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Lehnert, Kristina (1,2)*, Ronnenberg, Katrin (1), Weijs, Liesbeth (3,4), Covaci, Adrian (3), Das, Krishna (5), Hellwig, Veronika(2), Siebert, Ursula (1)

(1) Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation, 25761 Büsum, Germany
(2) Institute for Coastal Research, Helmholtz-Zentrum Geesthacht Centre for Materials and Coastal Research, 21502 Geesthacht, Germany
(3) Toxicological Center, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium
(4) National Research Centre for Environmental Toxicology (ENTOX), The University of Queensland, 39 Kessels Road, Coopers Plains, QLD 4108, Australia
(5) Laboratory of Oceanology - MARE Centre, B6C, University of LIEGE, 4000 Liege, Belgium

*corresponding author
Kristina.lehnert@tiho-hannover.de
Tel. +49 511 856 8171
Fax. +49 511 856 8181

Abstract

Harbor seals are exposed to increasing pressure caused by anthropogenic activities in their marine environment. Persistent organic pollutants (POPs) and trace elements are hazardous contaminants which accumulate in tissues of harbor seals. POPs and trace elements can negatively affect the immune-system and have been reported e.g. to increase susceptibility to viral infections in seals. Biomarkers of the xenobiotic metabolism, cytokines and heat-shock-protein as cell mediators of the immune-system were established to evaluate the impact of environmental stressors on harbor seals. Harbor seals (n=54) were captured on sandbanks in the North Sea during 2009 to 2012. Medicals including hematology were performed, RNAlater blood samples were taken and analyzed using quantitative Polymerase Chain Reaction. Normalized transcript copy numbers were correlated to hematology and POP burden in blood, and trace metals in blood and fur.

A significant correlation between xenobiotic markers and contaminant burden was found. Significant interrelationships between markers and POP compounds, as well as with season, weight and hematology values, indicate that biomarkers reflect pollutant exposure and effects. A significant relationship between cortisol levels and heat-shock-protein expression was observed, indicating stress experienced during restraint of the seals. Interleukin-10 transcription showed significant correlations with trace elements in fur pointing towards immune regulatory effects of metal exposure. The molecular markers prove to be an important non-invasive tool that reflects contaminant exposure and the impact of anthropogenic stressors in seal species. The connection between interleukin-2, xenobiotic markers and pollutants may indicate immune suppression in animals exposed to contaminants with subsequent susceptibility to inflammatory disease.

Keywords: *Phoca vitulina*, biomarker, mRNA, xenobiotic metabolism, ecotoxicology, POPs, trace metals, mercury, immune function, multiple stresses, pollution effects, seals

1. Introduction

Harbor seals are top predators in the upper trophic levels in the North and Baltic Seas (Burns 2002; Hammil 2002; Siebert et al. 2012). Although their population has been increasing over the last years (HELCOM 2013; CWSS 2014) unusual mortalities of European harbor seals occurred in 2014, resulting from infections with a novel influenza A H10N7 strain (Zohari et al. 2014; Bodewes et al. 2015).

Harbor seals are threatened by various anthropogenic impacts in their marine habitat, e.g. pollution by chemical and pharmaceutical substances, marine litter (plastics and nanoparticles), noise, changes in prey abundance, and climate change (Harwood 2001; Waterman et al. 2003; Weijs et al. 2009a). These constraints can have serious implications for the health status of marine mammals resulting in stress and immune suppression that may affect different species to a varying degree (Vinther & Larsen 2004). Two Phocine Distemper Virus (PDV) epidemics in 1988/89 reduced the harbor seal population by 40-60% (Jensen et al. 2002; Müller et al. 2004). The severity of the outbreak is thought to be, in part, related to a contaminant-related reduction in the seals' immune systems (Brouwer et al. 1989; Ross et al. 1996). Since then, the population has recovered and increased to highest numbers since counting was commenced. As top predators, harbor seals are characterized

by long life spans and can bioaccumulate high levels of contaminants such as Persistent Organic Pollutants (POPs; Sormo et al. 2003; Wolkers et al. 2004; Weijs et al. 2009b) and some trace metals (Teigen et al. 1999; Das et al. 2003; Kakuschke et al. 2007). When studied comprehensively, marine mammals can be considered as sentinels of pollution (Bossart 2006) and ecosystem health in the ocean (Reddy et al. 2001). POPs are known to negatively affect the immune system and have been reported to result in increased susceptibility to viral infections in seals (De Swart et al. 1996; Ross et al. 1996). Environmental exposure with metals is believed to affect marine mammal health adversely and to alter the modulation of immune homeostasis (Das et al. 2008; Dupont et al. submitted). Metals may change, among other functions, cell proliferation, phagocytosis or protein expression (Kakuschke et al. 2007; Das et al. 2008; Dupont et al. submitted) and result in acute or chronic inflammatory processes leading to hypersensitivities or autoimmune diseases (Kakuschke et al. 2006; 2008). Recent studies have indicated that environmental contaminants affect lipid metabolism and endocrine function in marine mammals (Tabuchi et al. 2006; Lyche et al. 2010; Fossi et al. 2014) and increasingly reveal that different stressors interact to affect the health of marine wildlife. This highlights the need to develop new tools to understand the relative contributions of different stressors. To secure a better understanding of the early biological effects of anthropogenic impacts on the physiology of marine mammals, recent studies established new molecular biomarkers which provide insight into toxicity and immunological health related impact in harbor seals, grey seals, and harbor porpoises from the North and Baltic Seas (Müller et al. 2013, Weirup et al. 2013, Lehnert et al. 2014) using blood samples as a minimally invasive method to establish health markers in protected wildlife. The aryl hydrocarbon receptor (AHR) and its dimerization partner the aryl hydrocarbon receptor nuclear translocator (ARNT) are activated by environmental pollutants and reported to mediate immune toxic contaminants (Barouki et al. 2007; Fujii-Kuriyama & Kawajiri 2010; Chopra & Schrenk 2011)**.** The transcription factor peroxisome proliferator-activated receptor (PPARα) regulates mRNA expression and detoxifying enzymes (van Raalte et al. 2004). Cytokines interleukin-2 (IL-2) and interleukin-10 (IL-10) are cell mediators of inflammatory disease in marine mammals (Fonfara et al. 2008; Boyman & Sprent 2012) and the acute-phase protein heat-shock-protein HSP70 is essential in measuring immune reactions and stress (De Maio 1995; Chen & Cao 2010).

Pollutant-associated biomarkers (AHR, ARNT & PPARα) were established in blood samples of harbor seal pups in rehabilitation and a decrease of infectious disease- and pollutant-induced markers after admission (IL-2, AHR, ARNT & PPARα) as well as a decrease of stress markers (HSP70) during rehabilitation was observed (Weirup et al. 2013). Differences in gene transcription between pups and adult grey seal were observed (Lehnert et al. 2014), probably related to the accumulation of pollutants. Therefore, more insight into the ecotoxicology of anthropogenic stressors is required to safeguard the health of endangered marine mammals by exploring molecular biomarkers (Byers et al. 2000).

The aim of this study was to use molecular biomarkers in free-ranging harbor seals to assess pollutant- and stress related immune response and to validate/correlate gene expression of relevant markers with total POPs and specific compounds, as well as with trace element concentration in blood and fur, taking into account hematology and life history parameters of the harbor seals.

2. Materials and Methods

2.1. Blood and fur sampling

North Sea, Germany: Harbor seals (n=54) were captured on two sandbanks (Lorenzenplate and Kolumbusloch) in the North Sea (supplementary material fig. 1) during spring (March/April) and autumn (August/September) from 2009 to 2012. 46 animals were adults (females n=30; males n=16) and 8 animals were yearlings (females n=3; males n=5) judging by their weight and length. Medicals including hematology were performed, the weight, length and blubber thickness of the animals were taken (supplementary table 1). The seals were captured in nets and restrained during the examinations and before their release back into the sea.

2.2. Blood status

As described by Dierauf & Gulland (2001) the blood was taken from the epidural intravertebral vein and collected in ethylenediaminetetraacetic acid (EDTA) tubes. Samples taken for hemogram profiles were measured with ScilVet ABC™ Animal Blood Counter (Scil Animal Care Company GmbH, D68519 Viernheim, Germany). Differential hematology profile of red blood cells (RBC), their derivatives hemoglobin (HGB), and white blood cells (WBC) and their derivatives lymphocytes (LYM), monocytes (MO) and granulocytes (GRA) and blood chemistry can be found in supplementary table 1.

2.3. Real time RT-PCR analysis

For analyses of mRNA transcription levels blood samples were taken in EDTA monovettes containing 3ml of RNA*later* (RiboPure-Blood Kit, Ambion Life Technologies). RNA samples were frozen at -20°C or isolated directly according to the manufacturers' protocol, from 500-700µL EDTA blood (RiboPure™-Blood Kit, Ambion Europe, Huntington, UK).

The Thermo Scientific Nanodrop 2000 unit (Peqlab Biotechnologie GmbH) was used with 2µl RNA to determine the RNA concentration and integrity. Murine reverse transcriptase (RT-PCR Core Kit™; Applied Biosystems, Weiterstadt, Germany) reverse transcribed 80-100ng/µL RNA under the following temperature conditions: 8min at 25°C, 15min at 42°C, 5min at 96°C and at 4°C until samples were removed and then stored at -20°C. Template cDNA was analyzed with the ThermocyclerMX4000™real-time PCR Systems (Stratagene Europe, Amsterdam, Netherlands). SYBR®Green QPCR Master Mix (Agilent Technologies) was used according to the manufacturers' protocol for real-time quantification. For each parameter standard curves with a dilution series from 10^8 to 10^2 DNA-copies/µl were run, with reactions performed in duplicate and with negative controls. The reaction volume of 20µl in each sample of the reaction batch included 0.3µl of reference dye, 8.3µl of DEPC-Aqua 10µl Mastermix a 0.2µl of sense and 0.2µl of antisense primer and 1.0µl cDNA. PCR initiated with 95°C for 3min, followed by 45 cycles with denaturation at 95°C for 5sec and a primer-specific annealing temperature for 20sec. At the end of the annealing and at the end of the dissociation program fluorescence was measured at a wavelength of 530nm. A dissociation program was run for 1min at 95°C, followed by 41 cycles during which the temperature was increased in each cycle, starting at 55°C and ending at 95°C, excluding the measurement of nonspecific PCR products and primer dimers and to determine true amplification. Primers, annealing temperatures and amplicon length are shown in table 1. As internal standards and after verification of their stability with geNorm software (version 3.5; March 2007) reference genes ß-actin, ß2M and YWAHZ were used to normalize biomarker mRNA transcription (Kok et al., 2005).

2.4. Trace element analyses

Concentrations of trace elements (Cd, Cu, Fe, Ni, Pb, Se, Zn) were measured in blood (n = 40) and fur (n = 10, n = 15 for Hg) of harbor seals sampled between 2009 and 2012 as described previously (Habran et al. 2012). About 0.2 g of freeze-dried blood, 0.25 g of washed and freeze-dried fur was weighed to the nearest 0.0001g. All these samples were subjected to

microwave-assisted digestion in Teflon^{TM} vessels with 4 ml HNO_3 (65%), 1 ml H_2O_2 (30%) and 3 ml of 18.2 MΩ-cm deionized water. After cooling, samples were diluted to 50 ml with 18.2 MΩ-cm deionized water in a volumetric flask. Cd, Cr, Cu, Fe, Ni, Pb, Se, V and Zn levels were determined by inductively coupled plasma mass spectroscopy (ICP-MS, PerkinElmer, Sciex, DCR 2). Multiple-elements (^{74}Ge, ^{103}Rh, ^{209}Bi, ^{69}Ga) internal standards (CertiPUR®, Merck) were added to each sample and calibration standard solutions. Quality control and quality assurance for ICP-MS included field blanks, method blanks, certified reference materials (CRMs) – Seronorm L-3, DOLT-3, NIES-13 and BCR-063. Certified reference material recovery (%) and the instrumental quantification limits for each element are listed in table 2. Reported concentrations for all elements in blood and fur are expressed on a dry weight basis in mg/g.

2.5. Total Hg analysis

About 30-50 mg of freeze-dried blood (n=40) and 1-4mg of fur (n=15) were accurately weighed and loaded into quartz boats. Total Hg (THg) levels were determined by atomic absorption spectroscopy, AAS (DMA-80, Direct Mercury Analyzer, Milestone). The method has been validated for solid samples using US EPA Method 7473. Quality assurance methods included evaluating by measuring blanks, duplicates and CRMs – Seronorm L-3 and NIES-13 with every 10 samples. Concentrations of all analyzed metals included in the statistical analyses are shown in supplementary material table 2.

2.6. Chemical analysis of organic contaminants

Target analytes

In all samples, target compounds were polychlorinated biphenyls (PCBs, IUPAC-numbers: 52, 74, 95, 99, 101, 105, 110, 118, 128, 138, 149, 151, 153, 156, 170, 171, 172, 174, 177, 180, 183, 187, 194, 196/203, 199, 206, and 209); polybrominated diphenyl ethers (PBDEs, IUPAC-numbers: 47, 100, 153); oxychlordane (OxC), *trans*-nonachlor (TN), hexachlorocyclohexanes (HCHs), alpha-, beta- and gamma HCH isomers; and DDT and metabolites. Standards were purchased from Dr Ehrenstorfer, Accustandard, Wellington and Cambridge Isotope Laboratories.

Sample preparation

The method for serum analysis was described by Weijs et al. (2009a) and Dirtu et al. (2010). Briefly, an accurate volume of serum (typically around 1.5ml) was spiked with internal standards (CB 143, epsilon-HCH, BDE 77 and BDE 128), diluted 1:1 with Milli Q water, mixed

with formic acid, sonicated for 20min and extracted using solid-phase extraction (SPE) cartridges (6 mL/500 mg Oasis HLB, Waters). Elution was done by 10ml of MeOH:DCM (1:1, v/v). After evaporation to near dryness, the analytes were reconstituted in 0.5ml hexane:DCM (1:1, v/v) and the extract was further cleaned on acidified silica (500 mg, 44% H2SO4, w/w). POPs were eluted with 10ml of hexane:DCM (1:1, v/v) and further evaporated to dryness under a gentle nitrogen stream and reconstituted in 100µl iso-octane.

Analysis

For the analysis of PBDEs and HCHs, a GC–MS operated in electron capture negative ionisation (ECNI) mode was equipped with a 30 m×0.25 mm×0.25 µm DB-5 capillary column (J&W Scientific). The ion source temperature was 170 °C. The MS was used in the selected ion monitoring mode (SIM) mode with two ions monitored for each HCH and CHL in specific windows, while ions m/z=79 and 81 were monitored for PBDEs during the entire run. Two µl of the extract was injected in cold pulsed splitless mode, splitless time 1.50 min. Helium was used at constant flow (1.0 ml/min).

For the analysis of PCBs and DDTs, a GC–MS operated in electron impact ionisation (EI) mode was equipped with a 25 m×0.22 mm×0.25 µm HT-8 capillary column (SGE). The ion source temperature was 230 °C. The MS was used in the SIM mode with two ions monitored for each PCB homologue group and for each individual pesticide in specific windows. Two µl of the extract was injected in cold pulsed splitless mode, splitless time 1.50 min. Helium was used at constant flow (1.0 ml/min). Concentrations of all analyzed POP compounds included in the statistical analyses are shown in supplementary material table 3.

QA/QC

Multi-level calibration curves ($r^2 > 0.999$) in the linear response interval of the detector were created for the quantification. Quality control (QC) was performed by regular analyses of procedural blanks, by random injection of standards, spiked samples and solvent blanks. The QC scheme is also assessed through regular participation to interlaboratory comparison exercises organized by AMAP (POPs in human serum). Obtained values were deviating with < 20% from consensus values. For analytes detected in procedural blanks, the mean procedural blank value was used for subtraction. After blank subtraction, the limit of quantification (LoQ) was set at 3 times the standard deviation of the procedural blank. For analytes not detected in procedural blanks, LoQs were calculated for a signal-to-noise ratio = 10. Results were expressed on a wet weight basis.

225 **2.7. Statistical analysis**

226 Chemical compounds analyzed in harbor seal below LoQ were not considered for statistical
227 analyses. Compounds with more than 50% of the samples below LoQ were excluded. POPs
228 tested are summarized in supplementary material table 3.

229 ***Multivariate analyses***

230 Non-metric multi-dimensional scaling (NMDS), derived from Euclidean distance was used to
231 visualize similarities between pollutant burdens in harbor seals. No attempt was made to
232 quantify toxicity differences among pollutants; however, equal weighting was guaranteed by
233 standardizing (dividing by maximum value) compounds. Two dimensions were sufficient to
234 minimize the stress factor to a good result of 0.07. From all tested pollutant compounds
235 sums were derived for relevant pollutant groups per blood sample (Table 1). The variable
236 "sum POPs" is the sum of all standardized pollutant compounds with detection frequency
237 >50% throughout all blood samples. It is heavily influenced by the group of PCBs with 28
238 compounds included. The other compound groups are only represented by 3, 2, 2, and 1
239 compounds (for PBDEs, DDTs, chlordanes (CHLs) and HCHs respectively, table 1). In order to
240 test significance between euclidean similarity matrix of standardized POP compounds and
241 explanatory variables (Table 2) PERMANOVAs were used with 999 permutations. Animals
242 caught in 2009 were not tested for trace elements, thus trace elements were not included in
243 the multivariate analyses.

244 ***Univariate analyses***

245 Trace elements showed best fit assuming normal distribution. To account for non-normal
246 errors and over-dispersion in organic pollutant groups, negative binomial regressions were
247 applied. For β-HCH, censored (or tobit) generalized regression models with the negative
248 binomial distribution were applied to account for the samples under limit of quantification
249 (LoQ). More specifically, we tested if pollutant loads of the pollutant groups PCBs, PBDEs,
250 DDTs, CHLs and β-HCH differed between tested years and if their concentration changed
251 with body weight. In a second model group we tested for differences in pollutant groups
252 between males and females and between spring and autumn samples. These effects were
253 also tested in trace elements in the blood. In the results, we only show significant effects of
254 trace elements. Once differences in organic pollutant burdens were established, biomarkers
255 were added to the models including sex and season in order to test whether xenobiotic
256 biomarkers reflect burdens of organic pollutant groups in the blood. To account for non-

normal errors Spearman rank correlations were used to test relationships between biomarkers and sum POPs. To avoid excess testing only the biomarkers that were found to be relevant in the explorative NMDS and PERMANOVAs were tested. To test whether stress effects are reflected in HSP70 levels in harbor seals a Spearman rank correlation was calculated with cortisol. All multivariate tests were run with the package vegan (Oksanen et al. 2013) in the R 3.0.2 environment (R Core Team 2014). The censored generalized regression model was run in the package VGAM (Yee 2014).

3. Results

POPs

A negative relationship between organic pollutant compound groups in the blood and the weight of the animals was apparent in all negative binomial regression models for most organic compound groups ($p<0.001$). Only β-HCH compounds showed no significant effect of weight ($p=0.14$) (Fig. 1). There was a non-significant trend of decreasing β-HCH levels within the 4 study years ($p=0.056$), whereas all other organic compound groups stayed at comparable levels ($p>0.1$) during the study period.

Sum POPs in blood taken in spring and autumn differed significantly ($p<0.05$) with higher loads in autumn except for DDT with a marginally significant difference ($p=0.087$). Males had higher loads ($p<0.05$) in all organic compound groups compared to females, but β-HCH (Fig. 2) showed no significant difference between sexes.

POPs and molecular biomarkers

The NMDS plot (Fig. 3) shows the relationship between molecular biomarkers, pollutant groups and hematology values and similarities in pollutant burden in blood samples of the first two rotated (thus showing the most important) NMDS axes. All significant effects tested in PERMANOVAs were plotted post-hoc over the NMDS (see also table 2). Sum POPs, as sum of all compounds per sample explains the highest proportion of variance of the first NMDS. PCBs, which are heavily influential on sum POPs and the primary matrix, are also highly correlated to sum POPs, as are PBDEs. CHLs and DDTs show a higher correspondence to the second NMDS axis and are almost orthogonal to the biomarkers AHR and ARNT. β-HCH is the least important of all pollutant groups in this context.

No clear grouping between spring and autumn samples is obvious (Fig. 3, grey upward triangles for spring and black squared symbols for autumn), however, on the positive side of the first axis there are more samples collected in autumn. Apart from pollutant groups the next important variables to explain the variance are, in order of decreasing magnitude: weight, SGPT, AHR, ARNT, total protein, IL-2 and creatinine. The hematology values SGPT, creatinine and total protein indicate a negative relationship to PCBs and PBDEs. The biomarkers AHR, ARNT and IL-2 are mostly explained by the first NMDS axis. Correspondingly, weak but significant Spearman rank correlations were found between sum POPs and AHR (rho=0.29, p=0.036; Fig. 4a) and ARNT gene transcription (rho=0.406, p=0.003; Fig. 4b).

A significant Pearson correlation between gene transcription of the xenobiotic biomarker AHR and pro-inflammatory cytokine IL-2 was found (r=0.92, p<0.001) (Fig. 4c). PPARα transcription showed a significant positive correlation with monocytes (r=0.58, p<0.001).

When testing the relationship between biomarkers and pollutant groups, a general pattern was observed (Fig. 5a-d). Apart from the difference between males and females in spring and autumn (shown in Fig. 2), a positive relationship between some biomarkers and some pollutant groups (PCBs and AHR, DDTs and ARNT, PBDEs and AHR, PBDEs and IL-2) in autumn and for males in spring was found. Females, in comparison, show a negative correlation in spring. The correlation between PCBs and AHR transcription is supported by a significant threefold interaction between sex, season and AHR (p=0.011). A twofold interaction between season and sex as shown above and sex and AHR transcription indicate that their interrelationships differ between females and males. The relation of PBDEs and AHR transcription was described by a significant main effect of season with higher values in autumn (p=0.011) and a significant positive correlation to AHR transcription was observed (p=0.019) (Fig. 5). A marginally higher PBDE contamination in males (p=0.065) was found. IL-2 transcription showed a similar non-significant pattern (p=0.084), the contribution of season and sex is similar to the previous model (season: p=0.020, sex: p=0.066). DDT presented the same overall pattern, however only the sex effect is significant (p=0.030). ARNT showed a non-significant positive relation to DDT (p=0.078) (Fig. 5). For DDT, no differences were found between season, nonetheless model fit was better including season as fixed effect, as found by a likelihood ratio test of competing models.

A weak but significant Spearman correlation was found between HSP70 gene transcription and cortisol values in adult harbor seals (rho=0.45, p=0.004) (supplementary material fig. 2).

Trace elements

Concentrations of Ni and Cu in blood samples taken in 2011 were significantly lower than in 2010 ($p<0.05$), but levels increased again in 2012. Fe in blood samples levels decreased in 2012 ($p<0.001$), whereas Cd showed a significantly higher concentration in 2012 ($p<0.001$). Cd was the only trace element showing some negative relation to body weight. This relation was however statistically non-significant ($p=0.072$). The trace element concentrations remained similar between seasons. However, Ni, Se, and Cu concentrations were lower in males ($p<0.05$) than in females.

Trace elements and molecular biomarkers

Significant correlations between trace element concentrations in blood and fur, biomarker transciption and hematology values were found (see table 3). IL-10 showed significant positive effects with Ni, Cu, Zn, and Pb concentrations measured in fur samples. Hg in blood and fur was positively correlated to hemoglobin. Fe was positively correlated with HSP70, PPAR alpha and monocytes, whereas it showed a negative correlation to AHR and IL-2 transcription. Selenium was negatively correlated with IL-2 transcription. Cadmium showed positive correlations with AHR and IL-2 transcription, but negative correlations with HSP70 and PPARα gene transcription. Lead was positively related to HSP70 and PPARα transcription.

4. Discussion

POPs

POP concentrations found in blood in this study were comparable to those found in recent studies in pinniped species (Weijs et al. 2009a). POP concentrations found in harbor seal serum remained stable over the study period, with only HCH showing a potentially decreasing trend. Blood samples of juvenile harbor seals showed higher POP contamination than blood samples of adults. The same trend was found for PBDEs in a study analyzing PCBs and PBDEs in serum of harbor seals sampled from 2006 to 2008 in the North Sea (Weijs et al. 2009a), where juveniles had higher levels than adult males and females, probably as a consequence of lactational transfer. Higher POP burdens were found in male harbor seals compared to females investigated in this study. This is supported by findings for PCBs

analyzed in serum of harbor seals sampled in 2006-2008 in the North Sea, where concentrations in adult males were slightly higher than in juveniles and lowest in juvenile females (Weijs et al. 2009a). This effect is probably caused by mobilization of lipophilic contaminants in harbor seals during lactation, and high lipid and contaminant transfer to offspring during that time period, which in fact influences pollutant load in blood depending on the season (Debier et al. 2006). The results of this study underline how pollutants are accumulated with age in marine top predators while some are lost during gestation and lactation by females to their young (Debier et al. 2003). Apart from reproductive status, general body condition, e.g. emaciation, may have an influence on the levels of chlorinated and brominated contaminants in serum of marine mammals (Weijs et al. 2009a). However, the animals investigated in this study were all estimated to be in good or moderate nutritional state.

In this study, both sampling locations were in immediate vicinity to each other, thus pollutant exposure and potential prey are assumed to be similar for all tested animals. There was a negative correlation between weight and contaminants in blood with heavier animals showing lower pollutant burdens, probably caused by a 'dilution effect' (Weijs et al. 2009a). The samples with the highest pollutant burden were sampled in autumn.

POPs and molecular biomarkers

The NMDS plot shows the relationship between molecular biomarkers, pollutant groups and hematology values (Borcard et al. 2011). Most of the pollutant groups were highly correlated with each other. Harbor seals with a high concentration of one pollutant group were more likely to be severely contaminated with the other pollutant groups. β-HCH was an exception, and was less correlated to the other compound groups. As it is a more water-soluble compound than the others it may represent different mobilization pathways than the other compound groups (Willett et al. 1998). The xenobiotic biomarkers AHR and ARNT contribute significantly to some of the pattern. Their correlation is supported by a weak but significant correlation between the sum POPs and AHR and ARNT (Fig. 4). This is supported by findings in other marine animals, in which AHR pathways were shown to be conserved and to play a critical role in the mediation of POP toxicology (Dougherty et al. 2008; Zhou et al. 2010). IL-2 is also highly correlated with AHR as observed in the NMDS plot. The hematology values for total protein, SGPT and creatinine show some negative response to pollutant burden.

In autumn, positive relations of POP compounds with biomarkers were found, while in spring, females also show a negative relation contrary to males. This relation is statistically relatively weak, nevertheless the same pattern was found for three POP groups (PCBs, PBDEs and DDTs) and two pollutant-induced (AHR and ARNT) and immune relevant (IL-2) biomarkers. It is assumed that this pattern in pollutant exposure and gene transcription reflects seasonal changes in mobilization of liposoluble contaminants and subsequent expression of transcription factors metabolizing toxic compounds, partly due to gestation and lactation of females in spring. Harbor and grey seal pups have been reported to show decreasing gene transcription of pollutant-induced markers AHR and ARNT during rehabilitation, after first lactating and then being fed a compensatory milk product containing bovine milk products, plant fat and egg powder (Weirup et al. 2013, Lehnert et al. 2014). This underlines that exposure to liposoluble contaminants induces gene transcription of AHR and ARNT which clearly correlates with sum POPs and POP compounds in this study.

Trace elements

Trace element concentrations found in blood and fur samples in this study were in a comparable range to those found in other studies (Kakuschke et al. 2008; Das et al. 2008; Habran et al. 2012). Trace elements analyzed in blood and fur samples of harbor seals were lower in males compared to females. This is concurrent with a study by Wenzel et al. (1993) who observed higher levels in Hg concentrations in fur from female compared with male harbour seals. Species- and gender related differences in Hg concentrations could be due to sexual dimorphism, diet and different energy requirement for gestation and lactation (Aubail et al. 2011). No clear age-dependent patterns in trace element concentrations of the harbor seals were observed. Metal concentrations in free ranging seals of the North Sea have been reported (Griesel et al. 2008; Das et al. 2008) with young seals showing high burdens of selected metals, probably caused by a transplacental transfer from the mother to fetus or through the milk during the lactation period (Kakuschke et al. 2008).

Recent studies suggested that the maternal transfer of Se was prominent during lactation, whereas the Hg transfer was larger during gestation in elephant seal mothers and pups (Habran et al. 2011). In grey seals pups all investigated trace elements (n=11) were shown to be transferred via lactation (Habran et al. 2012), except Cd which was transferred through the placenta.

Trace elements and molecular biomarkers

Anti-inflammatory cytokine IL-10 transcription showed high positive correlations to trace element concentrations of Ni, Cu, Zn and Pb in fur of harbor seals in this study. This probably indicates a negative influence of increased heavy metal loads on immune homeostasis (Kakuschke et al. 2007), and the influence of metals on the function of immunocompetent cells by immune-enhancement or immunosuppression effects. In ringed seal (*Pusa hispida saimensis*) pup fur samples from Finland, a relationship between Ni concentrations and stillbirths was found (Hyvärinen & Sipilä 1984). Other studies found increased IL-10 transcription correlated with increased susceptibility to infectious disease in harbor porpoises (Beineke et al. 2007) and inhibited lymphocyte proliferation in harbor seal pups from the North Sea by Be, Pb, Cd and Hg in newborn pups (Kakuschke et al. 2008).

Cd and Pb loads in blood showed significant correlations to xenobiotic biomarkers AHR and PPARα as well as immune-relevant IL-2 and acute-phase–protein HSP70 transcription, indicating clear effects of heavy metal concentrations on the xenobiotic metabolism, stress- and immune response. Cadmium showed a positive relation with AHR and IL-2 transcription, possibly reflecting negative impact of Cd concentrations and exposure to infectious disease. This is supported by a study measuring mRNA-expression of IL-2 and IL-4 in grey seal lymphocytes where Ni and Be induced lowest cytokine expression compared to other metals and the quotient IL-2/IL-4 was increased due to a strong down-regulation of the Th2 cytokine IL-4 (Kakuschke et al. 2006).

Cd in blood showed a negative correlation with HSP70 and PPARα transcription, while lead showed a positive relation with HSP70 and PPARα transcription in blood samples. Both relationships probably underline that effects of metals on the immune system may include immunosuppression or acute as well as chronic inflammatory processes leading to hypersensitivities or autoimmune diseases (Kakuschke et al. 2007).

Mercury concentrations in blood and in fur were positively correlated to hemoglobin values in the seals investigated. Hg is bound to hemoglobin in the red blood cells and therefore changes in hemoglobin concentration can affect Fe and Hg concentrations measured in whole blood (Habran et al. 2011). Although mercury did not show any significant relationships with immune-relevant or xenobiotic biomarkers in this study, high Hg concentrations were found associated with a prevalence of parasitic infections and pneumonia in harbor porpoises from the North and Baltic Sea (Siebert et al. 1999) and experimental inoculation with Hg has been shown to result in lethargy, weight loss and

446 death in harp seals (*Pagophilus groenlandicus*) (Ronald et al. 1977). Methylmercury altered
447 *in vitro* immunoproliferation in lymphocytes of harbour seals from the North Sea (Das et al.
448 2008).
449 Fe values in blood samples showed negative correlations to AHR and IL-2 biomarker
450 transcription, but positive correlations with HSP70, PPARα and monocytes. The correlation
451 of PPARα transcription with monocytes was observed in grey seals (Lehnert et al. 2014),
452 while a similar correlation between monocytes and ARNT and HSP70 gene expression was
453 observed in harbor seal pups (Weirup et al. 2013). This may indicate influences of the
454 xenobiotic metabolism (PPARa/ARNT) on immune status (monocytes) and reflect heightened
455 immune response due to exposure to environmental contaminants. Nickel in blood showed
456 a positive relationship to lymphocytes but a negative to monocytes, indicating possible
457 regulatory functions of blood cells towards trace element exposure (Alkahem 1994; Caicedo
458 et al. 2010). Selenium showed a weak negative correlation to IL-2, underlining the
459 interrelationships between selenium and immune cell modulation (Kiremidjian-Schumacher
460 et al. 1990).
461 ***Biomarkers***
462 IL-2 is highly correlated with AHR which may be due to possible immune-modulatory effects.
463 Increased AHR transcription is probably caused by exposure to environmental pollutants.
464 This is in accordance with preliminary results with primary harbor seal hepatocytes, which
465 were incubated with PCB mixtures (Behr et al. 2008; Hellwig 2011; Korff et al. submitted). A
466 subsequent up regulation of pro-inflammatory cytokine IL-2 may reflect immune
467 suppression and enhanced susceptibility to infectious disease.
468 HSP70 and cortisol showed a positive correlation in adult harbor seals, probably reflecting
469 stress experienced by the seals during capture. In previous studies on harbor and grey seal
470 pups HSP70 transcription decreased after admission and throughout rehabilitation, which
471 probably reflects habituation and underlines the use of this acute-phase-protein as a stress
472 marker in pinnipeds (Fonfara et al. 2008, Weirup et al. 2013, Lehnert et al. 2014).
473
474 **Conclusions:**
475 To our knowledge, this is the first study investigating gene transcription of immune-relevant
476 and xenobiotic molecular biomarkers to assess the impact of environmental pollutants on
477 the health of free-ranging harbor seals using minimally invasive blood and fur samples.

Transcription patterns observed suggest that organic pollutant compounds and trace elements have immune-modulatory effects on the harbor seals and that their impact may be detrimental to the seals health, potentially causing immune suppression and subsequent increased susceptibility to infectious disease. We conclude that the markers used in this study are useful to detect and assess pollutant-induced changes in vulnerable pinniped species.

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685

686 Table 1: Primer sequences, amplicon length, annealing temperature, accession numbers and
687 efficiencies for reference genes, cytokine, heat shock protein and xenobiotic markers
688
689

Gene	Primer sequences (5'-3')		Length of Amplicon (bp)	Annealing temperature (°C)	Accession number	Efficiency (%)
	s	as				
ß2M	GCT ACG TGT CAG GGT TCC AT	CAT GCT TTA CAC GGC AGC TA	177	58	GU046700	98.9
YWHAZ	AGA CGG AAG GTG CTG AGA AA	TCA TCA CCA GCA GCA ACT TC	208	58	GU046699	97.9
ß-actin	CCATCGGCAACGAGCGGTTCC	CGTGTTGGCGTAGAGGTCCTTCC	146	64	DQ212697	98.8
HSP70	GGG GCT GAA CGT GCT GAG G	CCG CTT GTT CTG GCT GAT GTC	280	61	DQ118386	98.7
IL-2	CTT GCA TCG CAC TGA CTC TT	GCT CCA ACT GTT GCT GTG TT	89	57	AY919322	93.2
Il-10	CCT GGG TTG CCA AGC CCT GTC	ATG CGC TCT TCA CCT GCT CC	206	64	AY919326	98.9
AHR	AACAGCTAGGCATTGATTTTGAAG	GTGGCTGCGGGTAGTCTGAACA	180	60	AB056700.1	100.1
ARNT	AGCACTGTCCCTGCCACCCA	GGGGTCCAAGTTGGGGCTGC	200	60	AB201467.1	96.6
PPAR α	GATCGTCCCGGCCTTCTAAAC	GCTGCGCGTGCTCTGTGAC	129	62	AF350327.1	98.5

690 Table 2: Explanatory variables tested in PERMANOVAs of Euclidean similarity matrix of POP
691 compounds. P-values are shown for significant effects p≤0.05 or marginally significant
692 effects p≤0.1 and p>0.05. (<tested effects with p values >0.1 were the following: Year, Sand
693 bank, Age group, Length, Sex, PPARα, IL-10, HSP70, Leukocytes, Erythrocytes, Thrombocytes,
694 Granulocytes, Lymphocytes, Monocytes, Kalium, Natrium, SGOT, Bilirubin, Urea).

explanatory Variable	p-values (PERMANOVAs)
Month	0.069
Season	0.036
Weight	0.005
AHR	0.003
ARNT	0.011
IL-2	0.014
Hemoglobin	0.062
Protein	0.008
Calcium	0.058
SGPT	0.003
Cholesterin	0.087
Creatinine	0.047

695
696

Table 3: Spearman rank correlations between trace elements (µg x g^{-1} dry weight) and biomarkers and hematology values. Table shows Spearman rho in bold for significant effects $p<0.05$.

	Hg	Fe	Ni	Cu	Zn	Pb
			fur samples (n=10, n = 15 for Hg)			
IL-10 (norm. copy numbers)	0.061	0.585	**0.809**	**0.799**	**0.912**	**0.851**
Hemoglobin (g/dl)	**0.547**	-0.5	-0.419	-0.326	-0.103	-0.14

	Hg	Fe	Ni	Se	Cd	Pb
			blood samples (n=40)			
AHR (norm. copy numbers)	0.038	**-0.591**	-0.123	-0.311	**0.608**	-0.091
HSP70 (norm. copy numbers)	-0.126	**0.473**	-0.366	-0.007	**-0.565**	**0.451**
IL-2 (norm. copy numbers)	0.059	**-0.664**	0.101	**-0.389**	**0.692**	-0.191
PPARα (norm. copy numbers)	-0.13	**0.487**	-0.19	-0.024	**-0.543**	**0.505**
Lymphocytes (#)	0.035	-0.068	**0.562**	0.016	0.301	-0.286
Monocytes (#)	-0.269	**0.495**	**-0.517**	0.016	-0.397	0.51
Hemoglobin (g/dl)	**0.348**	-0.084	0.163	0.1	0.172	-0.127

Figure Captions

Fig. 1: Pollutant load correlated to weight of the investigated harbor seals over the study years. Upward triangles show juveniles and dots adults. Indicated in grey are samples of b-HCH that were under the limit of quantification (LoQ) of 10 (pg/ml).

Fig. 2: Pollutant load in blood samples in male and female harbor seals during spring and autumn between 2009 and 2012.

Fig. 3: NMDS plot showing similarities between POP compounds and significant relationships to pollutant groups, sum pollutants, hematology values and biomarker gene transcription in spring and autumn (grey diamonds and black squares respectively) in harbor seal (n=54) blood samples (stress=0.07).

Fig. 4: Correlation between a) AHR, b) ARNT gene transcription and standardized total sum organic pollutants [excluding POPs > 50% under LoQ] in harbor seal blood samples, and c) correlation between IL-2 and AHR gene transcription in blood samples of harbor seals.

Fig. 5: Relationships between POP compounds PCBs, PBDEs, DDTs and biomarker gene transcription (AHR, IL-2) in male and female harbor seals during spring and autumn.