Global DNA methylation and persistent organic pollutants in ringed seals (*Phoca hispida*) from Svalbard and the Baltic Sea

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Abstract

It is not known whether exposure to persistent organic pollutants (POPs) cause epigenetic changes in marine mammals. Ringed seals are a key species at the higher trophic levels of arctic marine ecosystems, and may be particularly vulnerable due to bio-magnification of POPs in the food web. DNA methylation is a well-studied epigenetic mechanism, and is important for gene regulation and chromosomal stability. Changes in DNA methylation may lead to diseases such as cancer and developmental disruption.

The aims of this study are (i) to investigate whether sex, age and moulting status affect global DNA methylation measured as percent 5-methylcytosine (%5-mC) in tissues of ringed seals, (ii) to compare %5-mC levels in liver and kidney of ringed seals, (iii) investigate whether %5-mC differs in tissues of ringed seals from two differently polluted areas, Svalbard and the Baltic Sea, and (iiii) clarify whether there are associations between individual POPs and %5-mC in ringed seal liver and kidney from Svalbard and the Baltic Sea.

ELISA was applied to measure %5-mC. POPs were measured from liver and plasma by gas chromatography- mass spectrophometry (GC-MS). Univariate analysis were used for investigation of %5-mC in relation to biological factors and area, while principal components analysis (PCA) and non-parametric correlation analysis was used to investigate the relationship between %5-mC and POPs.

A significant difference in %5-mC was found between moulting and pre-moulting individuals, and between liver and kidney. Sex, age and area did not affect %5-mC. Several PCBs, PBDEs, CHLs, p,p’-DDE and 4'-OH-CB172 were significantly correlated with %5-mC, but the results were not the same between areas or tissues. Correlations between POPs and %5-mC were negative in liver and kidney from Svalbard, and in kidney from the Baltic Sea, while positive in liver from the Baltic ringed seals. The results indicated lower %5-mC levels in moulting seals compared to pre-moulting which could be caused by a reduction in dietary methyl donors due to fasting, leading to depletion of methyl donors in the cells. However, precaution must be taken due to small sample size. The lower %5-mC levels found in liver compared to kidney may be caused by a depletion of methyl-donors in hepatic cells as a result of enhanced xenobiotic metabolism and oxidative stress induced by POP exposure, in addition to reduced intake of dietary methyl-donors. Interaction by other contaminants than those measured here, mixture toxicity and non-linear relationships might also play a role in the relationship between %5-mC and POPs.

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1 Introduction

1.1 Persistent organic pollutants

Persistent organic pollutants (POPs) comprise a group of organic compounds generated either as commercial products or industrial byproducts. Several groups of chemicals, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs, used as flame retardants) and organochlorine pesticides (OCPs) including hexchlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT), chlordanes (CHLs) and toxaphenes (Toxs), all fall into this category, sharing similar physic-chemical properties such as persistence, capacity for long-range transport, lipophilicity and toxicity. Detrimental effects like cardiovascular diseases, cancer, immunological-, endocrine- and reproductive disruption have been associated with high levels of POP exposure in humans and wildlife (Hoppin et al. 2000, Engel et al. 2007, Sonne 2010, Lind et al. 2012, Sjöberg Lind et al. 2013, Villanger et al. 2013). POPs with the ability to induce xenobiotic metabolism in an organism are considered to have greater toxic potential than substances without this ability (Van den Berg et al. 1998). Metabolism of xenobiotics can create toxic metabolites from less toxic precursors (Guengerich et al. 1985) and affect concentrations and patterns of POPs within the organism.

Since 2004 the use and release of POPs have been highly constricted or banned completely by the Stockholm convention[1], but regulation of for example PCBs started already in the 1970's. Still, high levels of POPs are found in terrestrial and marine ecosystems, including biota, ranging from small invertebrates to humans (AMAP 2004, Fisk et al. 2005, Muir and de Wit 2010). The capacity for long-range transport relocates POPs from distant southern-more sources to northern polar regions via atmospheric and oceanic circulation and river run-off (Barrie et al. 1992, Lohmann et al. 2007). Persistency and lipophilicity promote accumulation and bio-magnification in marine food webs (Borgå et al. 2001, Fisk et al. 2002, AMAP 2004, Borgå et al. 2004, Letcher et al. 2009). There is great concern for the adverse health effects POPs may assert on marine mammals. Seals and whales are particularly vulnerable due to their high position in food webs, low metabolism and excretion of POPs, and maternal transfer of contaminants to their pups through lactation (Norstrom et al. 1992, Wolkers et al. 2006, Needham et al. 2010).

[1] An international agreement on POPs (http://chm.pops.int)

1.2 Ringed seals

Ringed seals (*Phoca hispida*) (Figure 1) are apex predators of marine ecosystems. They have a circumpolar distribution in the Arctic and are fully dependent on sea ice and snow cover for their survival. Some populations are found in southern industrialized areas, such as the Baltic Sea. After reaching maturity from around the age of four, females produce one pup a year, to which they give birth in subnivean birth lairs. The birth lairs are snow caves excavated over breathing holes in the sea ice, and are of vital importance to avoid hypothermia and predation, especially for pups.

Figure 1: Ringed seal. (Photo: Hans Wolkers/NorwegianPolar Institute)

To survive the cold, harsh conditions of the Arctic, ringed seals rely on thick insulating and lipid rich blubber. However, this makes them additionally susceptible to accumulation of POPs. Further, age is an important factor for accumulation of POPs in seals, and the increase is found to be greatest in males (Wolkers et al. 1998, Muir et al. 2000, Fisk et al. 2002). The lower tendency to accumulation of POPs in females is attributed to reproduction and lactation with subsequent transfer of POPs through the milk. However, unlike many pinnipeds, female ringed seals feed during lactation, resulting in comparatively low mobilisation of lipid-associated contaminants compared to other marine mammals (Ryg et al. 1990, Lydersen 1995). A natural, but important, stress factor in ringed seals is the annual moulting and fasting period. During this period the epidermis renews and hairs are shed. Routti et al. (2010) found

that moulting/fasting affects hormone, vitamin and POP levels, and that moulting status should be considered when using variables related to hormone, calcium or vitamin homeostasis as biomarkers for contaminant effects.

1.2.1 Svalbard and the Baltic Sea

Ringed seals can be divided into several subspecies, although little genetic variation is found between ringed seal subspecies, based on microsatellite studies (Palo et al. 2001).
Ringed seal inhabiting arctic waters (*Phoca hispida hispida*) is considered as less polluted compared to those from industrialized areas, owing to low industrial and human activity (Nyman et al. 2002, Routti et al. 2008a). Most of the POPs found in the arctic are attributed long-range transport (Macdonald et al. 2005). Located in the Arctic, the archipelago of Svalbard is surrounded by large water masses comprising the Arctic Ocean, the Barents Sea and Atlantic water entering from the North Sea. In contrast, the Baltic Sea, inhabited by the Baltic ringed seal subspecies (*Phoca hispida bothnica*), is regarded as a highly polluted area. Almost completely surrounded by land, a narrow passage in the south, between Denmark and Sweden, allows for limited exchange of water. This acts as a trap for the large amounts of contaminants released in to the shallow water basin from local industry, agriculture, shipping and river runoff from the nine countries surrounding the Baltic Sea (Rheinheimer 1998).

During the 20th century the Baltic ringed seal population declined from approximately 200.000 to about 5000 individuals due to hunting (Harding and Harkonen 1999). Regrowth of the population was stalled due to sterility, presumably caused by organochlorine contamination between the mid-1960s up until the 1990s (Olsson et al. 1994). Nyman et al. (2002) found that although concentrations of PCBs and DDTs in Baltic ringed seals had declined since the peak in the 1970s, the current load was still high enough to negatively impact their health, and that a large portion of females were still suffering from uterine occlusion causing sterility. Routti and co-workers have conducted several studies on the effects of POPs in ringed seal from Svalbard and the Baltic Sea, and found POP concentrations in the Baltic Sea ringed seals to be more than ten times higher than in ringed seal from Svalbard (Routti et al. 2008a, Routti et al. 2009a, Routti et al. 2009b). Further, contaminant exposure in the Baltic ringed seals has been related to changes in thyroid and vitamin A homeostasis, xenobiotic-metabolizing enzymes, as well as effects on genes related

to tumour growth, lipid metabolism and inflammation (Routti et al. 2008b, Routti et al. 2010, Castelli et al. 2014).

1.3 Epigenetics

Epigenetics is the study of heritable changes in gene expression, either mitotically or meiotically, that do not involve changes in the DNA sequence. DNA methylation is an epigenetic mechanism where a methyl group is added to the 5 position of cytosine (5-mC) in cytosine-guanine dinucleotides (CpG). The process is involved in regulation of gene expression and contributes to chromosomal structure and stability (Bird 1986). In general, methylation of the promotor region of a gene helps facilitating gene silencing, whereas removing of methylation from a promotor region helps facilitating gene activation (Jones and Takai 2001) (Figure 2).

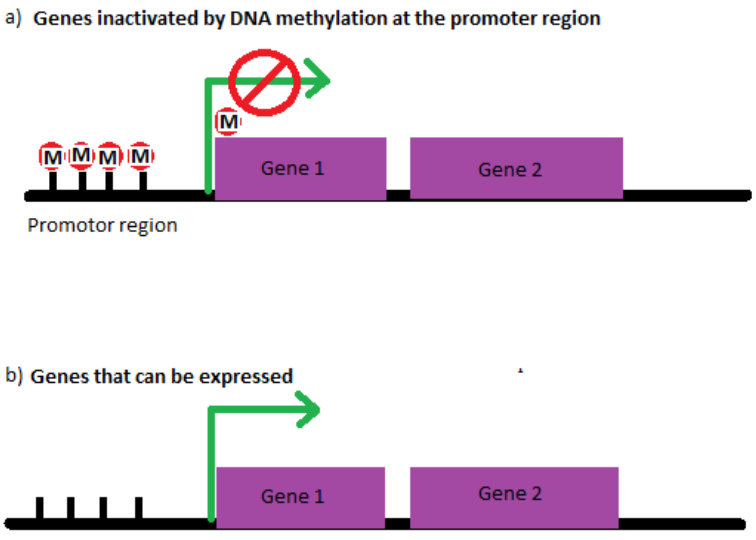


Figure 2: Inactivation and activation of genes by DNA methylation. a) depicts genes inactivated by DNA methylation in the promotor region. b) depicts genes with unmethylated promotor region, thereby accessible for expression

DNA methylation is considers to be both species- and tissue-specific (Gama-Sosa et al. 1983). Unintentional adding or removing of DNA methylation can disturb gene regulation and lead to adverse effects in the cell, with potential organ and systemic dysfunction. Metabolism is one of several cellular factors known to affect DNA methylation status (Ulrey et al. 2005). An important example is the growing evidence that perturbations in the methionine cycle, which

metabolic products include methyl-donors and homocysteine (Finkelstein 1998) cause epigenetic modulations. In addition, DNA methylation seems to be involved in illnesses caused by elevated cellular homocysteine levels (Yi et al. 2000, Ulrey et al. 2005). Methyl-donors needed for DNA methylation not only origin from endogenous processes such as the methionine cycle, but also from external sources. A study by Dolinoy et al. (2007) found that supplement of dietary methyl-donors e.g. folate, counteracted the demethylating effect of bisphenol a (BPA) in rats, thus, suggesting that dietary methyl-donors could play an important role in withholding the methylation status of DNA.

In relation to POPs, both *in vitro* and *in vivo* studies have shown alterations in DNA methylation after exposure to POPs and other environmental contaminants (Bollati et al. 2007, Desaulniers et al. 2009, Wang et al. 2009). Further, alterations in DNA methylation after exposure to contaminants have in some cases been found to occur not only in the individual originally exposed, but also in non-exposed offspring (Anway et al. 2005, Newbold et al. 2006). Reduction in DNA methylation has been linked to altered gene regulation and chromosomal instability, due to the role of DNA methylation as a genome-wide transcriptional regulator (Jackson-Grusby et al. 2001). Rusiecki et al. (2008) found decreasing global DNA methylation levels with increasing blood levels of POPs in Greenlandic Inuits. Similarly, Kim et al. (2010) found an inverse correlation between POPs and global DNA methylation in a South-Korean population. A negative association between tissue mercury concentrations, and global DNA methylation was observed for male polar bears by Pilsner et al. (2010). Both hyper- and hypomethylation of DNA have been associated with detrimental health effects, including various forms of cancer, cardiovascular diseases, reproductive dysfunction and mental retardation (Oberlé et al. 1991, Ehrlich 2002, Jirtle and Skinner 2007, Ordovas and Smith 2010, Wu et al. 2010).

A range of different methods for measuring global DNA methylation are available. Regarded as the ‘gold standards’ are the chromatography-based methods, such as high-performance liquid chromatography (HPLC), but these are less used to day due to demanding protocols and the need of relatively large amounts of DNA. A commonly used method for measurement of global DNA methylation, is a method based on bisulphite conversion and pyrosequencing of the highly methylated, -repetitive elements *Alu* and long interspersed nucleotide elements (LINE), which are considered as surrogates for global DNA methylation

(Yang et al. 2004). This method is species specific, and not feasible for species with a non-resolved genome. The enzyme-linked immunosorbent assay (ELISA) for global DNA methylation is a method where DNA is bound to assay wells, before capture antibodies sensitive to methylated cytosine are applied. Detection antibodies bind to the capture antibodies, creating a colour change proportional to the amount of methylated DNA, which can then be quantified in a plate reader.

1.4 Aim of study

In recent years several studies have revealed weight of evidence in the assessment of the potential health impacts from POP exposure on e.g. endocrine, vitamin, reproductive and immune systems of marine mammals (Fisk et al. 2005, Letcher et al. 2010, Villanger et al. 2011), but little is known about the mechanisms behind these responses. Epigenetic changes could potentially be such a mechanism, however, to my knowledge the present study is the first to investigate epigenetics in relation to POP exposure in marine mammals. Ringed seals are influenced by several environmental stress factors. In addition to POP exposure, climate change also poses a threat to ringed seal health and survival. In 2010, Kelly et al. suggested to classify ringed seal as a threatened species due to climate change, as shrinking sea ice leads to destruction of ringed seal habitat and causes decreased prey populations. With ringed seal being recommended as model species for arctic marine mammals (AMAP 2004), implementation of epigenetic studies could potentially bring forth new knowledge on ringed seal health and in addition give important implications for other marine mammals.

The aim of the present study was to use the ELISA method to investigate and compare global DNA methylation levels in liver and kidney of ringed seals from two differently polluted populations, Svalbard and the Baltic Sea, and to clarify whether there is a relationship between exposure to persistent organic pollutants (POPs) and global DNA methylation.

1.4.1 Questions being asked and null hypotheses:

- Does sex, age or moulting status affect global DNA methylation levels?
  H_0: There is no difference in global DNA methylation levels between ringed seal males, females or sub-adults, or between moulting and pre-moulting individuals.

- Do global DNA methylation levels differ between liver and kidney?
  H_0: There is no difference in global DNA methylation levels between liver and kidney.

- Do global DNA methylation levels differ between more and less polluted ringed seal populations, namely from Svalbard and the Baltic Sea?
  H_0: There is no difference in global DNA methylation levels between ringed seals from Svalbard and the Baltic Sea.

- Is global DNA methylation related to tissue concentrations of POPs?
  H_0: There is no relationship between concentrations of POPs and global DNA methylation levels.

2 Materials and methods

2.1 Sample origin

Sampling of ringed seals was conducted by the Norwegian Polar Institute and the Finnish Game and Fisheries Research Institute as part of a project on contaminant effects on ringed seals in 2006-2007. The seal samples were collected from two areas, Svalbard, Norway and the Baltic Sea. In Svalbard, ringed seals were sampled by the Norwegian Polar Institute granted special permission by the Governor of Svalbard, and during the local hunting season following the local hunting law of Svalbard. A total of 21 ringed seals were sampled in May and June 2007 from fjords on the west coast of Spitsbergen, Van Mijenfjorden and Tempelfjorden (77°47′N to 78°23′N, 17°00′E), 6 and 15 from Van Mijenfjorden and Tempelfjorden, respectively. The Finnish Game and Fisheries Research Institute were granted special permission by The Ministry of Forestry and Agriculture in Finland to sample ringed seals in the Baltic Sea. Sampling of 22 seals in total was conducted in April 2006 and 2007 (8 seals in 2006, and 13 seals in 2007), in the Bothnian bay, north-west of the island Hailuoto (65°10′N, 24°20′E). For both the Svalbard and Baltic areas the samplings took place during the moulting season of the seals. Moulting status (moulting/pre-moulting) was determined for all individuals by observing the hair. Individuals with no visible shedding were classified as pre-moulting, whereas individuals with visible shedding of hair were classified as moulting. Determination of age was conducted by counting annual layers from thin transverse sections of either the molar or canine tooth. Liver and kidney samples for effect studies were packed in aluminium foil and frozen in liquid nitrogen in the field and stored at -80˚C. Sampled for chemical analysis were stored at -20˚C. Seals ≥ 4 years old were considered as adults and < 4 years as subadults (Krafft et al. 2007). Number of individuals grouped according to area, sex and age group is given in Table 1.

2.2 DNA methylation

Liver and kidney samples were shipped from the Norwegian Polar Institute to the Department of Biosciences, University of Oslo, in a dryshipper. Each sample was packed in aluminium foil. For the transfer of tissue to eppendorf tubes (2 ml), a cooled working station was made

by putting a metal plate on top of dry ice in a styrofoam lid and then frequently pouring over liquid nitrogen, avoiding thawing of the samples. Forceps and scalpels were used for removing the aluminium foil and to cut in to the tissue pieces avoiding the outermost part of tissue, which was not sterile. dH_2O and 70% ethanol were used to clean the equipment between handling of each sample. The tissue samples were then stored at -80˚C until further processing.

Table 1: Number of liver and kidney samples of ringed seal from Svalbard and the Baltic Sea, divided area and sub-groups (males (adult males ≥4 years old), females (adult females ≥4 years old) and sub-adults (young males <4 years and young females <4 years) and moulting status (moulting/pre-moulting). Also, the total number for each area is included.

	Svalbard			the Baltic Sea		
	total	**moulting**	**pre-moult**	**total**	**moulting**	**pre-moult**
Liver	21			22		
Males		5	6		12	0
Females		6	0		7	0
Sub-adults		4	0		2	1
Kidney	21			10		
Males		5	6		6	0
Females		6	0		2	0
Sub-adults		4	0		1	1

2.2.1 DNA extraction

For DNA extraction a DNeasy Blood & Tissue Kit (QIAGEN Group, Netherlands) was used. Prior to the extraction 25 mg or less tissue was transferred to eppendorf tubes (2 ml) to stay within the maximum limit of 25 mg tissue for using the kit.

The DNA extractions were conducted following the kit protocol. The first step was sample lysing. Here different procedures could be followed dependent on sample type. The procedure for rodent tails was used. Each sample was added 180 µl Buffer ATL and 20 µl proteinase K and incubated at 56˚C for approximately 2 hours for lysing. During incubation the samples were intermittently vortexed. After lysing, the samples were vortexed for 15 seconds before the addition of 200 µl buffer AL and another round of vortexing. Then 200 µl of 96% ethanol was added and mixed by vortexing before pipetting the samples into spin columns with collection tubes (2 ml) for centrifugation at 6000 x g for 1 minute. The addition of ethanol

precipitates DNA, while the centrifugation in spin columns traps the DNA to enable cleaning. Next the samples were cleaned twice, first with 500 µl buffer AW1 and then 500 µl Buffer AW2. In the last step DNA was eluted by adding 200 µl buffer AE to the spin columns (which were placed in new eppendorf tubes (2 ml)) and incubate for 1 minute in room temperature before centrifugation at 6000 x g for 1 minute. All centrifugations were at room temperature. All buffers were included in the DNeasy Blood & Tissue Kit (QIAGEN Group, Netherlands).

DNA concentrations were quantified by UV spectrophotometry, measuring the absorption of light by DNA at $A_{260/280}$ in a plate reader (SynergyMx, BioTek Instruments, USA). dH_2O were used as laboratory blanks and all samples were run twice with two laboratory blanks for each batch of seven samples. A mix sample containing 10µl of each sample was also measured. The DNA concentrations were calculated as the mean of the two runs for each sample. The DNA samples were stored at -20˚C until further processing.

2.2.2 DNAs global methylation (ELISA)

An Imprint® Methylated DNA Quantification Kit (MDQ1 Sigma, Sigma-Aldrich, USA) was used to measure the methylation levels of global DNA. The kit detects methylated cytosine nucleotides (5-mC) by the use of capture and detection antibodies in pre-coated 96-well plates. Quantification was done colorimetrically at A_{450} and the amount of methylated DNA calculated from the absorbance measured.

Prior to ELISA analysis all DNA samples were diluted in AE elution buffer from the DNeasy Blood & Tissue Kit (QIAGEN Group, Netherlands) to a concentration of 50 ng/µl. The ELISA analysis was conducted following the kit protocol. First all DNA samples, including methylated control DNA, was diluted in DNA Binding Solution to a concentration of 5 ng/µl. Then 30 µl of all samples were added to the 96-well plates for a total of 150 ng DNA. Control DNA was added at four different concentrations for a total of 25, 50, 150 and 200 ng. DNA Binding Solution was used as blank. All samples, control DNA and blank were run in triplicate on three 96-well plates. After samples were applied the plates were incubated at 28˚C for 50 minutes and 37˚C for 30 minutes, before the addition of 150 µl Block Solution and further incubation at 37˚C for 30 minutes. Then the plates were washed three times with 1xWash Buffer (diluted in MilliQ water). Further, Capture Antibody was diluted 1:1000 in 1xWash Buffer and an amount of 50 µl were added to each well and incubated at room

temperature for 60 minutes. The Capture Antibody was removed and the plates washed four times with 1xWash Buffer. Then Detection Antibody was diluted 1:1000 in 1xWash Buffer and 50 µl added to each well and incubated in room temperature for 30 minutes. After removing the Detection Buffer the plates were washed five times with 1xWashbuffer. Next 100 µl of Developing Solution were added to each well and the plates were covered with aluminium foil to protect from light. A light blue color developed and after 5 minutes 50 µl of Stop Solution was added to each well to stop the reaction. The reaction then turned yellow and the plates were ready for absorption reading in a plate reader (SynergyMx, BioTek Instruments, USA) using the program Gen5 1.10 to read the plates at $A_{450.}$
To calculate the relative global methylation levels the Single Point Method described in the kit protocol was used. First, the absorbance readings for the samples, control DNA and blanks were averaged. Then the following calculation was applied to calculate DNAs global methylation:

$$\%5\text{-mC} = ((A_{450\ av}\ \text{Sample} - A_{450\ av}\ \text{Blank})/(A_{450\ av}\ \text{Methylated Control DNA} - A_{450\ av}\ \text{Blank})) \times 100$$

which is the measured DNA methylation level relative to the methylated control DNA.

2.3 Chemical analysis

To establish the contamination levels of persistent organic pollutants (POPs) in the seals, liver and plasma samples from all individuals were analysed for PCBs, PBDEs and the following OCPs: HCB, DDE, CHLs and Toxs. Also the levels of OH- and $MeSO_2$-metabolites of the POPs were analysed. Analysis of POPs was conducted previously and analytical methods are described in Routti et al. (2008a, 2009a). Sample extractions were conducted by H. Routti and quantification by H. Björnhoft (University of Örebro) and S. Chu (Environment Canada). Briefly described, the methods were as following:

2.3.1 POPs and POP metabolites in liver

For the analysis of liver samples, PCBs, PBDEs and OCPs were extracted using a Suprex Autoprep/Accutrap Supercritical Fluid Extraction machine (Suprex, USA). Elution of the POPs was done with combination mini silica columns using n-hexane as elution solvent. The solvent was change to tetradecane before the samples were analysed by gas chromatography – mass spectrometry (GC-MS) (Agilent 6890, Agilent 5973, Agilent, Germany).
For the analysis of PCB metabolites in liver an ASE 200 accelerated solvent extraction system (Dionex, Canada) was used with dichloromethane/n-hexane (50:50) for extractions. The samples were cleaned by a gel permeation chromatography Autoprep 2000 (OI Analytical, USA) and concentrated by a LC-Si solid phase extraction (SPE) cartridge (500 mg, 6 mL, Baker, USA). The solvent was changed to isooctane before GC-MS analysis.

2.3.2 POP metabolites in plasma

For plasma analysis the POP metabolites were extracted from the plasma by combined silica columns, and eluted with n-hexane. The solvent was changed to tetradecane before analysis by GC-MS.
For the analysis of PCB and PBDE metabolites in plasma all contaminants other than the ones in question were removed with methyl tert-butyl ether MtBE/HEX (50:50). After further cleaning and change of solvent to isooctane the samples were analysed with GC-MS.

2.3.3 Quality control

All samples were spiked with an internal standard (IS) prior to analysis. For quality assurance and quality control (QA/QC) measures, human adipose tissue was used as standard reference material (SRM) and run together with a laboratory blank sample with each batch of 7-10 samples.
Hexane, ethanol and dicloromethane were used to clean all glassware and extraction vessels.

2.4 Data analysis

All analysis were conducted using the software JMP 8.0.2. (SAS, Cary, USA) GraphPad Prism 5 (GraphPad Software, La Jolla, USA) was used for graphical displaying of data. Shapiro-Wilk test was used to test variables for normality (Shapiro and Wilk 1965). For normally distributed variables parametric test were used, for non-normally distributed variables non-parametric test was used. Two-way analysis of variance (ANOVA) was used to test differences in %5-mC against sex and age (defined as sub-groups: males, females and sub-adults), and moulting status (Zar 1999).
Levene's test of homogeneity was applied to verify that the assumption of equal variance between groups, required by ANOVA, was met (Levene 1952). The non-parametric Wilcoxon rank-sum test was used to test the difference between moulting and pre-moulting males from Svalbard (Wilcoxon 1945). To confirm the results for sub-groups, i.e. that age did not affect %5-mC, the non-parametric Spearman's ρ correlation test was used on the variables %5-mC and age (years) (Spearman 1904).
To investigate possible differences between tissue types, the parametric t test was used (Zar 1999). This was also done to investigate possible differences between areas.

Further, the relationships between POP exposure and DNA methylation were investigated. First, POP's were divided into their respective classes and summed according to compound group (Table 3). Only the POP's that were detected in all individuals were included. Four individuals were lacking POP data entirely (three sub-adults from Svalbard and one sub-adult from the Baltic Sea) and were excluded from statistical analysis regarding POPs. Most samples from the Svalbard ringed seal population were below the limit of detection for the OH and $MeSO_2$-metabolites. Thus only metabolite levels from the Baltic population were used in statistical analysis. In addition, only metabolites found in all Baltic individuals were included in analysis, resulting in nine PCB metabolites (Table 6).

Principal component analysis (PCA) was used to investigate possible correlations between %5-mC and POPs (Wold et al. 1987). PCA is a means for reducing complex data by transforming many possible correlating variables in to fewer principal components (PC). Loading values on the PCs describe the influence of the variable on the total variation. Values close to 1 have high influence on the variation, whereas values close to zero have little influence. In the loading plot variables located close to each other indicate positive correlation

and variables with opposite location are negatively correlated (Wold et al. 1987). PCA is a robust analysis, resistant to non-normal distributions and skewness, therefor no transformation of data have been conducted (Wold et al. 1987).
A PCA was performed separately for liver and kidney from each area. The same procedure was used for PCB metabolites in Baltic ringed seals.
POPs with loading >0.75 on PC1 (the component explaining most of the variability of the data) were investigations further for possible correlations with %5-mC, using Spearman's ρ correlation analysis. The same criteria were used for PCB metabolites.

When performing multiple correlation analysis, the probability of producing false positives increases i.g. rejecting H_0 when H_0 is true (type I error). Bonferroni correction (Cabin and Mitchell 2000) can be used to reduce type I errors when multiple test are conducted. On the other hand, applying Bonferroni correction increase the probability of producing false negatives, i.g. not rejecting H_0 when H_0 is false (type II error) (Nakagawa 2004). When investigating potentially harmful substances, such as in the present study, precautionary should be taken, as the consequences of type II errors (not discovering a harmful substance) would be more serious than that of type I errors (claiming a substance harmful when it's not). Therefore Bonferroni corrections were not applied in the present study.

Level of significance was set to $\alpha = 0.05$ for all statistical analysis.

3 Results

3.1 Global DNA methylation in relation to biological factors

Table 2 presents the levels of %5-mC found in liver and kidney, grouped according to area, sex and age (males (adult males ≥4 years old), females (adult females ≥4 years old) and sub-adults (young males <4 years and young females <4 years). Further, males from Svalbard are divided according to moulting status (moulting/pre-moulting).

3.1.1 Effect of sex, age and moulting status

No significant difference was found in %5-mC in liver or kidney between sub-groups (males, females, sub-adults) from Svalbard (two-way ANOVA, p= 0.72). Further, no significant difference in liver %5-mC were found between sub-groups from the Baltic Sea (two-way ANOVA, p=0.79) (Table 2, Figure 3). There were too few individual to compare %5-mC in kidney between the sub-groups from the Baltic Sea (Table 2). Spearman's ρ correlation test between %5-mC and age confirmed that age did not affect %5-mC (Spearman's correlation, p = 0.99 and p = 0.48 for Svalbard and the Baltic Sea, respectively). Pre-moulting individuals were not included in the analysis as they differed significantly from the rest of the population (two-way ANOVA, p=<0.01). To further investigate the difference in %5-mC between moulting and pre-moulting individuals Wilcoxon rank-sum test was used. Because of small sample size, only males from Svalbard could be tested (Table 2). A statistically significant difference was found for both liver and kidney (Wilcoxon, p=0.04 for both tissues). Pre-moulting males had significantly higher %5-mC than moulting males (Figure 3).

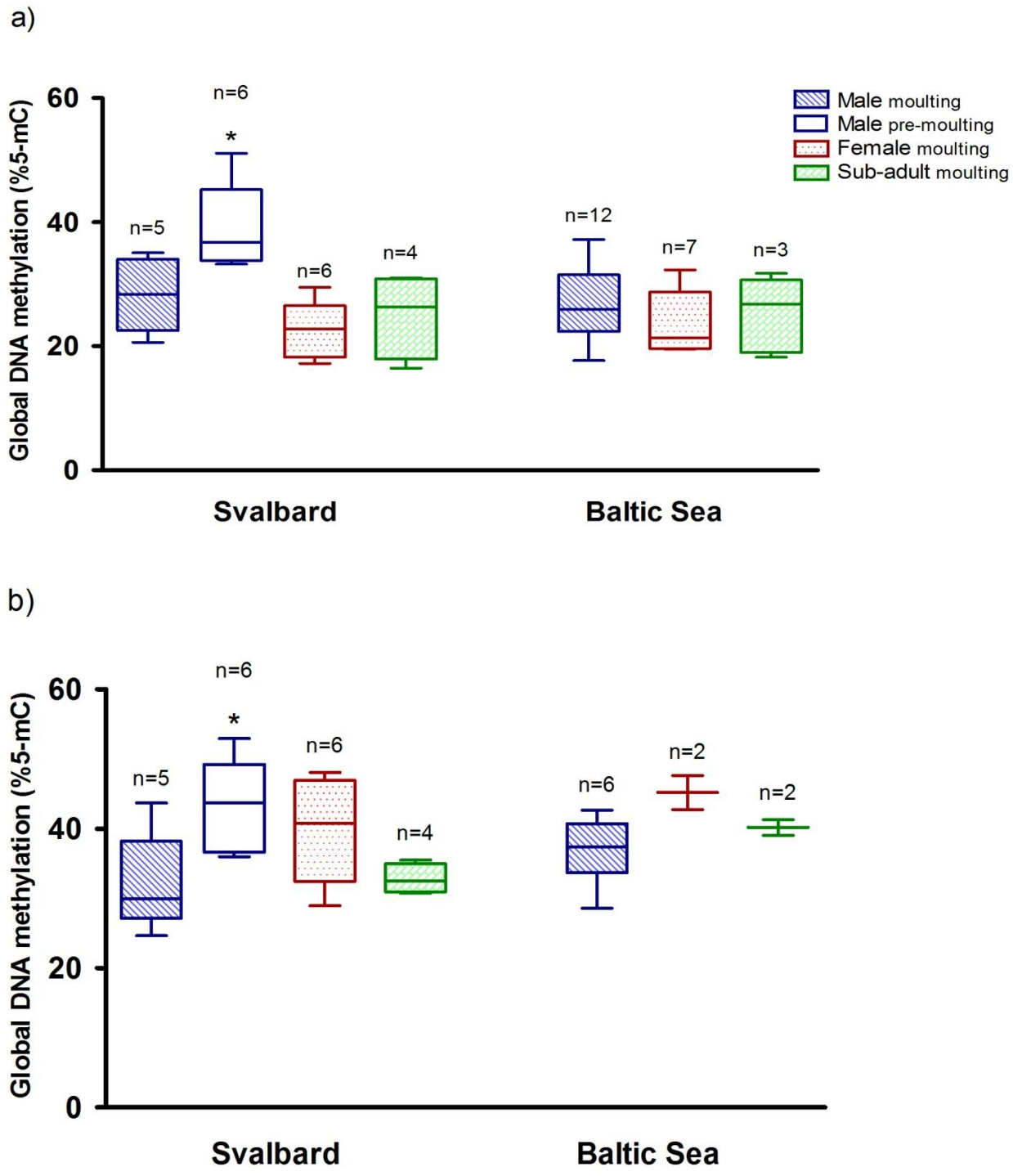


Figure 3: %5-mC in a) liver (upper plot) and b) kidney (lower plot) from moulting and pre-moulting males from Svalbard, female and sub-adult individuals from Svalbard and the Baltic Sea; mean, 2.5 and 97.5 percentile. * indicates p= ≤ 0.05.

Table 2: %5-mC in liver and kidney of ringed seals from Svalbard and the Baltic Sea. Sample size (n) from each area and their sub-groups, mean and standard deviation (sd), minimum and maximum values (min-max).

	Liver			Kidney		
	n	**mean ± sd**	**min - max**	**n**	**mean ± sd**	**min - max**
Svalbard	21	29.2 ± 8.60	16.4 - 51.1	21	37.7 ± 7.49	24.7 - 53.0
Males, moulting	5	28.3 ± 5.31	20.6 - 35.1	5	32.1 ± 6.3	24.7 - 43.7
Males, pre-moult	6	39.2 ± 6.32	33.2 - 51.1	6	43.5 ± 5.94	36.0 - 53.0
Females	6	22.7 ± 4.11	17.2 - 29.5	6	39.8 ± 6.74	29.0 - 48.1
Sub-adult	4	25.0 ± 6.04	16.4 - 31.0	4	32.8 ± 1.84	30.8 - 35.5
Baltic	22	25.5 ± 5.42	17.7 - 37.2	10	39.2 ± 4.88	28.6 - 47.6
Males	12	26.3 ± 5.65	17.7 - 37.2	6	36.9 ± 4.44	28.6 - 42.6
Females	7	24.0 ± 4.58	19.5 - 32.3	2	45.2 ± 2.46	42.7 - 47.6
Sub-adults	3	25.6 ± 5.58	18.2 - 31.7	2	40.2 ± 1.1	39.1 - 41.3

3.1.2 Effect of tissue

There was a significant difference in %5-mC between liver and kidney in ringed seals from Svalbard (t-test, $p = < 0.001$). Similarly, the Baltic population had a significant difference in %5-mC between liver and kidney (t-test, $p = <0.001$). In both populations liver %5-mC was lower than in kidney (Figure 4). Pre-moulting males from the Svalbard population were not included in the analysis.

Wilcoxon rank-sum test between liver and kidney in pre-moulting individuals revealed no significant difference (Wilcoxon, $p = 0.23$).

3.1.3 Comparison between Svalbard and the Baltic Sea

When comparing %5-mC in liver and kidney between Svalbard and the Baltic Sea, no significant difference was found (t test, $p = 0.89$ and $p= 0.94$, for liver and kidney, respectively). Pre-moulting individuals from Svalbard was not included in the analysis.

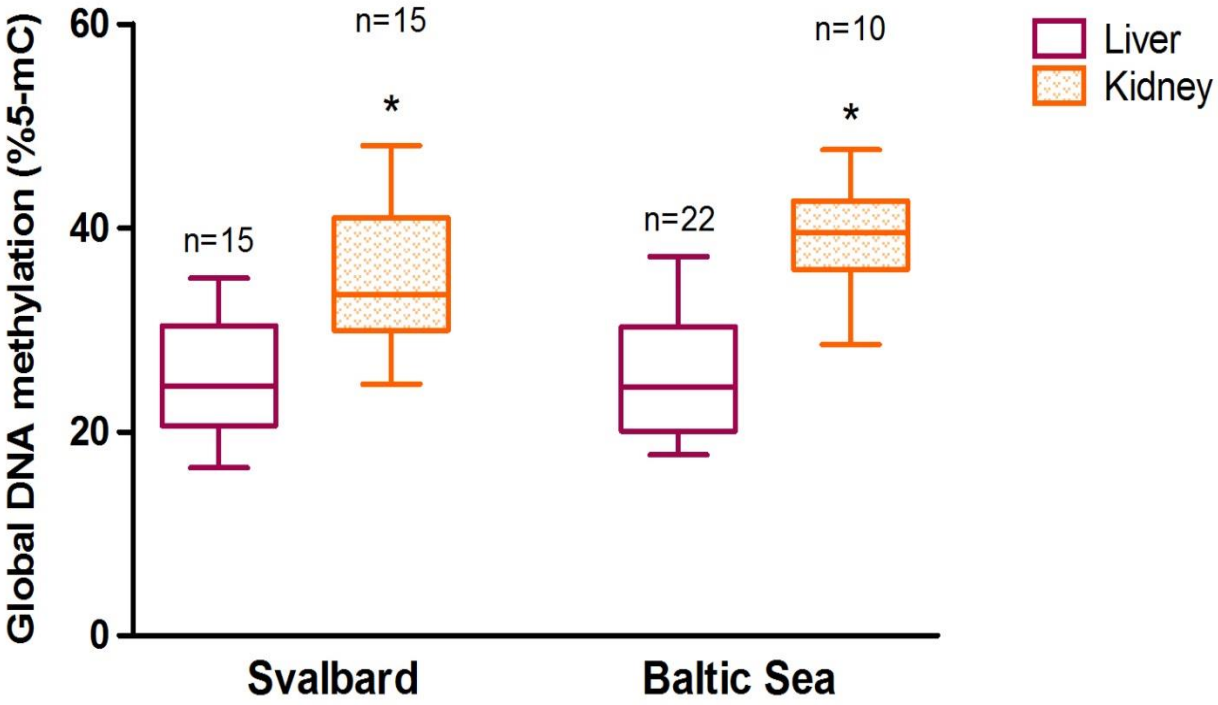


Figure 4: %5-mC in liver and kidney of ringed seals from Svalbard and the Baltic Sea (pre-moulting individuals were removed from the Svalbard population); mean, 2.5 and 97.5 percentile. * indicates p= ≤ 0.05 between tissues.

3.2 Global DNA methylation in relation to POPs

To give an indication of the level of POPs in Svalbard and Baltic ringed seals, a list of the different POP classes (summed) and PCB metabolites in ringed seals from the Baltic Sea are presented in Table 3 and Table 6, respectively. A complete list of the levels of individual POPs can be found in the Appendix.

3.2.1 Svalbard

For both liver and kidney PCAs, PC1 and PC2 explained approximately 67% and 7% of the variation in the data, respectively. Similar for both plots, all POPs had a positive loading on PC1, whereas the %5-mC variables had a negative loading on PC1, thus indicating a negative association between %5-mC and the POPs (Figure 5). The POPs had varying loading on PC2,

while both %5-mC variables had a positive loading. To further investigate possible associations between %5-mC and POPs, Spearman's correlation analysis was used on POPs with loading value > 0.75. The loading values of the POPs were the same for liver and kidney PCAs. This excluded all Toxs, HCB, PCB66, PCB74, PCB110, PCB172/192 and PCB201/204 from further investigations. Several of the associations indicated by PCA were confirmed by correlation analysis for kidney, whereas only few were significant for liver. A list of all significant correlations between %5-mC and POPs based on the PCA findings is presented in Table 4.

Table 3: Concentrations of persistent organic pollutants found in liver of ringed seal from Svalbard and the Baltic Sea, in ng/g wet weight (sample size (n), mean, standard deviation (sd), minimum and maximum values (min-max)).

	Svalbard			Baltic		
	n	mean ± sd	min-max	n	mean ± sd	min-max
∑PCB	18	45,3 ± 38,7	9,0 - 130,5	22	581 ± 362	140 - 1379
∑PBDE	18	1,25 ± 0,96	0,30 - 4,12	22	6,0 ± 5,39	1,95 - 28,3
∑CHL	18	13,3 ± 10,2	4,39 - 33,3	22	61,0 ± 54,0	10,3 - 252
∑Tox	18	3,05 ± 1,25	1,36 - 5,96	22	7,39 ± 7,85	0,59 - 27,3
HCB	18	0,28 ± 0,18	0,12 - 0,93	22	0,45 ± 0,24	0,14 - 0,98
p,p'-DDE	18	19,5 ± 17,1	5,16 - 59,7	22	121 ± 90,6	25,7 - 437
∑POPs	18	82,9 ± 66,4	23,8 - 230	22	778 ± 499	180 - 2130

[a] $\sum_{36}$PCB: -153, -178, -182/187, -183, -172/192, -180/193, -189, -202, -201/204, -197, -196/203, -194, -199, -208, -207, -206, -209, -138, -128, -170/190, -177, -195, -66, -74, -118, -114/122, -105, -156, -157, -52, -47/48, -99/113, -101, -110, -141, -174.

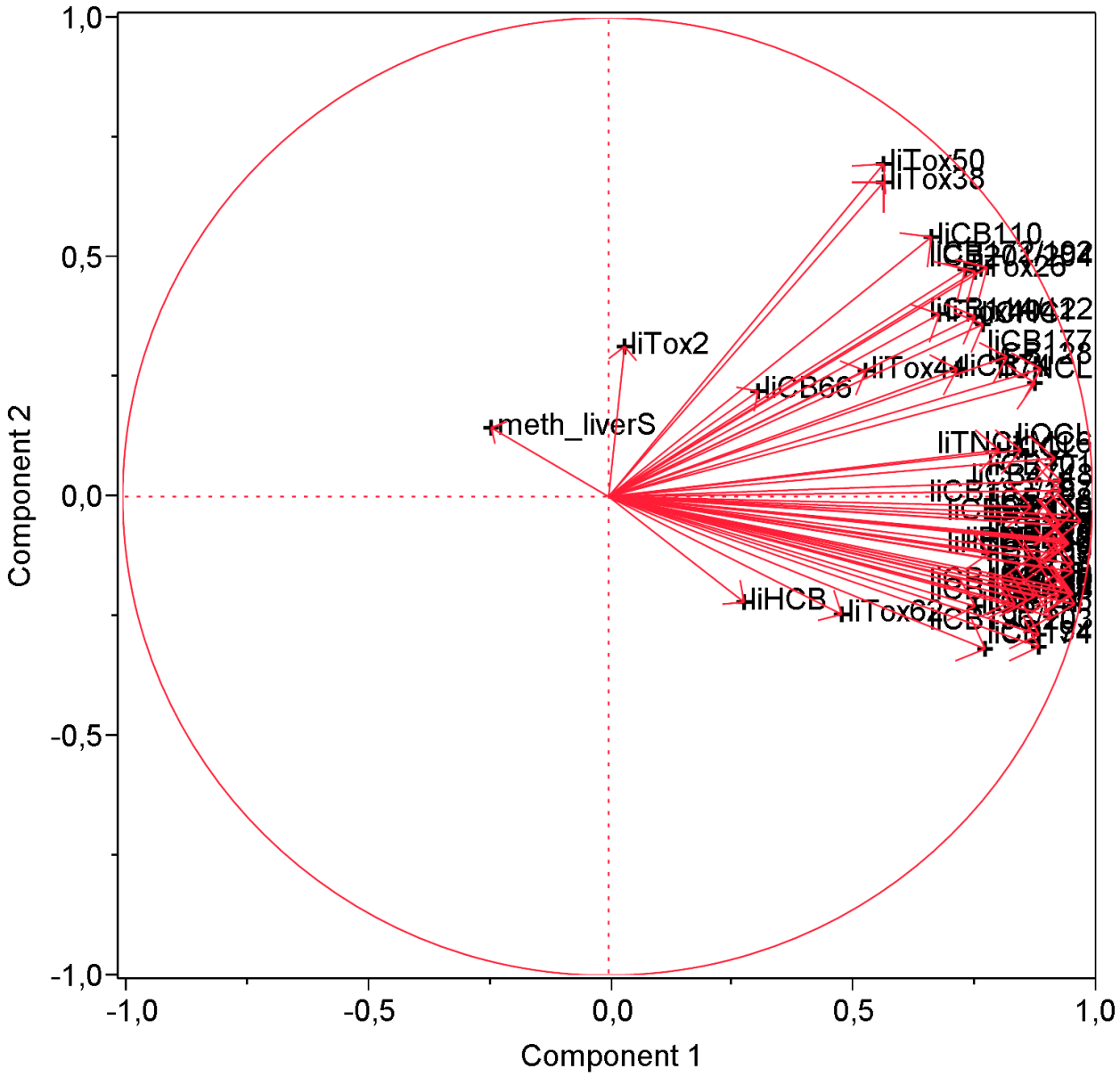
[b] $\sum_{2}$PBDE: -47, -100.

[c] $\sum_{5}$CHL: cis-heptachlorepoxide, cis-chlordane, oxy-chlordane, trans-nonachlor/MC6 and cis-nonachlor.

[d] $\sum_{7}$Tox: Tox 2, Parlar-26, -38, -40/-41, -44, -50, -62.

[e] $\sum_{52}$POPs: $\sum_{36}$PCB, $\sum_{2}$PBDE, $\sum_{5}$CHL, $\sum_{7}$Tox, HCB, p, p'DDE

a)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liCB66
liTox4
meth_liverS
liHCB
liTox62

b)

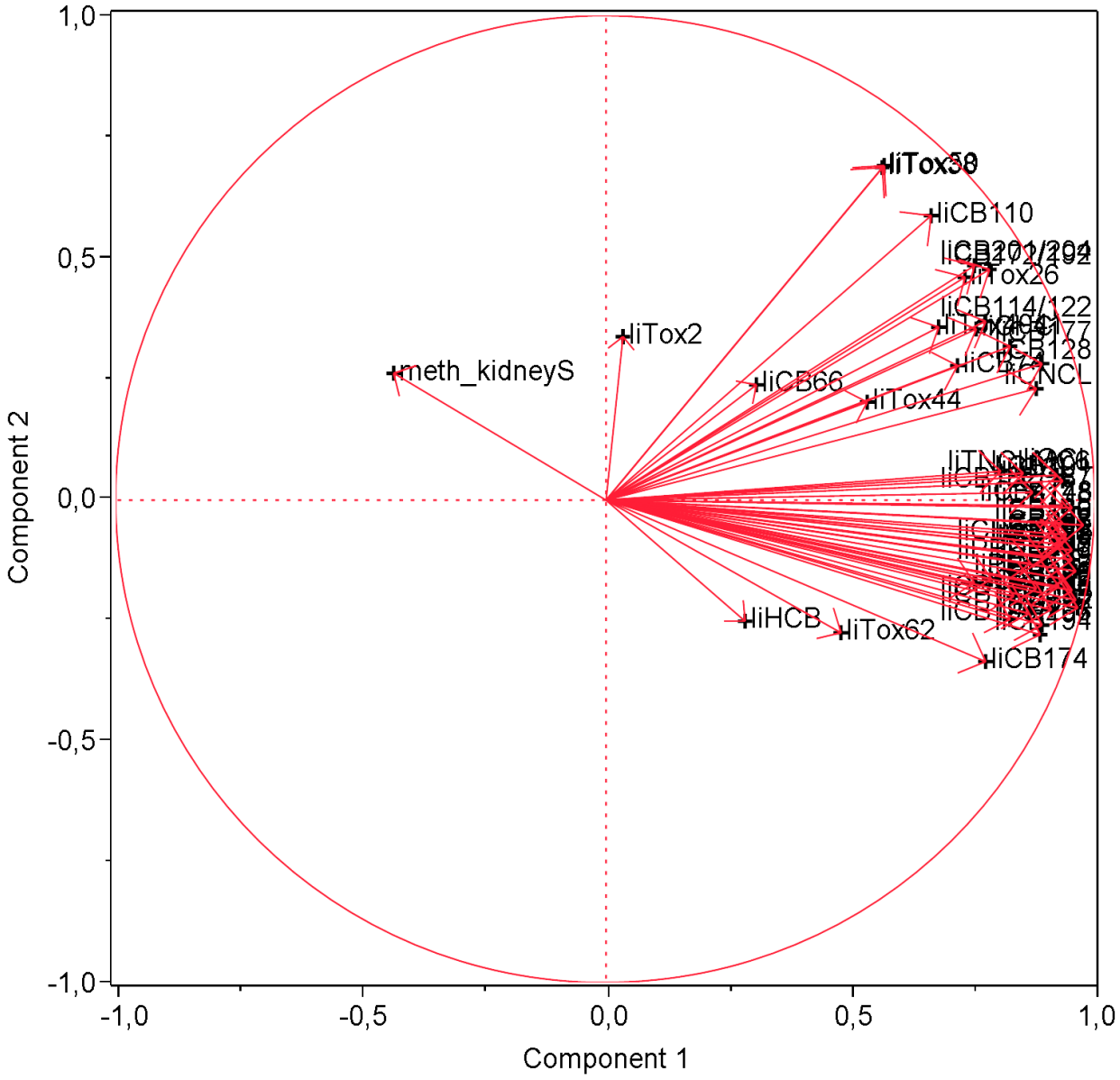


Figure 5: PCA loading plots for POPs and %5-mC in a) liver and b) kidney of ringed seals from Svalbard (all sub-groups, including pre-moulting males, n=18 for liver, n=18 for kidney). Pops (PCBs, PBDE47, PBDE100, CHLs, Toxs, HCB and p,p' DDE (Table 3)) were measured in liver. Labels 'meth_liverS' and 'meth_kidneyS' represent the vectors for %5-mC in liver and kidney, respectively. The plots show component 1 and 2, explaining 67% and 7%, and 68% and 8% of the variance in the data, for liver and kidney respectively.

Table 4: Spearman's ρ correlation analysis for %5-mC in liver and kidney and selected POPs in liver in ringed seals from Svalbard (all ages and both sexes). The individual POPs tested were selected based on a loading value > 0.75 in PCA. Only p-values ≤0.05 within one or both tissues were included in the table.

Svalbard		**Liver** (n=18)		**Kidney** (n=18)	
Variable	**by Variable**	**Spearman ρ**	**Prob>|ρ|**	**Spearman ρ**	**Prob>|ρ|**
PCB153	%5-mC	-0,3168	0,20	-0,5232	0,03*
PCB178	"	-0,3725	0,13	-0,517	0,03*
PCB180/193	"	-0,3746	0,13	-0,5315	0,02*
PCB202	"	-0,3684	0,13	-0,4861	0,04*
PCB194	"	-0,4881	0,04*	-0,5273	0,02*
PCB138	"	-0,356	0,15	-0,4861	0,04*
PCB170/190	"	-0,4014	0,10	-0,5439	0,02*
PCB177	"	-0,4757	0,05*	-0,4943	0,04*
PCB156	"	-0,387	0,11	-0,5253	0,03*
PCB157	"	-0,4262	0,08	-0,5335	0,02*
PCB110	"	-0,5315	0,02*	-0,3168	0,20
PCB141	"	-0,5562	0,02*	-0,3065	0,22
PCB183	"	-0,2797	0,26	-0,5851	0,01*
PCB172/192	"	-0,4014	0,10	-0,4716	0,05*
PCB209	"	-0,42	0,08	-0,4964	0,04*
PCB114/122	"	-0,3437	0,16	-0,5088	0,03*
PCB105	"	-0,3251	0,19	-0,5046	0,03*
CNCL	"	-0,226	0,37	-0,5108	0,03*
TNCL/MC6	"	-0,195	0,44	-0,4861	0,04*

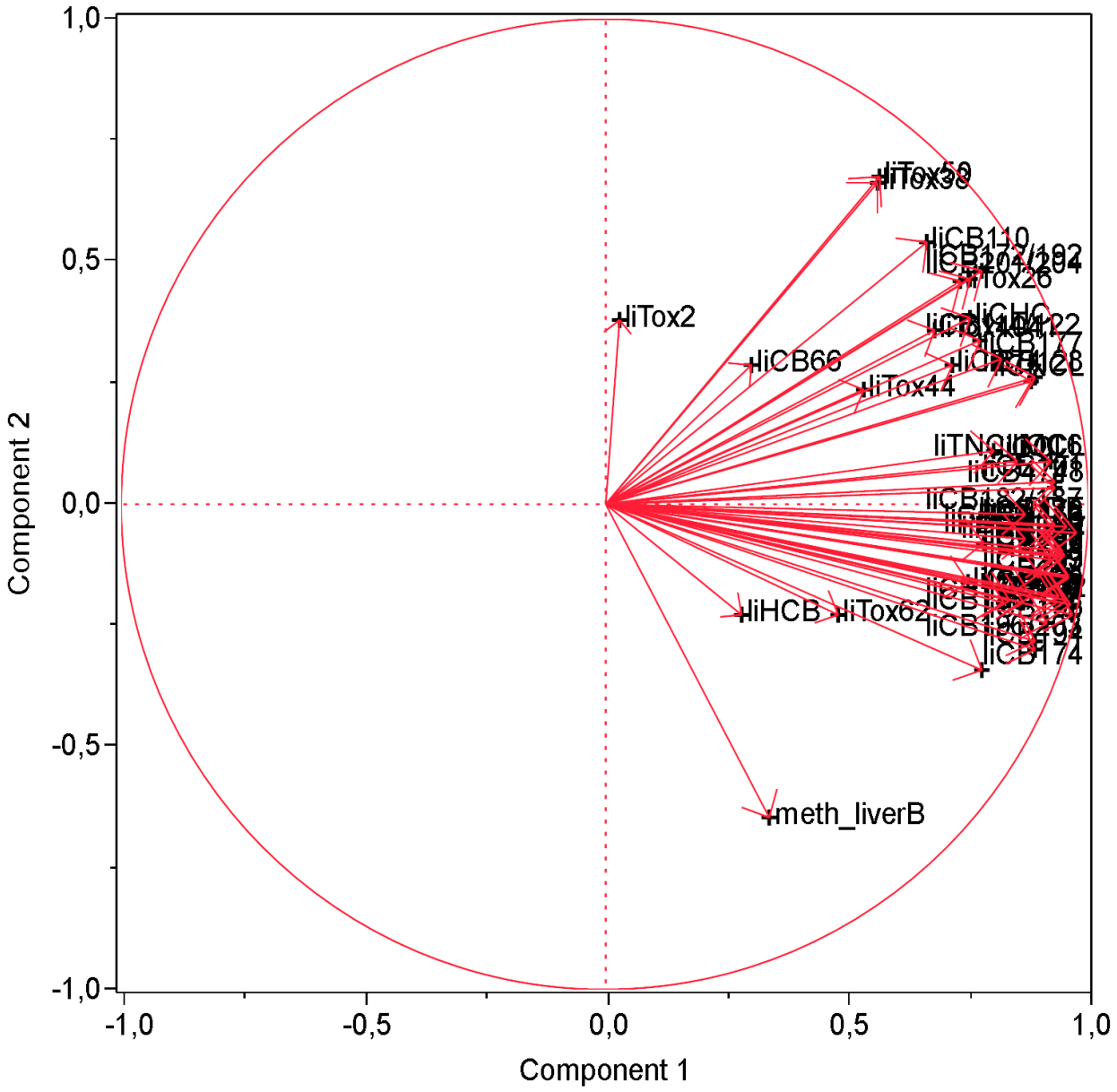
* indicate p-values ≤ 0.05

3.2.2 The Baltic Sea

For both liver and kidney PCAs, PC1 and PC2 explained 68% and 8% of the variation of the data, respectively. In the PCA plot for liver all variables (%5-mC and all POPs) had positive loadings on PC1, revealing a positive association between %5-mC and the POPs (Figure 6). In the PCA plot for kidney, all POPs had a positive loading on PC1, while the %5-mC variable had a negative loading on PC1, reflecting a negative association between %5-mC and all the POPs. The loading values for the POPs were similar in both plots despite the opposite direction of the %5-mC variables (Figure 6). All POPs, except all Toxs, HCB, PCB66, PCB74 and PCB110 had loading values > 0.75, and were further investigated for possible associations with %5-mC using Spearman's correlation. The findings in PCA were in large

confirmed by the correlation analysis regarding both liver and kidney. A list of significant correlations is presented in Table 5.

a)



b)

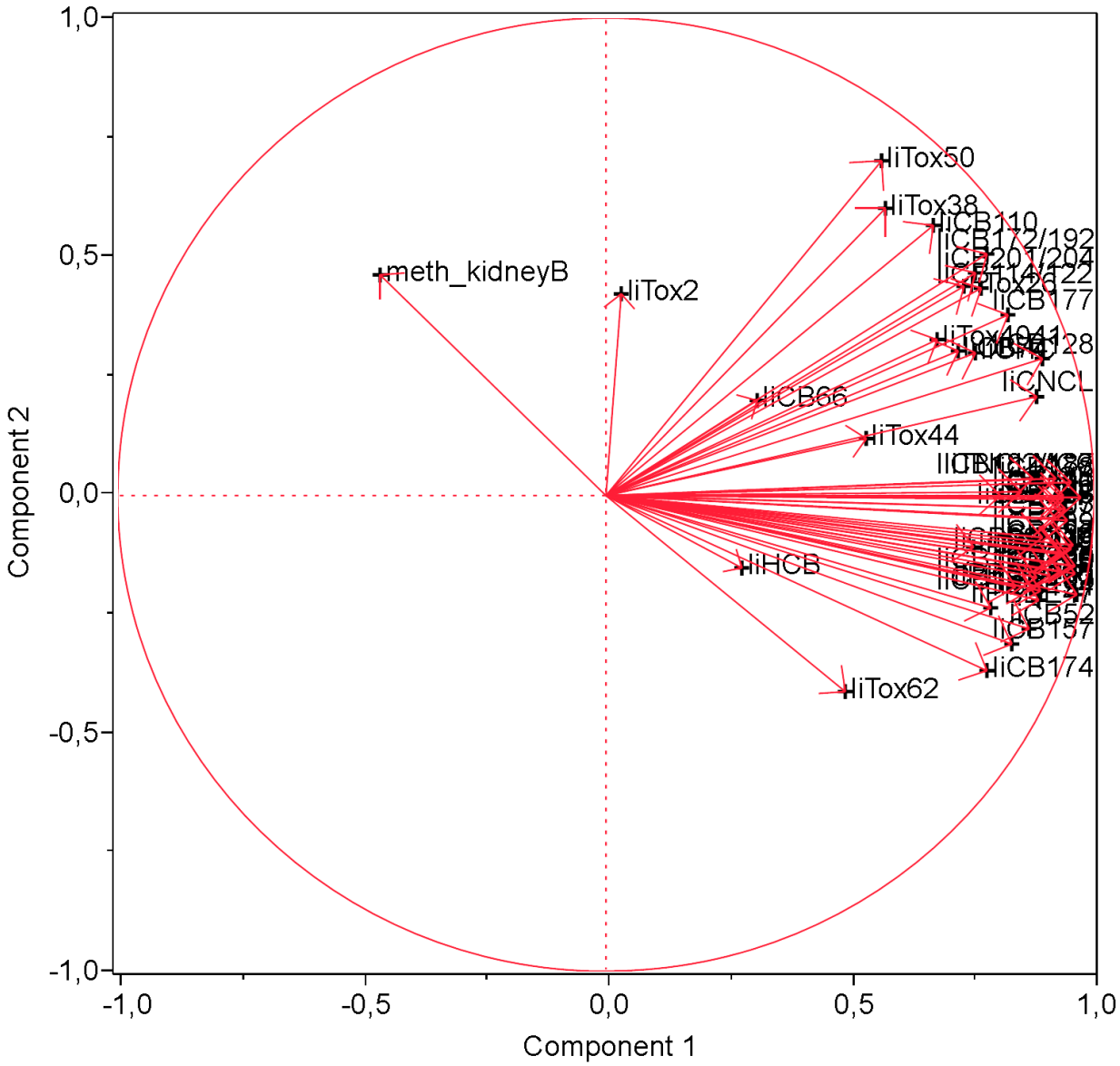


Figure 6: PCA loading plots for POPs and %5-mC in a) liver and b) kidney of ringed seals from the Baltic Sea (all sub-groups, n=21 for liver, n=9 for kidney). POPs (PCBs, PBDE47, PBDE100, CHLs, Toxs, HCB, p,p' DDE (Table 3)) were measured in liver. Labels 'meth%_liver' and 'meth%_kidney' represent the vectors for %5-mC in liver and kidney. The plots show component 1 and 2, explaining 68% and 8% of the variance in the data for both liver and kidney.

Table 5: Spearman's ρ correlation analysis for %5-mC in liver and kidney and selected POPs measured in liver in ringed seals from the Baltic Sea (all ages and both sexes). The individual POPs tested were selected based on a loading value > 0.75 in PCA. Only p-values ≤0.05 within one or both tissues were included in the table.

the Baltic Sea		**Liver** (n=21)		**Kidney** (n=9)	
Variable	**by Variable**	**Spearman ρ**	**Prob>|ρ|**	**Spearman ρ**	**Prob>|ρ|**
PCB153	%5-mC	0,4675	0,03*	-0,7167	0,03*
PCB178	"	0,5039	0,02*	-0,55	0,13
PCB180/193	"	0,4857	0,03*	-0,6833	0,04*
PCB189	"	0,4935	0,02*	-0,2833	0,46
PCB202	"	0,4597	0,04*	-0,3667	0,33
PCB118	"	0,4078	0,07	-0,7667	0,02*
PCB197	"	0,4584	0,04*	-0,4167	0,26
PCB194	"	0,487	0,03*	-0,35	0,36
PCB182/187	"	0,4195	0,06	-0,7167	0,03*
PCB138	"	0,461	0,04*	-0,7167	0,03*
PCB170/190	"	0,4623	0,03*	-0,5333	0,14
PCB195	"	0,4935	0,02*	-0,35	0,36
PCB156	"	0,561	0,01*	-0,6	0,09
PCB52	"	0,3961	0,08	-0,7167	0,03*
PCB99/113	"	0,3831	0,09	-0,7167	0,03*
PCB101	"	0,3961	0,08	-0,8833	0,002*
PCB157	"	0,5039	0,02*	-0,7667	0,02*
PCB174	"	0,5766	0,01*	-0,6	0,09
PBDE47	"	0,3883	0,08	-0,8667	0,003*
PBDE100	"	0,2026	0,38	-0,9333	0,0002*
CCL	"	0,4506	0,04*	-0,6	0,09
OCL	"	0,4506	0,04*	-0,6167	0,08
CNCL	"	0,1805	0,43	-0,7167	0,03*
p,p'-DDE	"	0,3766	0,09	-0,75	0,02*

* indicate p-values ≤ 0.05

3.2.3 PCB metabolites in Baltic ringed seals

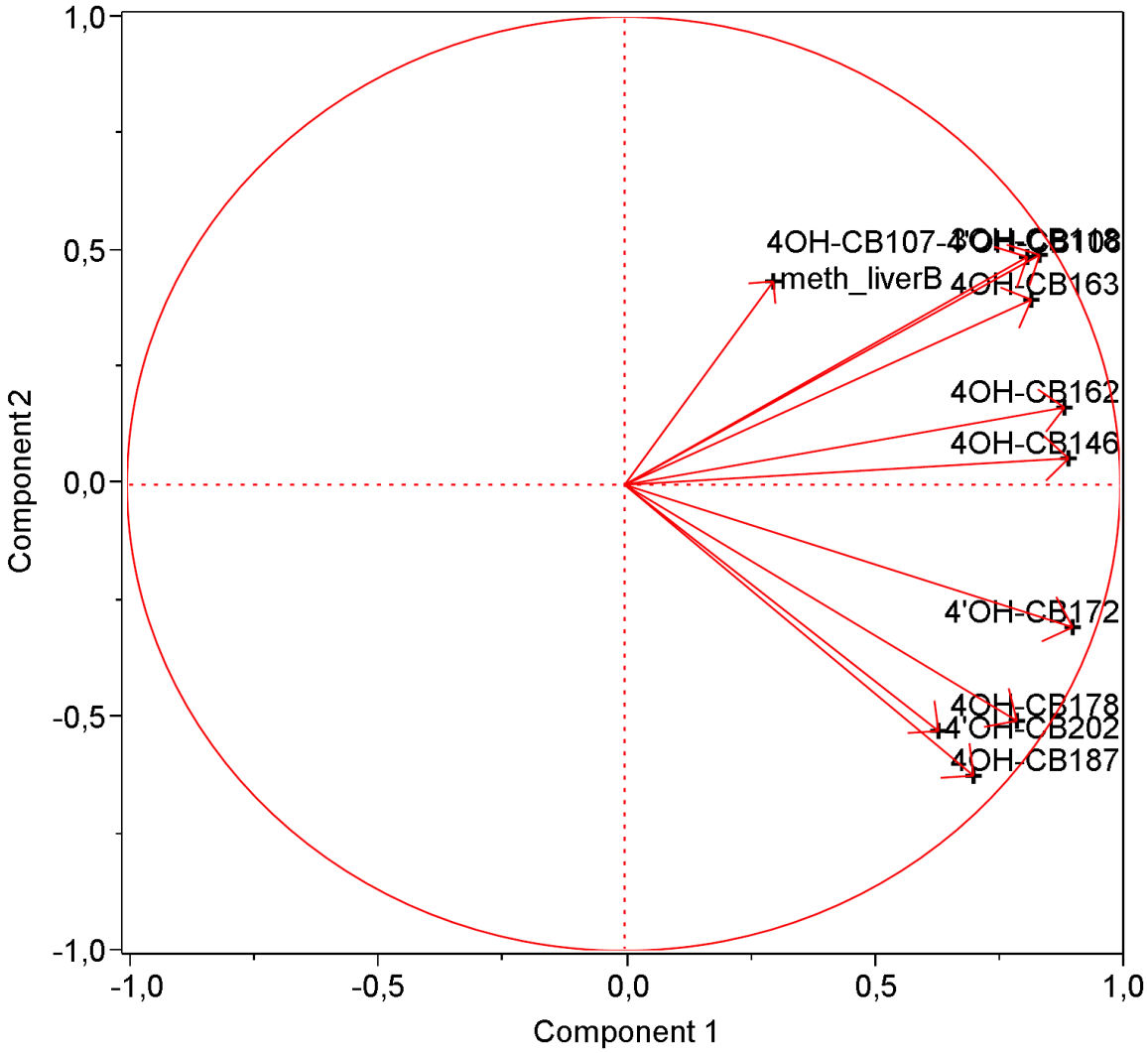
For liver the PCA's PC1 and PC2 explained about 61% and 19% of the variation in the data. For kidney PC1 and PC2 explained 62% and 23% of the variation. For both PCA plots all metabolite variables had positive loading on PC1, while some had positive and others negative loading on PC2 (Figur 7). The %5-mC variable for liver had a positive loading on PC1, suggesting a positive association with the metabolites. The %5-mC variable for kidney

had a negative loading on PC1, suggesting a negative association with the metabolites. For the liver PCA, all metabolites except 4'OH-CB202 had loading values >0.75, and were thus further investigated. For the kidney PCA, five out of nine metabolites had loading values >0.75. No significant correlations were found between %5-mC in liver and PCB metabolites. For %5-mC in kidney, one significant correlation was found with 4'-OH-CB172 (Spearman's correlation, ρ= -0.8908, p=0.001).

Table 6: Concentrations of PCB metabolites in ringed seals from the Baltic Sea, in ng/g plasma weight (sample size (n), mean, standard deviation (sd), minimum and maximum values.

	n	mean ± sd	min-max
4OH-CB107-4'OH-CB108	21	7.28 ± 3.89	1.28 - 13.2
3OH-CB118	21	0.38 ± 0.19	0.08 - 0.64
4OH-CB146	21	0.41 ± 0.18	0.08 - 0.68
4OH-CB163	21	1.76 ± 0.57	0.78 - 3.01
4OH-CB162	21	0.15 ± 0.08	0.03 - 0.31
4'OH-CB172	21	0.13 ± 0.07	0.03 - 0.29
4OH-CB178	21	0.16 ± 0.09	0.04 - 0.40
4OH-CB187	21	0.43 ± 0.23	0.11 - 0.96
4'OH-CB202	21	0.18 ± 0.11	0.05 - 0.51

a)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4OH-CB178
4'OH-CB202
4OH-CB187

b)

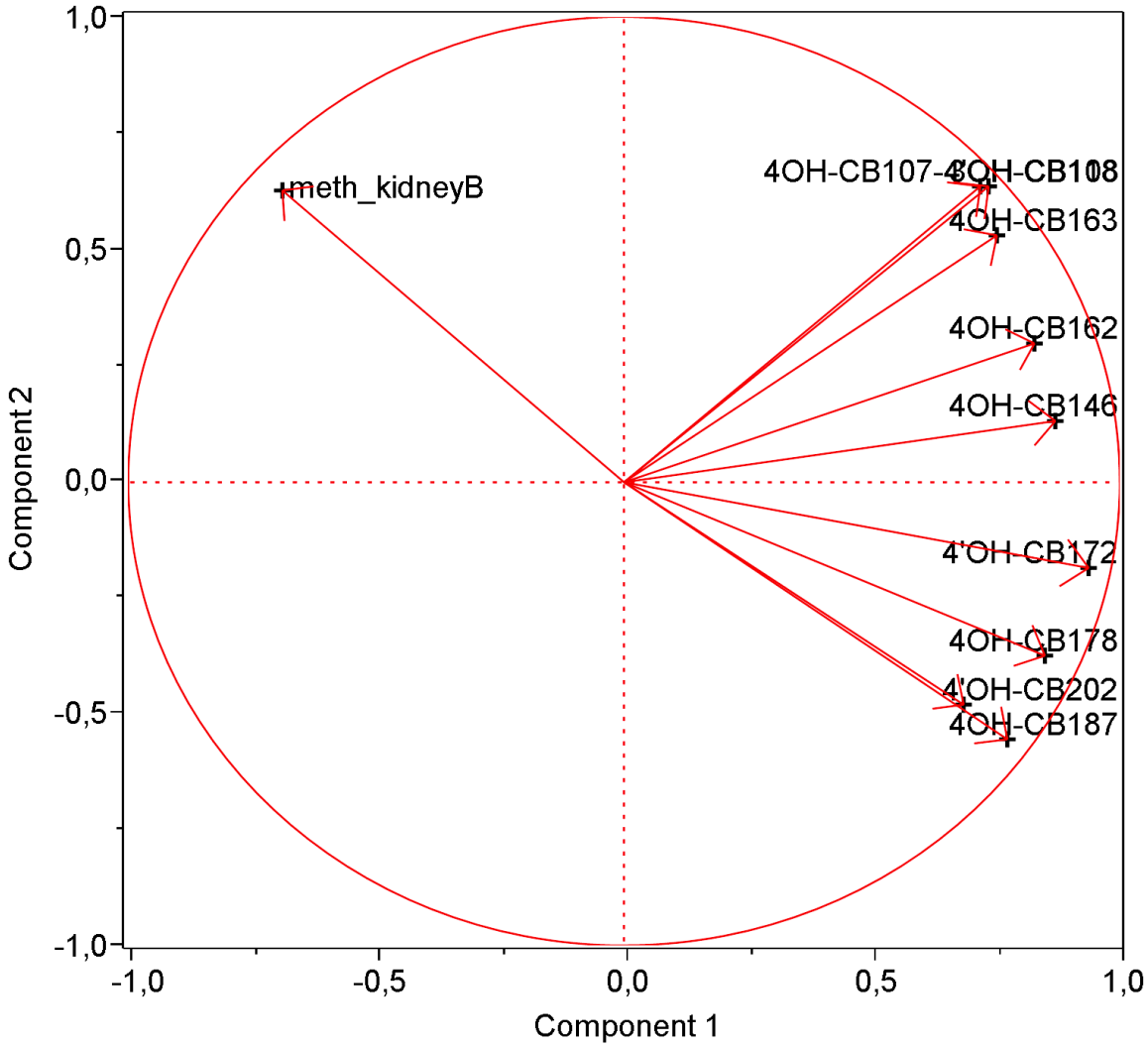

Figure 7: PCA of PCB metabolites and %5-mC in a) liver and b) kidney of ringed seals from the Baltic Sea (all sub-groups, n=21 for liver, n=9 for kidney). PCB metabolites were measured in plasma. Labels 'meth_liverB' and 'meth_kidneyB' represent the vectors for %5-mC in liver and kidney respectively. The plots show component 1 and 2, explaining 61% and 19%, and 62% and 23% of the variance in the data, for liver and kidney, respectively.

4 Discussion

4.1 Effect of sex, age and moulting status on global DNA methylation

No significant difference in global DNA methylation (%5-mC) levels was found when comparing males, females and sub-adults in either of the tissues. Further, no significant difference was found in %5-mC levels between adults and sub-adults. This is in contrast to several studies of effects of sex and age on DNA methylation in humans and rodents (Anway et al. 2005, Zhu et al. 2010, Zhang et al. 2011, Huen et al. 2014), where males usually have higher levels of DNA methylation than females, and age is associated with lower methylation levels. Diet has been a proposed cause of the different methylation level between sexes found in humans, where the intake of dietary folate, a methyl-donor, was lower in women than men (Zhang et al. 2011).

The cause of the results in the present study, regarding DNA methylation in relation to sex and age, is not clear. Due to low sample sizes of the different sub-groups, caution must be used when interpreting the results. Still, as all individuals in sub-groups tested were moulting, the stress exerted on moulting seals might be of importance in explaining why there was no apparent sex or age difference in %5-mC. The moulting/fasting period is a physiological stressful time for the seals, with reduced feeding, renewal of epidermis and changes in metabolism (Ashwell-Erickson et al. 1986). During the moulting period feeding is reduced, thus the intake of dietary methyl-donors is reduced as well. Additionally, increased plasma levels of lipid soluble POPs due to mobilization of lipids from blubber during moult (Ryg et al. 1990, Kleivane et al. 1997, Routti et al. 2010), could potentially inflict deviation from normal DNA methylation status. Plasma levels of POPs have been negatively associated with global DNA methylation in studies on humans and rodents (Rusieckie et al. 2008, Desaulniers et al. 2009, Kim et al. 2010).

Alternatively, there could be species-specificity in the relationship between DNA methylation and biological factors, such as sex and age, explaining the discrepancy between the ringed

seals in the present study and previous findings in humans and rodents (Rusieckie et al. 2008, Desaulniers et al. 2009, Kim et al. 2010).

4.2 Effect of tissue

A significant difference was found in %5-mC levels between liver and kidney in moulting individuals, with kidney having higher levels of %5-mC. This could be attributed to tissue-specificity in gene methylation patterns (Rakyan et al. 2004, Eckhardt et al. 2006). Because different tissues have different characteristics and functions, different genes need to be activated or silenced depending on the tissue. As mentioned in the introduction, gene silencing and activation is facilitated by DNA methylation. On the other hand, the main part of mammalian DNA do not consists of genes, but non-coding DNA, with introns, transposons and repetitive elements, where DNA methylation acts as a mechanism for long-term silencing (Jones and Takai 2001) and chromosomal stability (Bird 1986). Further, with the importance of metabolism on DNA methylation status and the methionine cycle in mind, it could be hypothesized that a cellular metabolic response to POPs could indeed be a contributing mechanism behind the low methylation status in liver in moulting seals.

In the methionine cycle, methionine (an essential amino acid) is converted to S-adenolsyl-methionine (SAM) (cells primary methyl donor and necessary for DNA methylation), which in turn becomes homocysteine through the step of S-adenosylhomocysteine (SAH) subsequent to methyl donation. Homocysteine is then either methylated back to methionine or is catabolized to synthesize cysteine (Finkelstein 1998, Ulrey et al. 2005). Cysteine is essential for phase II metabolism of xenobiotics as it is the sulphydryl source for 3'-phosphoadenosyl-5'-phosphosulfate (PAPS) and is one of three constituents of glutathione. In addition, glutathione can be depleted by oxidative stress, which has been associated with POP exposure (Kumar et al. 2014).

High demands for cysteine by phase II enzymes and oxidative stress in response to contaminants could potentially up-regulate the methionine cycle under conditions of low dietary uptake of cysteine, as the methionine cycle is the only source of homocysteine, the precursor for cysteine. A study by Kanerva et al. (2012) revealed higher glutathione metabolism in Baltic ringed seals compared to ringed seals from Svalbard. In a step between

SAM and homocysteine in the methionine cycle, SAM is converted to S-adenosylhomocysteine (SAH). SAH is an inhibitor of SAM-dependent methyltransferases, thereby blocking the cells demands for methylation (Hoffman et al. 1980). Even though cysteine is common in the diet, fasting, phase II metabolism and oxidative stress could be hypothesized to lead to depletion of cysteine and subsequent hypomethylation of DNA in hepatic tissue exposed to high levels of contaminants.

4.3 Comparison between Svalbard and the Baltic Sea

The finding of no significant difference in %5-mC levels in liver and kidney of ringed seals sampled in Svalbard and the Baltic Sea is interesting, as it could be expected that the differences in environmental factors, such as pollution, would affect the DNA methylation levels of the two populations differently. Baltic ringed seals had 10-20 times higher levels of POPs compared to ringed seals from Svalbard (Routti et al. 2008a, Routti et al. 2009a, Routti et al. 2009b). Based on previous studies on global DNA methylation in relation to POPs in humans and rodents (Rusieckie et al. 2008, Desaulniers et al. 2009, Kim et al. 2010), it would be expected that Baltic ringed seals had lower levels of DNA methylation than ringed seals from Svalbard, due to the higher levels of POPs in Baltic ringed seals. Reasons why this was not the case in the present study, could be due to species-specificities resulting in ringed seals responding differently than humans and rodents in response to POPs, or it could indicate that POPs do not have a linear relationship with %5-mC. As the difference between areas were tested on moulting individuals, it would be interesting to investigate possible differences in %5-mC between the two areas on pre-moulting individuals.

4.4 Global DNA methylation in relation to POPs

For Svalbard, all significant correlations between %5-mC and POPs in liver and kidney were negative, indicating decreasing %5-mC levels with increasing contaminant concentrations. This is in accordance with previously reported findings of hypomethylation in mammalian tissues in association with contaminants (Rusiecki et al. 2008, Desaulniers et al. 2009, Kim et al. 2010, Pilsner et al. 2010). Interestingly, despite the lower %5-mC in liver compared to kidney, only four individual PCBs correlated with liver %5-mC in contrast to seventeen

individual POPs (fifteen PCBs and two CHL) correlating with %5-mC in kidney. Further, only two POPs (PCB194, 177) had significant correlation with %5-mC in both tissues. This could indicate a difference in tissue baseline %5-mC levels as well as tissue-specific sensitivity to contaminants.

Correlations between POPs and %5-mC in liver and kidney for Baltic ringed seals displayed a very different pattern than that from Svalbard. Both liver and kidney had about the same number of significant correlation between %5-mC and POPs (15 and 13, respectively). These included PCBs, PBDEs, CHL and p,p'-DDE. Surprisingly though, all significant correlations with liver %5-mC were positive, while the opposite was true for POP correlations with kidney %5-mC. For liver, this is in contrast to the expectation of a negative association between %5-mC and contaminants. Similar to the Svalbard pattern, only a few POPs correlated significantly with %5-mC in both tissues (PCB153, 180/193, 138, 157). The main part of all significant correlations with %5-mC for both Svalbard and the Baltic Sea were PCBs. From the PCA plots it was evident that the various PCBs correlated strongly with each other, it is therefore not surprising that they have similar associations to the %5-mC variables in each plot. Interestingly, nine of the PCBs correlating with %5-mC in the present study were also correlated with %5-mC in the study by Rusiecki et al. (2008) on blood from Greenlandic Inuits (Table 7).

In order to further investigate the PCBs from the present study, they were grouped into five groups (I-V) according to Cl-substitution pattern as suggested by Boon et al. (1997) (Table 7) (I: PCBs with no vicinal hydrogen (H)-atoms. II: PCBs with vicinal H-atoms in the *ortho*- and *meta*-positions in combination with ≥ 2 *ortho*-Cl. III: PCBs with vicinal H-atoms in the *ortho*- and *meta*-positions in combination with ≤1 *ortho*-Cl. IV: PCBs with vicinal H-atoms in the *meta*- and *para*-positions in combination with ≤ 2 *ortho*-Cl. V: PCBs with vicinal H-atoms in the *meta*- and *para*-positions in combination with ≥ 3 *ortho*-Cl). In addition, the PCBs were grouped into four groups (1-4) according to their potential toxicity as suggested by McFarland and Clarke (1989) (Table 7), with group 1 having greatest toxic potential and group 4 having least toxic potential (1a: close resemblance to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (2, 3, 7, 8-TCDD) in structure and function, i.e. inducing xenobiotic metabolism systems. 1b: mixed-type inducers of aryl hydrocarbon metabolizing mixed-function oxidases (MFOs) and frequently reported in environmental samples. 2: phenobarbital-type inducers prevalent in the

environment. 3: weak- or non-inducers, frequently reported in environmental samples. 4: mixed-type inducers, but are infrequently reported in environmental samples and only in low concentrations).

Interestingly, no pattern was found among the %5-mC -correlating PCBs. They were of varying groups with respect to both Cl-substitutions and toxicity. In addition, several PCBs were not included in any of the toxicity groups, which suggest that they are generally considered as non-toxic, mainly due to their inability to induce xenobiotic metabolising systems and their low concentrations in the environment and animal tissues. The fact that these PCBs were found to be correlated with %5-mC in the present study might imply that they exert an epigenetic toxicity that is not connected with the ability to induce xenobiotic metabolizing systems. Similar to the PCBs, no specific pattern was found between the %5-mC-associated CHLs either. All associations with %5-mC were negative except with %5-mC in liver from Baltic seals. Only CNCL (*cis*-nonachlor) was associated with %5-mC in kidney from both Svalbard and the Baltic Sea, otherwise the CHLs were only associated with %5-mC in one tissue from one area. PBDE47, PBDE100 and p,p'-DDE was only associated with %5-mC in kidney from Baltic seals, negatively so. For p,p'-DDE, this is in contrast to findings by Lind et al. (2013) whom found an positive association between p,p'-DDE and DNA methylation in a study on humans using the LUMA method for %5-mC measurement in blood samples. In addition, they found that high toxic equivalency (TEQ) value (a measure of toxic potential in a mixture of) was associated with DNA hypermethylation. No such pattern was evident in the present study.

For PCB metabolites in Baltic seals, one was negatively correlated with %5-mC in kidney (4'-OH-CB172). This metabolite had the lowest concentration of all the PCB metabolites included in the analysis of the present study, suggesting that a response in DNA methylation may not be dependent on a high dose of the effector substance. Although not significant, all other correlations between PCB metabolites and %5-mC in both liver and kidney were negative.

Table 7: PCB congeners found to significantly correlate with %5-mC in one or both tissues (ringed seal liver and kidney) in one or both areas (Svalbard and the Baltic Sea), and in the study by Rusiecki et al. (2008) (human blood). Regular x indicate negative correlation, while bold **x** indicate positive correlation. Cl I-V indicates group based on Cl-substitution pattern (Boon et al. 1997). Toxicity 1b-4 indicates potential toxicity (McFarland and Clarke 1989).

				Svalbard		the Baltic Sea	
Congener	**Cl**	**Toxicity**	**Rusiecki**	**Liver**	**Kidney**	**Liver**	**Kidney**
PCB153	I	2	x		x	**x**	x
PCB178	I				x	**x**	
PCB182/187	I	3					x
PCB183	I	2	x		x		
PCB172/192	I				x		
PCB180/193	I	2	x		x	**x**	x
PCB189	I	4				**x**	
PCB202	I				x	**x**	
PCB197	I					**x**	
PCB194	I	2		x	x	**x**	
PCB209	I				x		
PCB138	II	1b	x		x	**x**	x
PCB170/190	II	1b	x		x	**x**	
PCB177	II	3		x	x		
PCB195	II					**x**	
PCB118	III	1b	x				x
PCB114/122	III	4			x		
PCB105	III	1b	x		x		
PCB156	III	1b	x		x	**x**	
PCB157	III	4			x	**x**	x
PCB52	IV	3					x
PCB99/113	IV	2	x				x
PCB101	IV	2					x
PCB110	IV			x			
PCB141	IV			x			
PCB174	V					**x**	

Cl: I: no vicinal hydrogen (H)-atoms. II: vicinal H-atoms in the *ortho*- and *meta*-positions in combination with ≥ 2 *ortho*-Cl. III: vicinal H-atoms in the *ortho*- and *meta*-positions in combination with ≤1 *ortho*-Cl. IV: vicinal H-atoms in the *meta*- and *para*-positions in combination with ≤ 2 *ortho*-Cl. V: vicinal H-atoms in the *meta*- and *para*-positions in combination with ≥ 3 *ortho*-Cl

Toxicity: 1b: mixed type inducers, frequently found in environmental samples. 2: phenobarbital type inducers. 3: weak inducing or non-inducing, but occur frequently in environmental samples. 4: mixed type inducers, infrequently reported in environmental samples, low concentrations

A possible explanation for the inconsistency in the association between %5-mCand POPs in the present study could be that other contaminants than those measured here affects %5-mC. For instance, mercury and lead were found to have inverse associations with %5-mC in studies by Desaulniers et al. (2009) and Pilsner et al. (2009, 2010). Lead has also been shown to affect homocysteine levels, associated with decreased DNA methylation (Yi et al. 2000). Further, cadmium was found to affect global DNA methylation in an *in vitro* exposure study with rat liver cells by Takiguchi et al. (2003).Wang et al. (2009) found that both tributyltin (TBT) and triphenyltin (TPT) were inversely correlated with hepatic global DNA methylation and the methyl donor SAM, in false kelpfish (*Sebastiscus marmoratus*). Kelly et al. (2007) addressed the need for more knowledge about a large group of environmental contaminants with low octanol-water partition coefficient ($K_{ow} < 10^5$), which are not considered to bio-magnify in aquatic food webs (due to their low K_{ow}), and thus have not been considered as environmentally threatening. But because of high octanol-air partition coefficient (K_{oa}), these substances may potentially bio-accumulate in air-breathing animals, and could therefore be present in ringed seals. Several other contaminants than those measured in the present study, such as perfluorooctane sulfonate (PFOS) and organotin compounds, have been detected in marine mammals (Fant et al. 2001, Kannan et al. 2001, Ciesielski et al. 2004).

In addition, the concept of mixture toxicity could apply here, and would not be revealed by correlation analysis with individual POPs. A general principal of mixture toxicity is that in a mixture, substances with similar mode of action will add up to a combined effect larger than that of each substance's individual effect (Carpenter et al. 2002). Thus, although each substance might be present below its no effect concentration (NOEC), when added together they could exceed this limit and cause a joint effect (Silva et al. 2002). Further, substances in a mixture could interact by potentiation, synergism or antagonism, making the outcome difficult to predict, unless all is known about the concentrations and modes of action of all substances present in the mixture. An example of mixture toxicity is the exposure study on rats by Desaulniers et al. (2009), which found that a mix of PCBs, organochlorine pesticides (OCP) and methylmercury (meHg) affected hepatic global DNA methylation, whereas the three groups (PCBs, OCPs, meHg) by themselves did not.

In the present study, the concentrations and pattern of POPs differed between the two areas and additional contaminants other than those measured here might be present (Kelly et al.

2007, Kannan et al. 2001, Fant et al. 2001, Ciesielski et al. 2004). Thus, mixture toxicity might be an underlying factor leading to the differences in association between %5-mC and individual POPs observed between tissue and area. In the present study possible associations with %5-mC were investigated with individual POPs, not summed POP classes, e.g. ∑PCBs or ∑Tox. It could be meaningful to group POPs according to mode of action and investigate associations between these groups and %5-mC. Still, more knowledge about how individual POPs, and other contaminants, might affect DNA methylation is warranted, in order to assess potential joint effects. Further, the relationship might not be linear.

The method for measuring %5-mC could also lead to inconsistent findings with regards to hypomethylation in association with POPs. In the present study, the ELISA method was applied, whereas most of the studies reporting an inverse correlation between %5-mC and POPs have been using the bisulphite conversion and pyrosequencing methodology, focusing on *Alu* and LINE elements. In both Rusiecki et al. (2008) and Kim et al. (2010) only *Alu* elements had significant negative correlations with POPs, not LINE. None of the studies report if the associations with POPs were still significant if %5-mC measures of both *Alu* and LINE elements were pooled. It could be questioned if Alu and LINE repetitive elements are in fact good measures of %5-mC, and if findings in just one of the two (such as inverse correlation with *Alu* elements only) are enough to conclude with evidence on the global DNA level. Lisanti et al. (2013) found LINE to be the assay for %5-mC measurement best comparable to HPLC when comparing *Alu*, LINE and LUMA assays, although LINE only accounts for about 17% of all CpG sites in the human genome. Further, the bisulphite conversion method is only sensitive to methylated cytosines in CpG dinucleotides, whereas ELISA is sensitive to all methylated cytosines, thereby capturing a wider spectrum of DNA methylation. Despite the common view that only CpG cytosines can be methylated, a study by Yan et al. (2011) on various mammalian tissues, found that 7-13% of non-CpG cytosines were in fact methylated. It is important to remember though, that in the present study the methylation levels were calculated relative to control methylation levels, and cannot be directly compared with methylation levels from other studies.

5 Conclusions

Global DNA methylation levels (%5-mC) were not influenced by sex or age. Species-specific DNA methylation pattern could be an explanation. Additionally, physiological stress caused by moulting might also be of importance. Moulting status seemed to affected %5-mC levels, with moulting individual having significantly lower %5-mC levels compared to pre-moulting individuals. Reduction of dietary methyl-donors due to fasting during the moulting period might be an explanation. However, caution must be used when interpreting the results due to low sample sizes of the sub-groups. Liver had significantly lower %5-mC levels compared to kidney, which could be due to tissue-specific DNA methylation patterns. In addition, it's possible that xenobiotic metabolism and oxidative stress induced by high levels of POP exposure in the liver, could lead to depletion of methyl-donors and subsequent decrease in hepatic DNA methylation.

Several POPs were found to correlate with %5-mC in liver and kidney from both Svalbard and the Baltic Sea. All correlations were negative between POPs and %5-mC for both tissues and areas, with the exception of all correlations between POPs and %5-mC in liver from the Baltic Sea, which were positive. The main group of POPs which significantly correlated with %5-mC for both populations were PCBs. PCB metabolites were all negatively associated with %5-mC in liver and kidney from the Baltic ringed seals, although there was only one was significant correlation. No pattern was found between the PCBs or between the CHLs that correlated with %5-mC, indicating a complex nature in their associations to DNA methylation, which is yet to be unraveled. The unclear reasons for the findings in the present study could be due to interactions of other contaminants than those measured here. Different contaminant sources for the two ringed seal populations and varying ability to metabolize xenobiotics, could lead to dissimilar patterns of POPs with potential mixture toxicity effects. Further, caution should be taken when comparing results from studies using different methodologies for measuring %5-mC.

Although sex and age were not found to affect %5-mC levels in the present study, it would be interesting to investigate the effect of these factors on DNA methylation in larger sample sizes in future studies. In future studies, measures of cysteine levels in addition to %5-mC levels

could help identify a possible association between depletion of cysteine through fasting and contaminant exposure, and %5-mC levels. In addition, establishment of baseline %5-mC levels in various tissues from ringed seal is warranted in order to identify possible deviations in relation to contaminant exposure. Future studies should be conducted with larger sample size of both moulting and pre-moulting individuals in order to investigate possible differences in their %5-mC levels associated with POPs. Potential non-linear relationships should also be investigated.

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Appendix

Attachment 1: Data for calculation of global DNA methylation levels (%5-mC) in liver and kidney from ringed seals from Svalbard. Abs1-3 indicates the absorbance (A_{450}) readings from the three replicates of ELISA.

	Liver					Kidney				
Individual	**Abs1**	**Abs2**	**Abs3**	**Average**	**%5-mC**	**Abs1**	**Abs2**	**Abs3**	**Average**	**%5-mC**
S1-07	0,60	0,59	0,65	0,61	**51,06**	0,45	0,50	0,53	0,49	**36,82**
S2-07	0,51	0,55	0,47	0,51	**38,59**	0,51	0,52	0,64	0,56	**44,41**
S3-07	0,45	0,50	0,48	0,48	**34,82**	0,45		0,53	0,49	**35,98**
S4-07	0,43	0,46	0,50	0,46	**33,20**	0,55	0,61	0,61	0,59	**47,99**
S5-07	0,57	0,52	0,55	0,55	**43,27**	0,46	0,60	0,58	0,55	**42,96**
S6-07	0,44	0,49	0,48	0,47	**33,99**	0,56	0,69	0,65	0,63	**52,95**
S7-07	0,40	0,47	0,45	0,44	**30,41**	0,36	0,51	0,45	0,44	**30,76**
S8-07	0,37	0,43	0,39	0,40	**25,53**	0,47	0,58	0,53	0,53	**40,52**
S9-07	0,32	0,35	0,31	0,33	**17,19**	0,42	0,47	0,51	0,47	**33,60**
S10-07	0,30	0,31	0,36	0,32	**16,44**	0,47	0,45	0,48	0,47	**33,48**
S11-07	0,42	0,48	0,43	0,44	**31,00**	0,41	0,49	0,54	0,48	**35,48**
S12-07	0,35	0,38	0,39	0,37	**22,34**	0,60	0,55	0,62	0,59	**48,07**
S13-07	0,37	0,41	0,39	0,39	**24,43**	0,35	0,40	0,43	0,39	**24,67**
S14-07	0,34	0,40	0,39	0,38	**23,13**	0,36	0,43	0,49	0,43	**28,95**
S15-07	0,34	0,36	0,38	0,36	**20,61**	0,54	0,55	0,56	0,55	**43,71**
S16-07	0,39	0,46	0,42	0,42	**28,36**	0,40	0,43	0,47	0,44	**29,98**
S17-07	0,32	0,34	0,36	0,34	**18,57**	0,51	0,61	0,61	0,58	**46,58**
S18-07	0,40	0,45	0,45	0,43	**29,46**	0,55	0,57	0,47	0,53	**40,99**
S19-07	0,39	0,51	0,48	0,46	**32,93**	0,41	0,42	0,47	0,43	**29,62**
S20-07	0,36	0,35	0,40	0,37	**22,27**	0,42	0,44	0,49	0,45	**31,51**
S21-07	0,44	0,51	0,49	0,48	**35,05**	0,46	0,45	0,47	0,46	**32,65**

Attachment 2: Data for calculation of global DNA methylation levels (%5-mC) in liver and kidney from ringed seals from Svalbard. Abs1-3 indicates the absorbance (A_{450}) readings from the three replicates of ELISA.

	Liver					Kidney				
Individual	**Abs1**	**Abs2**	**Abs3**	**Average**	**%5-mC**	**Abs1**	**Abs2**	**Abs3**	**Average**	**%5-mC**
N1-07						0,54	0,50	0,52	0,52	**40,05**
N2-07	0,43	0,48	0,39	0,43	**29,86**	0,34	0,42	0,51	0,42	**28,56**
N3-07	0,37	0,42	0,40	0,40	**25,22**	0,62	0,59	0,54	0,59	**47,64**
N4-07	0,31		0,36	0,34	**18,21**	0,52	0,49	0,53	0,51	**39,06**
N5-07	0,41	0,44	0,50	0,45	**31,51**	0,54	0,52	0,57	0,54	**42,64**
N6-07	0,40	0,43	0,44	0,43	**28,72**	0,53	0,54	0,56	0,54	**42,72**
N7-07	0,40	0,40	0,46	0,42	**28,21**	0,52	0,48	0,54	0,51	**38,67**
N8-07	0,46	0,55	0,47	0,50	**37,18**	0,43	0,55	0,48	0,49	**36,07**
N9-07	0,39	0,39	0,35	0,38	**23,17**	0,47	0,48	0,49	0,48	**35,44**
N10-07	0,46	0,43	0,46	0,45	**31,71**	0,50	0,58	0,52	0,53	**41,31**
N2-06	0,33	0,37	0,34	0,35	**19,51**					
N3-06	0,32	0,33	0,40	0,35	**19,67**					
N4-06	0,32	0,36	0,36	0,35	**19,59**					
N5-06	0,39	0,42	0,42	0,41	**26,79**					
N6-06	0,25	0,36	0,38	0,33	**17,70**					
N7-06	0,37	0,39	0,37	0,38	**23,09**					
N8-06	0,45	0,46	0,45	0,46	**32,30**					
N9-06	0,38	0,35	0,36	0,36	**21,20**					
N10-06	0,34	0,36	0,36	0,35	**20,18**					
N11-06	0,44	0,44	0,42	0,43	**29,66**					
N12-06	0,38	0,36	0,36	0,36	**21,36**					
N13-06	0,39	0,37	0,39	0,38	**23,60**					
N14-06	0,43	0,54	0,38	0,45	**31,47**					

Attachment 3: Concentrations of persistent organic pollutants found in liver of ringed seal from Svalbard and the Baltic Sea, in ng/g wet weight (mean, standard deviation (sd), minimum and maximum values (min, max) Sample size n= 18 and n= 22 for Svalbard and the Baltic Sea, respectively.

	Svalbard				the Baltic Sea			
	mean	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**
PCB153	11.44	10.17	2.24	36.07	126.39	77.92	35.57	299.95
PCB178	0.28	0.29	0.04	1.07	4.91	3.43	1.26	14.74
PCB182/187	1.72	1.58	0.32	4.93	36.00	26.34	7.71	127.90
PCB183	0.98	0.87	0.15	3.07	22.72	19.26	3.31	79.77
PCB172/192	0.16	0.14	0.00	0.51	4.00	3.60	0.55	15.77
PCB180/193	4.10	3.63	0.44	12.40	79.45	63.29	14.83	229.80
PCB189	0.02	0.02	0.00	0.05	0.48	0.32	0.13	1.08
PCB202	0.17	0.15	0.03	0.45	3.33	2.98	0.51	10.48
PCB201/204	0.02	0.02	0.00	0.09	0.86	0.95	0.01	3.05
PCB197	0.03	0.03	0.00	0.09	0.96	0.90	0.04	3.36
PCB196/203	0.48	0.44	0.06	1.50	15.09	15.31	1.50	65.57
PCB194	0.26	0.28	0.02	1.12	9.14	9.19	1.59	39.01
PCB199	0.23	0.24	0.04	0.88	6.22	5.11	0.86	21.30
PCB208	0.04	0.04	0.00	0.17	1.31	1.22	0.13	4.18
PCB207	0.01	0.01	0.00	0.05	1.61	1.58	0.09	5.71
PCB206	0.09	0.11	0.02	0.50	2.41	2.29	0.24	8.44
PCB209	0.10	0.14	0.01	0.60	1.62	1.47	0.14	5.13
PCB138	9.21	8.41	1.55	26.28	122.71	71.24	34.44	267.71
PCB128	0.13	0.16	0.02	0.49	7.19	4.16	0.93	15.43
PCB170/190	1.34	1.27	0.17	4.28	36.86	27.97	6.42	108.62
PCB177	0.17	0.18	0.01	0.54	7.17	5.92	1.28	26.83
PCB195	0.05	0.05	0.00	0.15	2.76	2.90	0.37	11.70
PCB66	0.55	0.60	0.05	2.10	0.72	0.45	0.14	1.96
PCB74	0.75	0.59	0.21	2.37	2.78	1.50	1.28	6.66
PCB118	2.67	2.66	0.47	10.22	8.57	4.34	1.18	20.37
PCB114/122	0.07	0.06	0.01	0.23	0.49	0.28	0.15	1.19
PCB105	1.17	1.20	0.21	4.31	5.80	3.16	1.35	14.35
PCB156	0.43	0.40	0.08	1.32	5.98	4.06	1.60	15.97
PCB157	0.13	0.12	0.02	0.39	1.83	1.70	0.25	6.47
PCB52	1.06	0.95	0.23	3.34	4.89	3.52	1.24	14.98
PCB47/48	0.27	0.24	0.05	0.75	1.70	1.25	0.49	6.22
PCB99/113	3.40	3.11	0.82	10.12	18.38	12.54	4.68	56.52
PCB101	3.07	2.73	0.70	9.06	15.63	6.77	5.76	30.30
PCB110	0.41	0.40	0.10	1.81	2.76	1.72	0.35	7.22
PCB141	0.18	0.22	0.04	0.85	1.46	0.68	0.59	3.91
PCB174	0.11	0.10	0.00	0.33	6.56	7.05	0.50	21.64
PBDE47	1.18	0.91	0.28	3.86	5.51	5.28	1.71	26.27

PBDE100	0.07	0.06	0.01	0.26	0.57	0.38	0.20	1.99
HCB	0.28	0.18	0.12	0.93	0.47	0.24	0.14	0.98
CHC	1.23	0.83	0.36	3.28	2.52	2.39	0.32	11.85
CCL	0.22	0.15	0.07	0.64	0.43	0.37	0.13	1.90
OCL	7.35	5.56	2.34	18.39	17.61	14.88	4.72	66.87
CNCL	0.17	0.25	0.02	1.13	1.81	1.75	0.08	8.18
TNCLMC6	4.35	4.45	1.20	19.10	35.03	34.53	4.19	163.26
p,p'-DDE	19.47	17.14	5.16	59.72	119.79	92.46	25.68	437.88
Tox2	0.04	0.02	0.01	0.08	0.07	0.07	0.01	0.23
Tox26	0.27	0.21	0.10	0.87	2.08	2.33	0.10	8.35
Tox38	0.03	0.03	0.00	0.12	0.19	0.25	0.01	1.11
Tox40/41	0.17	0.08	0.05	0.40	0.33	0.26	0.05	1.07
Tox44	1.85	0.58	0.88	3.07	1.58	2.44	0.08	10.92
Tox50	0.06	0.06	0.01	0.22	1.28	1.65	0.02	5.52
Tox62	0.65	0.44	0.07	1.91	1.16	1.38	0.14	6.08