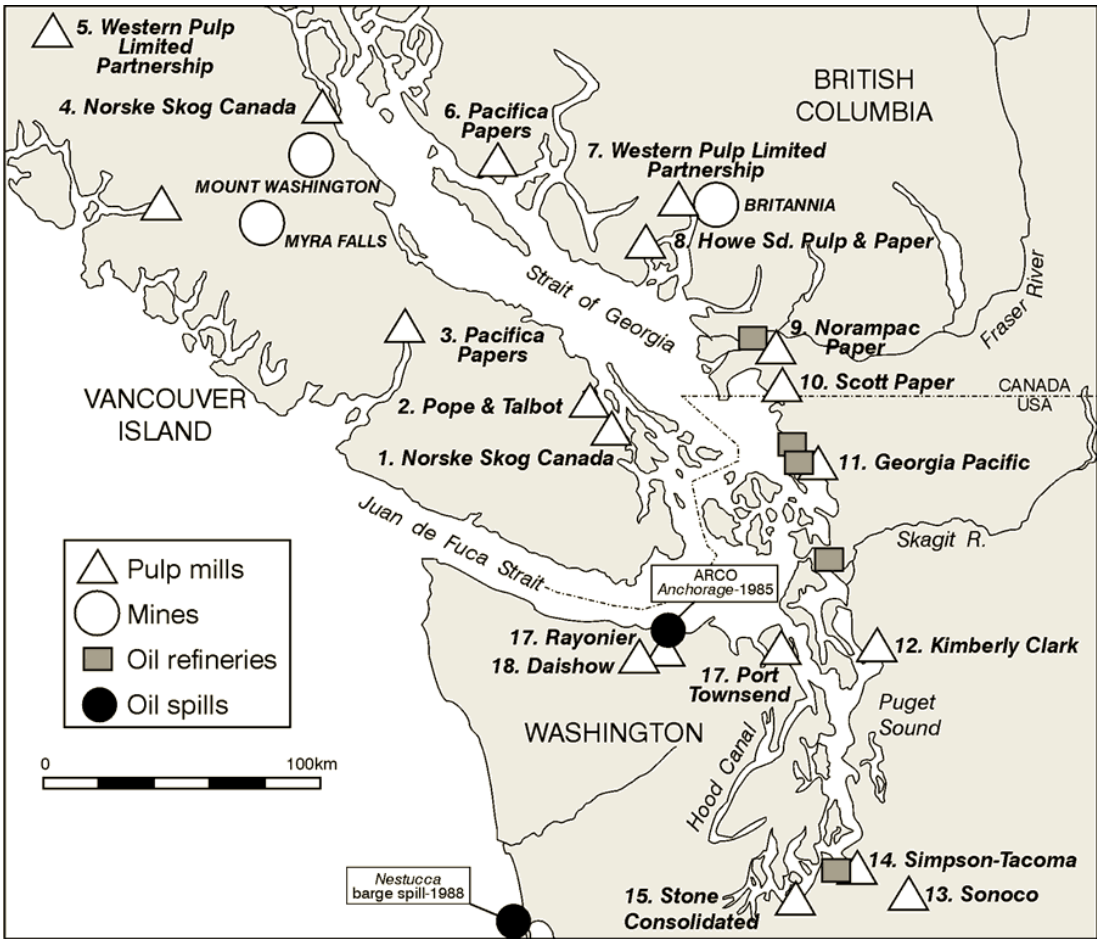
BDE was efficiently absorbed from the diet and could be stored in the blubber (Thomas *et al.* 2005). The analytical difficulties associated with the accurate determination of BDE-209 in environmental samples may introduce some uncertainties. However, our results for harbor seal pups were similar to previous studies where they reported an average contribution of BDE-209 of 0.1 – 2% in harbor seals from Spitsbergen (Jenssen *et al.* 2007) and polar bears from the Norwegian Arctic (Verreault *et al.* 2005). As explained previously, its low contribution at the top of the food chain can be attributed to its low bioavailability but also to its potential debromination (Ross *et al.* 2009; Tomy *et al.* 2008).

Dioxins and Furans

ΣPCDD and ΣPCDF concentrations in harbor seal pups were six orders of magnitude lower than PCB levels. This reflects the lower environmental (prey) concentrations of PCDDs and PCDFs (Cullon *et al.* 2005), but also their relatively reduced bioaccumulation. A preferential ability to metabolize and excrete dioxin compounds has also been observed in previous studies (Boon *et al.* 1988; Ross *et al*. 2000). The PCDD spatial pattern was different than the one reported for the other contaminants with harbor seal pups from the South Sound having the highest levels; Hood Canal (south) was still one of the cleanest sites. Before the implementation of regulations in the 1990s, pulp and paper mills were an important source of dioxins and furans to this region (Grant and Ross 2002; Ross *et al*. 2004). There were eight mills in Washington State in the 1990s producing bleached pulp and / or paper (Figure 5). A shift away from liquid chlorine bleaching to chlorine dioxide bleaching resulted in a diminished production and release of dioxins and furans in the 1990s (Yake *et al.* 1998). The presence of three mills in the South Sound area may explain the relatively high PCDD concentrations in harbor seal pups from this area. In 2004, Ross *et al*. reported a decreasing trend in PCDD concentrations in harbor seal pups from the Strait of Georgia to the South of Puget Sound. However, seal pups were only sampled at one site in Puget Sound. Our present results give an insight into the PCDD trend within four regions of Puget Sound and reveal increasing concentrations from north to south.

PCDD levels in harbor seal pups from Puget Sound were about half the concentrations reported in Baikal seals (Imaeda *et al.* 2009), and in the same range as the levels reported for ringed seals from the Arctic (Ikonomou *et al.* 2002; Table 10). The present PCDD levels observed in harbor seal pups collected from the South Sound were, on average, an order of magnitude lower than the levels reported in pups collected in 1996-1997 from the same area (Ross *et al*. 2004) reflecting the implementation of regulations on dioxin emissions in 1990s.

The association of PCDD contamination of harbor seal pups with pulp mills was confirmed by the congener pattern. 1,2,3,6,7,8 HxCDD was highly dominant and this congener has been attributed to the historical use of elemental chlorine by pulp mills, and to woodchips contaminated by pentachlorophenol (Addison *et al.* 2005).



- From Grant and Ross, 2002

Figure 5: Eight pulp and paper mills that may have been a source of PCDDs to inland waters including Puget Sound before the implementation of regulations.

Organochlorine Pesticides

Harbor seal pups from the four sites had similar OC pesticide concentrations, with DDT accounting for over 90% of the total. Most agriculture uses of DDT ended in the United States in 1972 and in Canada between 1970 and 1978. The uniform distribution in the marine environment probably reflects their ban more than 30 years ago. This was supported by the pesticide pattern which was dominated by p,p'DDE, the main degradation product of DDT. The ratio p,p'DDE/ sum DDTs is an indicator of the time since DDT was released in the environment. In the present study, an average ratio of 0.95 confirms that there are no new major DDT inputs into Puget Sound (Aguilar 1984). Compared to levels in harbor seal pups sampled from the South Sound in 1990 (Hong *et al.* 1996), our present levels of p,p'DDE were about six times lower once again reflecting the regulations on DDT usage.

Mercury

Mercury was measured in harbor seal pup fur which has been reported to be a good surrogate for internal organs (Ikemoto *et al.* 2004; Brookens *et al.* 2008). Similar to the PCB and PBDE spatial patterns, harbor seal pups from the Main Basin had the highest levels of mercury probably reflecting once again the proximity to the city of Seattle. A study on the loading of contaminants into Puget Sound revealed that the Elliott Bay study area had the greatest unit area loading rate for mercury (Herrera Environmental Consultants, 2010). A study on rockfish also showed that fish collected from Elliott Bay had the greatest mercury concentrations when compared to non urban rockfish (West and O'Neil 1995).

Table 10: PCB, PBDE, PCDD, sum DDT, and Hg concentrations in pinnipeds from around North America.

Species	Location	Year	Age	PCBs (µg/g lw)	PBDEs (µg/g lw)	PCDD (pg/g lw)	Sum DDTs (µg/g lw)	Hg (µg/g ww)	Reference
Harbor seal (*Phoca vitulina*)	Hood canal (south)	2009	pup	1.57 ± 0.21	0.25 ± 0.05	6.01 ± 0.96	0.41 ± 0.04	3.52 ± 0.32	Our study
	Whidbey Basin			2.59 ± 0.63	0.48 ± 0.10	6.10 ± 2.26	0.72 ± 0.15	4.46 ± 0.36	
	Main Basin			6.34 ± 1.53	0.82 ± 0.14	7.64 ± 1.97	0.88 ± 0.31	11.11 ± 2.12	
	South Sound (east)			4.02 ± 0.73	0.66 ± 0.13	12.37 ± 1.87	0.54 ± 0.09	5.47 ± 0.60	
Harbor seal	South Puget Sound	1996 -1997	pup	18.13 ± 3.08		119 ± 16			Ross et al., 2004
Harbor seal	Southern California, USA	1994 - 2006	pup	9.59 ± 8.67			85.52 ±42.37		Blasius et al., 2008
Harbor seal	Northwestern Atlantic, Canada	1991 - 2001	pup	43.00 ± 11.66			21.10 ± 9.86		Shaw et al., 2005
Harbor seal	Northern Gulf of Alaska, USA	2000 - 2001	pup	0.131					Wang et al., 2007
Ringed seal (*Phoca hispida*)	Northern Alaska, USA	1996	juvenile / adult				0.56 ± 0.26		Kucklick et al., 2002
Ringed seal	Holman, Northwest Territories, Canada	2000	juvenile / adult		0.004	11.6			Ikonomou et al., 2002
Harbor seal	California, USA	1989 - 1998	juvenile / adult		1.73 ± 2.48				She et al., 2002
Harbor seal	Northwest Atlantic, Canada	1991 - 2005	pup		3.60 ± 7.40				Shaw et al., 2008
Harbor seal	California, USA	2006	pup					8.20 ± 0.60	Brookens et al., 2008
Harbor seal	Western Hudson Bay, Canada	1999 - 2006	pup					0.66 ± 0.13	Young et al., 2009
Steller sea lion (*Zalophus californianus*)	Prince William Sound, USA	1998 - 2000	pup					1.50 ± 0.50	Beckmen et al., 2002

Impacts of Contaminants on Harbor Seal Pup Health

Organic contaminants are a concern for the health of marine mammals, with observations of impaired reproduction, skeletal lesions, kidney damage, tumors, premature birth and skin lesions in populations inhabiting contaminated areas (Bergman *et al.* 2001; Olsson *et al.* 1994; Beckmen *et al.* 1997). Elevated PCB concentrations have been linked to decreased immune function in field studies of harbor seals (Mos *et al.* 2006), bottlenose dolphins (*Tursiops truncatus*) (Lahvis *et al.* 1995) and polar bears (Lie *et al.* 2005), as well as captive feeding studies of harbor seals (De Swart *et al.* 1994; Ross *et al.* 1996). In addition, contaminant-related immunotoxicity has been, in part, blamed for serious outbreaks of infectious disease in marine mammals (Osterhaus *et al.* 1995).

Different thresholds have been published for health effects in marine mammals. First, toxic equivalency factors (TEFs) have been developed for dioxins and dioxin-like compounds based on their relative toxicity compared to the highly toxic 2,3,7,8-TCDD and are used to calculate the total toxic equivalents (TEQ). In the 1990s, the World Health Organization evaluated previously developed TEFs and published revised values in 1998 (Van den Berg *et al*. 1998). TEFs were then further revised in 2005 (Van den Berg *et al*. 2006). A value of 209 pg/g TEQ was associated with immunotoxicity and endocrine disruption in harbor seals (De Swart *et al.* 1994; Ross *et al.* 1995). Our present results showed that using the 2005 TEFs lead to total TEQs 5 times lower than when using the 1998 TEFs. This difference was mainly attributed to CB-114, 118, 126, 157, and 157. If the 209 pg/g threshold were adjusted to new 2005 TEF values, it would be reduced by approximately 79%, to 44 pg/g. None of the harbor seal pups in this study surpassed this threshold (Table 4). However, calculation of TEQs only takes into account dioxin-like PCBs (i.e. 12 congeners out of the 209 possible) and other thresholds have been developed strictly based on total PCB concentrations. PCB levels above 17µg/g lw have been determined to be the PCB counterpart to the 209 pg/g TEQ threshold for immunotoxicity in captive harbor seals (Ross *et al*. 1996). A 10 µg/g lw PCB threshold was associated with an increase in calf mortality in bottlenose dolphin (Hall *et al.* 2006). Recently a more protective value of 1.3 µg/g PCBs was proposed for the protection of marine mammal health based on the 95% confidence interval for endocrine disruption and immunotoxicity in multiple studies of free-ranging harbor seals (Mos *et al.* 2010). 71% of our harbor seal pups surpassed this latter threshold suggesting a potential risk for adverse health effects.

Mercury is also a concern for the health of marine top predators since its methylated form bioaccumulates. However, since marine mammals have been exposed to heavy metals throughout their evolutionary history they have developed mechanisms to either control the internal concentrations of Hg or to mitigate its toxic effects. For instance, cetaceans and pinnipeds have developed a tolerance to mercury based on a protective interaction with selenium (Dietz *et al.* 2000). However, even though they might be able to tolerate higher mercury levels than terrestrial mammals, mercury, and especially methymercury, are still toxic and at some point concentrations are thought to exceed the capacity to detoxify. Methylmercury is well known for its neurotoxicity leading to sensory, motor deficits and behavioural impairment. MeHg is readily transported through the placenta and concentrates in the developing fetal brain (Clarkson 2002). Liver and kidney damage have been reported in bottlenose dolphins and polar bears with elevated mercury concentrations (Woshner *et al.* 2002; Sonne *et al.* 2007). In addition, in vitro studies on white

blood cells isolated from beluga whales (*Delphinapterus leucas*) and harbor seals showed that mercury exposure could result in immunotoxicity (De Guise *et al.* 1996; Das *et al.* 2008). Pools (1994) reported that total mercury level of 40 µg/g in hair of adult terrestrial mammals is considered "toxic". Total mercury levels in our seal pups fell below this threshold. However, other studies have shown that because seal pups have a limited ability to demethylate mercury, high percentages of methylmercury which were not measured in the present study have been reported in pup tissues elsewhere (Wagemann *et al.* 1988). Since methylmercury is considered the most toxic form of mercury, it cannot be ruled out that mercury levels reported in this study may indeed still represent a concern for the health and development of Puget Sound harbor seal pups.

Thyroid Hormones

Thyroid hormones play a significant role in growth regulation, cell differentiation, perinatal development of the nervous system, as well as in energy homeostasis and numerous key metabolic pathways (Jugan *et al.* 2010). Several studies have reported that organic contaminants and mercury are able to disrupt the thyroid hormone system. In our study, even though harbor seal pups from the four different sites had different contaminant levels, TH hormone levels were similar among sites. The only significant relationships observed between TH hormones and contaminants were negative correlations between TT3, FT3 and total PCDD. In a previous study comparing thyroid hormones in harbor seals from the same area and other nearby Canadian sites, Tabuchi *et al*. (2006) found a decrease of circulating TT4 with increasing PCBs. As PCDD levels appeared pretty low in our harbor seal pups, it remains unclear why other contaminants, and especially PCBs, did not correlate with the present thyroid hormone levels. However, it should be noted that in their study, Tabuchi *et al.* (2006) had a wider range of PCB concentrations, and that PCB levels in Puget Sound harbor seals have dropped appreciably during the intervening years.

A decrease in circulating thyroid hormones has been associated with PCB, PBDE, dioxins and mercury concentrations in several studies (Letcher *et al.* 2010; Mori *et al.* 2006). Even though the major form of thyroid hormone in the blood is T4, T3 is the most active form and is known to play a role in the maintenance and function of adipose tissue (Ailhaud *et al.* 1992). In a study treating young rats with T3, Grimaldi *et al.* (1982) found an induction of adipocyte cell proliferation, fat cell cluster formation, and lipid accumulation. Tabuchi *et al.* (2006) concluded that a decrease in thyroid hormones in blubber may alter metabolism within adipocytes and therefore present a risk to the structural and functional integrity of blubber, a tissue essential for energy storage and insulation in marine mammals.

Vitamin A

Vitamin A is involved in a wide variety of physiological functions including growth and development and is also essential for the immune system (Ball *et al.* 1992; Rolland 2000). In seals, blubber stores a large proportion of vitamin A (40-60%) (Mos and Ross 2002; Schweigert and Stobo 1994). There is limited placental transfer of vitamin A so nursing is an important period for the pups to acquire vitamin A from milk (Debier and Larondelle 2005). Simms and Ross (2000) concluded that nursing may represent a confounding factor in determining the effects of contaminant exposure on vitamin A dynamics as they found different relationships between organic contaminants and vitamin A in nursing and weaned pups. In the present study, all pups

were approximately the same age (four to six weeks old) and therefore all nearly weaned. Vitamin A levels in blubber were decreasing with contaminant concentrations even though only significant with ΣPBDEs and Hg. In addition, harbor seal pups from Hood Canal (south), which appears to be the least contaminated site, had significantly higher vitamin A levels. Similarly, Simms and Ross (2000) found that harbor seals from the less contaminated British Columbia, Canada, had higher circulatory vitamin A levels than harbor seals from Washington State, USA. In addition, PBDEs have been shown to negatively affect vitamin A levels both in experimental studies (Brouwer *et al.* 1989; De Swart *et al.* 1994) and in harbor seals, grey seals (Nyman *et al.* 2003; Vanden Berghe *et al.* 2010), and California sea lions (Debier *et al.* 2005).

Two mechanisms of action underlying the disruption of vitamin A by PCBs and related compounds have been proposed. Vitamin A disruption has been found to be in part an aryl hydrocarbon receptor (AhR) mediated response; the increase of AhR usually associated with contaminants induces the transcription of proteins involved in the regulation of vitamin A storage and catabolism. The second possible mechanism of action is an interference with the blood transport system of vitamin A. T4 and vitamin A are co-transported as the thyroxine – transhyretin (TTR) – retinol – retinol binding protein (RBP) complex. PCB metabolites compete with T4 and can bind to TTR resulting in a decrease in TTR affinity for the retinol – RBP complex which is then more easily excreted and leads to a decrease in vitamin A levels (Rolland 2000). The lower levels of vitamin A reported here in the most contaminated pups may be of concern for their health as disruption of vitamin A have been associated with immune deficiency (Brouwer *et al.* 1989).

Gene Expression

A total of 11 (including three normalizer genes) harbor seal specific primers were selected and designed for their potential to provide evidence of a health impact associated with environmental exposure to contaminants. We evaluated these genes in inner blubber, outer blubber, and skin. Most of the correlations between the expression of the different target genes and contaminants were found in inner blubber. Blubber is not a homogeneous tissue. Recently, it was found that in ringed seals there are three chemically distinct layers in terms of fatty acid composition. It was suggested that it might result in an associated stratification of fat soluble hormones and contaminants. The outer blubber layer has been described as relatively stable and mainly ensures insulation and buoyancy. In contrast, the inner blubber layer is more involved in the storage and mobilization of lipids therefore being a more metabolically active layer (Strandberg *et al.* 2008). In addition, higher organochlroine concentrations were found in the inner blubber of yearling harbor porpoises (Tilbury *et al.* 1997) and grey seals. These factors might help to explain why most of our relationships with contaminants were observed in the inner blubber layer.

POPs interfere with thyroid hormone physiology at different levels including hormone synthesis, circulatory transport, and TH metabolism (Zoeller 2005; Jugan *et al.* 2010). Above, we reported that levels of TT3 and FT3 were negatively correlated with PCDD concentrations. Studies have reported that contaminants are able to affect the thyroid hormone receptor activity as well as the thyroid hormone responsive gene expression (Zoeller 2005). In the present study, we did not find any correlation between TRα expression and contaminant levels. While this runs counter to the observations of Tabuchi *et al.* (2006), who found a positive correlation between TRα expression in inner blubber and PCB concentrations in harbor seal pups sampled in the same area,

contaminant levels have dropped appreciably since the earlier study. This may indicate an improvement in the health of harbor seals as contaminant levels decline.

The estrogen receptor alpha belongs to a group of receptors that are activated by the hormone 17β-estradiol (estrogen). The main function of the estrogen receptor is as a DNA binding transcription factor that regulates the transcription of genes involved in cell differentiation and proliferation. In the present study, we found a positive correlation between ERα expression and Hg concentrations. Mercury is known to increase the concentrations of reactive oxygen species therefore creating an oxidative stress. Studies have found that estrogens can have antioxidant effects by inducing the synthesis of protective molecule via activation of the estrogen receptor (Olivieri *et al.* 2002). A potential oxidative stress caused by mercury might therefore have triggered the up regulation of ERα in our harbor seal pups. Even though several studies have linked ER expression alterations with exposure to PCBs and / or other organic contaminants, no correlations were found in the present study. However, the up regulation of ERα in association with mercury might be a concern for harbor seal pups from Puget Sound since alterations of the ER signalling pathways have been reported to interfere with sexual development and the endocrine system.

Heat shock proteins are also referred to as stress proteins. Up-regulation of heat shock proteins can be triggered by a wide variety of physical and chemical stressors such as exposure to metals, oxidative stressors, hypoxia, and several carcinogens, mutagens, and teratogens. It represents the first cell response to physiological stress (Schlesinger 1990). In harbor seal pups from Puget Sound, WA, the expression of hsp 70 was positively correlated with Hg concentrations. Most studies investigating the impacts of mercury exposure on hsp expression were carried out on invertebrate species or fish and reported an increase in hsp 70 expression with exposure to Hg (Duffy *et al.* 1999; Franzellitti and Fabbri 2005). Limited data is available concerning mammals. An experimental study on rats reported an increase in hsp 70 expression with exposure to Hg. The observed up-regulation of hsp 70 was suggested to be a response to the oxidative stress caused by Hg exposure in order to protect and / or repair cellular damage (Schlesinger 1990; Reus *et al.* 2003).
Sample size and variability may have constrained a full evaluation of these relationships, since correlations between hsp 70 expression and ΣPCBs, ΣPBDEs, and ΣOC pesticides were nearly significant ($p = 0.06$). In recent studies, Fossi *et al.* (2010) also found an increase in hsp 70 expression in striped dolphin (*Stenella coeruleoalba*) skin slices exposed to a mixture of organochlorine compounds, PBDEs, and PAHs. Similarly, they found higher expression of hsp 70 in more contaminated male fin whales (*Balaenoptera physalus*) (Fossi *et al.* 2010), perhaps due to an influence of organic contaminants on the expression of this stress related protein.

The peroxisome proliferator-activated receptor γ belongs to a group of receptors that, upon activation, acts as transcription factors regulating the expression of genes mainly involved in the differentiation of adipocytes and lipid metabolism (Jiang *et al.* 1998). In the present study, PPARγ expression was positively correlated with ΣPCBs, ΣOC pesticides and Hg. As those three contaminants were also correlated with each other, it is difficult to make any conclusion on which of those contaminants is influencing PPARγ expression. This receptor is usually used as an indicator of nutritional stress and there are limited studies investigating the potential impacts of

contaminant exposure on PPARγ expression. Contrary to our present results, a study on rats reported a suppression of PPARγ following a TCDD treatment.

Conclusions and Recommendations

→ Samples collected from harbor seal pups are ideally suited for use in contaminant trend analyses because they are highly contaminated, represent an integration of concentrations in a broad selection of prey in a region, reflect health and physiological consequences from contaminant exposure and, with the utilization of non-emaciated live-captured pups, provide minimal inter-sample variability allowing sensitive detection of changes over space and time.

→ Harbor seal pups from the Main Basin of Puget Sound are more PCB- and PBDE-contaminated than seal pups from three other Puget Sound locations (Hood Canal (south) < Whidbey Basin < South Sound (east)), likely reflecting their proximity to urban contaminant sources in Seattle and surrounding areas, as well as possible differences in feeding ecology.

→ Harbor seal pups from Hood Canal (south) had the lowest PCB and PBDE concentrations, likely reflecting a combination of a less contaminated terrestrial environment and/or different feeding ecology.

→ PCBs dominated the organic contaminant composition in harbor seal pups (on average 6 times higher than PBDEs > OC pesticides > PCDDs > PCDFs). These results, adjusted by the relative toxicity of the different contaminant constituents, clearly indicate that despite regulations imposed in the mid 1970s, PCBs remain the top ranked contaminant of concern in harbor seals.

→ Despite the continued dominance of the PCBs as a top POP concern, other studies suggest that PBDE concentrations have been doubling every 3.5 years in the aquatic environment and are rapidly emerging as a concern to aquatic wildlife.

→ Vitamin A levels were lower in harbor seal pups from contaminated sites, and some gene expression endpoints (Erα, hsp70, and PPARγ) were associated with contaminant concentrations, suggesting that harbor seal health is affected by persistent contaminants in Puget Sound.

→ Further health-oriented research will provide more insight into the effects of persistent contaminants of concern on marine mammals in Puget Sound, and document trends for POP contaminananants in response to regulations and mitigation measures over time.

→ In addition, further research into the influence of variable feeding ecology of harbor seals at different locations will clarify the role that trophic level plays in shaping POP contaminant exposure, and strengthen the utility of harbor seals as sentinels of the health of the Puget Sound environment.

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