Environmental Pollution 247 (2019) 783–791

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Cytokine expression and lymphocyte proliferative capacity in diseased harbor porpoises (*Phocoena phocoena*) – Biomarkers for health assessment in wildlife cetaceans☆

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ARTICLE INFO

Article history:
Received 25 October 2018
Received in revised form
17 January 2019
Accepted 19 January 2019
Available online 29 January 2019

Keywords:
Harbor porpoise
Blood samples
Lymphocyte proliferation
Mitogens
Xenobiotics
Cytokine expression

ABSTRACT:

Harbor porpoises (*Phocoena phocoena*) in the North and Baltic Seas are exposed to anthropogenic influences including acoustic stress and environmental contaminants. In order to evaluate immune responses in healthy and diseased harbor porpoise cells, cytokine expression analyses and lymphocyte proliferation assays, together with toxicological analyses were performed in stranded and bycaught animals as well as in animals kept in permanent human care. Severely diseased harbor porpoises showed a reduced proliferative capacity of peripheral blood lymphocytes together with diminished transcription of transforming growth factor-β and tumor necrosis factor-α compared to healthy controls. Toxicological analyses revealed accumulation of polychlorinated biphenyls (PCBs), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyltrichloroethane (DDT) in harbor porpoise blood samples. Correlation analyses between blood organochlorine levels and immune parameters revealed no direct effects of xenobiotics upon lymphocyte proliferation or cytokine transcription, respectively. Results reveal an impaired function of peripheral blood leukocytes in severely diseased harbor porpoises, indicating immune exhaustion and increased disease susceptibility.

1. Introduction

Harbor porpoises (*Phocoena phocoena*) are the only reproducing cetacean species of the eastern North and Baltic Seas (Scheidat et al., 2008; Gilles et al., 2009). Their populations are exposed to anthropogenic impacts in their habitat including acoustic stress and hazardous substances (Kannan et al., 2000; Weijs et al., 2010; Siebert et al., 2001). Organochlorine compounds, such as polychlorinated biphenyls (PCBs), and heavy metals can have detrimental effects on the health and physiology of odontocetes (Vos et al., 2000; Law et al., 2012). For instance, harbor porpoises from German waters exhibit a higher incidence of bacterial infections compared to whales from less polluted arctic waters (Wünschmann et al., 2001). Elevated PCB levels were found in harbor porpoises suffering from infectious diseases, suggesting an adverse effect of environmental contaminants on the immune defense (Jepson et al., 1999, 2005). Contaminant analysis revealed a correlation between increased body burdens of xenobiotics, such as PCBs, and depletion of lymphoid organs in harbor porpoises (Beineke et al., 2007) and hypothyroidism in bottlenose dolphins (Schwacke et al., 2011). Although blubber PCB concentrations initially declined in some marine mammal populations following a mid-1980s EU ban (Aguilar et al., 2005, Ross, 2002), they have since stabilized in UK harbor porpoises and recent studies show that the effects of PCB pollution continue to affect cetaceans in European waters (Jepson &

☆ This paper has been recommended for acceptance by Christian Sonne.

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https://doi.org/10.1016/j.envpol.2019.01.079
0269-7491/

Law, 2016; Desforges et al., 2018), causing e.g. reproductive failure (Jepson et al., 2016) and increasing the risk of infectious disease (Hall et al., 2006). However, the impact of PCB exposure on marine mammals is still largely unknown (Jepson et al., 2016) and it remains undetermined whether immunological changes are directly contaminant-induced or a sequel of concurrent infectious diseases and poor health status, respectively.

Lymphocyte proliferation assays are well-proven tools to quantify immune responses in marine mammals *ex vivo* and can help to assess the health status in vulnerable wildlife species (Beineke et al., 2010; Desforges et al., 2016). Immune cells of beluga whales exposed to *in vitro* mixtures of organochlorines were observed to have reduced proliferation (De Guise et al., 1996) and contaminant cocktails derived from polar bear and killer whale blubber modulated marine mammal immune cells underlining the cytotoxic effects of real world contaminant mixtures (Desforges et al., 2016).

Suppressive effects of marine xenobiotics on lymphocyte proliferation have been observed in free-ranging bottlenose dolphins (*Tursiops truncates*) (Lahvis et al., 1995) and several seal species (de Swart et al., 1994; Ross et al., 1995, Sormo et al., 2003; Mos et al., 2006), and confirmed in rodent models (Fournier et al., 2000). In meta-analysis, a clear dose–response relationship for the effects of PCBs and heavy metals on lymphocyte proliferation in marine mammals was found (Desforges et al., 2016).

Pro- and anti-inflammatory cytokines decisively orchestrate innate and adaptive immune responses and represent surrogate markers (biomarkers) in human and animal cohort studies to determine the immunotoxic potential of xenobiotics (Bilrha et al., 2003; Imbeault et al., 2012; Cigliano et al., 2016). However, while these mechanisms are well documented in human medical science and standardized animal experiments, cytokine research in marine mammals also reflect inter-individual and interspecies variability and inconsistent results across studies, underlining the complex interactions in cytokine signaling and the need for further studies (Desforges et al., 2016). Blood cytokine expression analyses have been used to investigate health and stress status in harbor porpoises (Beineke et al., 2007a; Müller et al., 2013; Fonfara et al., 2007a,b) and seals (Fonfara et al., 2008, Lehnert et al., 2010). Decreased proliferative ability and changed cytokine transcription of lymphocytes in association with environmental contaminants and immune-relevant effects of xenobiotics using gene transcription were observed in harbour seals from the North Sea (Weirup et al., 2013; Lehnert et al., 2014).

For ethical and juridical reasons experimental studies cannot be performed in protected species like the harbor porpoise, resulting in the need for thorough and critical investigations of wildlife populations. Therefore, studies using minimally invasive sampling and measuring contaminant-driven effects to assess health effects in marine mammals are highly relevant, especially when correlating environmental organochlorine load to immune endpoints (Brown et al., 2017; Lehnert et al., 2018). The aims of the present study were to (i) evaluate immune responses in healthy and diseased harbor porpoise cells by the aid of cytokine expression analyses and lymphocyte proliferation assay, and (ii) to determine whether real world xenobiotic levels are associated with immune dysfunction in this cetacean species.

2. Materials and methods

2.1. Sampling

Blood samples from 24 harbor porpoises were taken *intra vitam* and investigated. The animals were divided into three different groups: (1) Harbor porpoises (animals H1-H9) sampled at the Dolfinarium Harderwijk (the Netherlands). They had been kept permanently in human care (1–13 years) and were healthy on clinical examination. These animals had been trained for medical purposes to allow voluntary handling to minimize stress induced changes during blood sampling. (2) Free-ranging animals (animals B1-B11), accidentally by-caught in Danish pound-nets, were sampled during field trials including tagging and recording of audiograms of the animals before release into the wild. (3) Stranded harbor porpoises (animals S1-S4) found diseased/incapacitated at the coastline and brought to the rehabilitation center SOS Dolfijn in Harderwijk. The age, sex and background data are shown in Table 1.

Handling and sampling of the free-ranging porpoises (animals B1-B11) were carried out directly on board the fishing boats after the harbor porpoises had been lifted out of the pound nets. Experimental research on the study animals followed internationally recognized guidelines and was approved by an appropriate ethics committee with permits from the Danish Forest and Nature Agency SN 343/SN-0008 and Ministry of Justice 1995-101-62. Blood sampling of animals in permanent human care (animals H1-H9) and in rehabilitation (animals S1-S4) was performed during routine medical examinations in the facilities. Sampling was conducted immediately after the animals were taken out of the water.

2.2. Post mortem examination

Since the stranded harbor porpoises (animals S1-S4) were diseased and euthanized due to animal welfare reasons, post mortem investigations were performed as described by Siebert et al. (2001). All organ systems were examined macroscopically and organ samples were taken. The nutritional state was judged from muscle condition and blubber thickness. Formalin fixed (10% formalin) and paraffin embedded, 5 μm thick tissue sections were stained with hematoxylin and eosin. Lung, liver, kidney, spleen, and intestine were also sampled for mycological and bacteriological culture. In addition, all pathological lesions suspected to contain bacterial infections were analyzed (Siebert et al., 2001).

3. Lymphocyte transformation assay

3.1. Isolation of peripheral blood lymphocytes

EDTA blood samples were taken from the tail fluke as described before. Cooled tubes were transported within 18 h. Blood was diluted in a ratio of 1–2 with minimal essential medium Eagle with Earle's salts with 10% fetal calf serum and 1% penicillin/streptomycin(culture medium; ICN Biochemicals, Meckenheim, Germany; PAA Laboratories, Linz, Austria; Seromed, Berlin, Germany). By one-step gradient centrifugation (25 min; 800×*g*; 15 °C) peripheral blood mononuclear cells (PBMC) were isolated using Percoll® (1.073 mg/ml; Pharmacia Fine Chemicals, Uppsala, Sweden). After washing of PBMC twice, cells were re-suspended in culture medium. Following Percoll® isolation, the lymphocyte yield was increased above 90% in each sample as determined microscopically by Pappenheim stain (Carl Roth GmbH, Karlsruhe, Germany). Number of viable cells used for cell culture experiments were microscopically determined by trypan blue staining (Carl Roth GmbH, Karlsruhe, Germany).

3.2. Cell culture

Pokeweed mitogen (PWM) and concanavalin A (Con A) were diluted in culture medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). 2×10^5 viable harbor porpoise PBMC in duplicates were treated with 100 μg/ml of the respective mitogen in a 96-well microculture plate (Falcon, New jersey, USA). Control

Table 1
Group, age, sex, origin, and contaminant levels in the blood of investigated harbor porpoises.

Animal ID	Group	Age	Sex	Origin	PCB [ng/g lipid]	p,p'-DDE [ng/g lipid]	p,p'-DDT [ng/g lipid]
H1	human care	juvenile	male	The Netherlands	2000	690	130
H2	human care	adult	male	The Netherlands	3300	670	90
H3	human care	adult	female	The Netherlands	n.d.	n.d.	n.d.
H4	human care	adult	female	The Netherlands	n.d.	n.d.	n.d.
H5	human care	juvenile	female	The Netherlands	n.d.	n.d.	n.d.
H6	human care	adult	male	The Netherlands	n.d.	n.d.	n.d.
H7	human care	juvenile	male	The Netherlands	4700	490	180
H8	human care	adult	female	The Netherlands	40000	6700	1300
H9	human care	adult	female	The Netherlands	13000	2300	1500
B1	bycaught	juvenile	male	The Netherlands	n.d.	n.d.	n.d.
B2	bycaught	adult	male	Denmark	57000	2200	240
B3	bycaught	juvenile	male	Denmark	44000	4600	370
B4	bycaught	juvenile	male	Denmark	14000	2300	370
B5	bycaught	juvenile	male	Denmark	19000	4300	640
B6	bycaught	juvenile	female	Denmark	4900	710	110
B7	bycaught	juvenile	female	Denmark	n.d.	n.d.	n.d.
B8	bycaught	juvenile	female	Denmark	15000	2500	170
B9	bycaught	juvenile	male	Denmark	47000	2600	430
B10	bycaught	juvenile	female	Denmark	27000	4200	520
B11	bycaught	juvenile	male	Denmark	2780	644	209
S1	stranded	juvenile	male	The Netherlands	n.d.	n.d.	n.d.
S2	stranded	juvenile	female	The Netherlands	10100	1240	545
S3	stranded	neonate	female	The Netherlands	2200	150	74
S4	stranded	juvenile	female	The Netherlands	26700	3470	1610

PCB = polychlorinated biphenyls (sum of congeners 99, 138, 149, 153, 180, and 187); DDE = p,p'-dichlorodiphenyldichloroethylene; DDT = p,p'-dichlorodiphenyltrichloroethane.

PBMC were incubated with mitogen free culture medium. PBMC suspensions (final volume: 200 µl per well) were incubated for 72 h at 37 °C with 5% CO_2 (Beineke et al., 2004).

3.3. 5-bromo-deoxyuridine assay

The 5-bromo-deoxyuridine (BrdU) assay was performed according to the manufacturer's instructions (Roche; Mannheim, Germany) as described previously (Beineke et al., 2004). Briefly, following stimulation for 72 h with PWM or ConA, 20 µl of the BrdU-labeling solution was added to each well. After an incubation period of 2 h incubation at 37 °C with 5% CO_2, microtiter plates were centrifuged (300×*g*, 15 °C, 10 min). Subsequently, cells were dried, fixed, and the DNA was denatured. The anti-BrdU peroxidase conjugated antibody was added and incubated at room temperature for 90 min. Following three washing steps (washing solution) bound peroxidase was visualized by substrate reaction. After adding 1M H_2SO_4, the optical density (OD) of the yellow product was measured at a wavelength of 450 nm (reference wavelength: 630 nm) using an ELISA reader (Titertek® Multiskan Plus, Flow Laboratories, Switzerland). The stimulation index (SI) was calculated as follows: *value of mitogen-stimulated cells* divided by *value of non-stimulated cells* multiplied by 100.

4. Reverse-transcription polymerase chain reaction

4.1. RNA analysis

RNA was isolated from 150 µl of whole blood samples using the RNaid Plus Kit (Bio 101, LaJolla, CA) immediately upon arrival (within 18 h after blood collection). RNA purity and concentration were measured by a spectrophotometer (GeneQuant™ pro, Amersham Biosciences Europe GmbH, Freiburg, Germany). Following DNase-treatment, 200 ng RNA was reverse transcribed with reverse transcriptase (RT-PCR Core Kit, Perkin Elmer, Applied Biosystems, Weiterstadt, Germany) as described before (Beineke et al., 2007). Resulting cDNA served as a template for PCR using a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany). For quantification by real time, the Brilliant III Ultra-Fast SYBR®Green QPCR Master Mix (Agilent Technologies) was applied. DNA amplification was performed using primer pairs specific for interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative amounts of cytokine mRNA were determined by calculating the ratio between cytokine mRNA and mRNA of the housekeeping gene GAPDH (units cytokine mRNA/units GAPDH mRNA x 100 = relative amount of cytokine mRNA in %GAPDH). Amplicon specificities were confirmed by melting curve analyses (Table 2).

4.2. Contaminant analysis

Blood contaminant levels were measured in five harbor porpoises in human care, nine bycaught animals, and three stranded animals (Table 1). 0.1 g–9 g of blood was added to a fivefold quantity of sodium sulfate. Soxhlet extraction was performed for 6 h in a 1:1 mixture of dichloromethane and n-hexane. The extractable lipid was measured gravimetrically in an extract aliquot as described previously (Beineke et al., 2004). A separate extract aliquot was used for analysis of polychlorinated biphenyls (PCBs), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyltrichloroethane (DDT). Internal standard was added ($^{13}C_{12}$ labeled *p,p'*-DDE and $^{13}C_{12}$ labeled PCB congeners 28, 52, 101, 138, 153, and 180) and the extracts were purified on an alumina/silica gel column. PCBs, DDT, and DDE were analyzed using GC/MS-MS (EI). The sum of dominant PCB congeners present in the blood (IUPAC # 99, 138, 149, 153, 180, and 187) was used in the statistical evaluation. All contaminant concentrations were normalized to the extractable lipid content.

4.3. Statistical analyses

For statistical analysis of non-normal distributed data, multiple Mann-Whitney U-tests were performed to determine any difference between groups. The relationships between values obtained by cytokine analyses, lymphocyte proliferation assay, and

Table 2
Primer sequences and annealing temperatures used for analysis of mRNA by real time PCR as well as specific melting point temperatures of the amplicon.

Gene	Primer	Sequence (5′-3′)	Accession No.	Annealing temperature	Melting point temperature
IL-2	forward	GCA CCT ACT TCA AGC TCT AC	AF 346296	58 °C	81.7 °C
	reverse	TAG CAC ATC CTC CAG AGG TT			
IL-4	forward	GCA TGT ACC AGC AAC TTC GT	AF 346295	57 °C	85.4 °C
	reverse	TTG GCT TCA TTC ACA GAA CAG			
IL-6	forward	GCA AGG AGG CAC TGG CAG AA	AF 346297	60 °C	85.5 °C
	reverse	CCT CAG GCT GAA CTG CAG GA			
IL-10	forward	CCT GGG TTG CCA AGC CCT GTC	AF 346294	58 °C	88.1 °C
	reverse	ATG CGC TCT TCA CCT GCT CC			
TNF-α	forward	CCA AGT GAC AAG CCA GTA GC	AF 346298	58 °C	89.2 °C
	reverse	TCT TGA TGG CAG AGA GTA GG			
TGF-β	forward	TTC CTG CTC CTC ATG GCC AC	AF 346299	66 °C	92.5 °C
	reverse	GCA GGA GCG CAC GAT CAT GT			
GAPDH	forward	GCC AAA AGG GTC ATC ATC TC	AF 346300	61 °C	89.8 °C
	reverse	GGG GCC ATC CAC AGT CTT CT			

Accession No. = GenbankTM/NCBI accession number; IL = interleukin; TNF = tumor necrosis factor; TGF = transforming growth factor; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Table 3
Summary of post mortem findings in stranded harbor porpoises.

Animal ID	Main pathological findings	Nutritional state	Thymus	Microbiology (infected tissue)
S1	• disseminated ulcerative dermatitis • multifocal necrotizing hepatitis	emaciated	severe atrophy	• β-hem. *E. coli* (liver, spleen)
S2	• disseminated suppurative dermatitis • multifocal suppurate bronchopneumonia	emaciated	severe atrophy	• Pseudomonas sp.(lung) • Streptococcus sp. (skin)
S3	• pulmonary atelectasis	normal	normal	• β-hem. *E. coli* (lung)
S4	• diffuse fibrino suppurative pneumonia • diffuse fibrinous pericarditis	emaciated	severe atrophy	• β-hem. *E.coli* (pleura, lung lymph node) • *Aspergillus sp.* (lung)

β-hem. *E. coli* = β-hemolytic *Escherichia coli*.

contaminant concentrations were expressed as the rank correlation coefficient of Spearman (r_s). A p-value of $\leq$0.05 was considered as statistically significant. Analyses were performed by SPSS for windows (SPSS Inc.). Graphs were created with GraphPad Prism® (GraphPad Software).

5. Results and discussion

5.1. Poor prognosis in stranded harbor porpoises is associated with generalized inflammatory disease

Free-ranging harbor porpoises (animals B1-B11) showed normal behavior and clinical signs upon examination and were immediately released into the wild after clinical examination and blood sampling. Animals in human care (animals H1-H9) showed no clinical signs and were clinically healthy after an observation period of at least 6 months after sampling. Stranded animals (animals S1-S4) exhibited poor body condition with stupor, indicating poor clinical prognosis and were euthanized due to animal welfare reasons and submitted to necropsy.

The main post mortem findings and microbiology results for stranded porpoises are summarized in Table 3. Animals S1, S2, and S4 showed severe inflammatory lesions in skin, lung, pericardium, and liver associated with bacterial infection. In addition mycotic infection was found in harbor porpoise S4. These three porpoises also exhibited poor nutritional status and severe thymic atrophy, indicating chronic disease processes. By contrast, the neonatal animal S3 exhibited pulmonary bacterial growth without overt inflammatory lesions and an unaffected nutritional state, suggestive of acute neonatal infection.

Lymphoid atrophy, either primary or secondary, is commonly observed in harbor porpoises suffering from prolonged infectious diseases and poor health status (Beineke et al., 2007b). Thymic atrophy occurs frequently in human patients with systemic bacterial infection, and this has also been observed in various rodent models of infectious diseases. Malnutrition also causes depletion of lymphoid organs in human beings and animals, which leads to disturbed immune responses and increased susceptibility to additional infections (Alwarawrah et al., 2018; Savino and Dardenne, 2010).

5.2. Reduced lymphocyte responsiveness indicates poor prognosis in diseased harbor porpoises

ConA and PWM have been used to stimulate proliferation of cultured lymphocytes in a variety of marine mammals, including

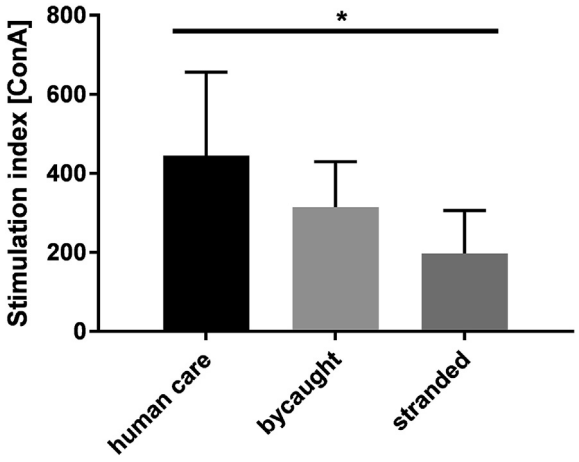

Fig. 1. Lymphocyte stimulation assay: concanavalin A (ConA)-induced lymphocyte proliferation in harbor porpoises. Column bars display median and maximum values. * = significant difference p $\leq$ 0.05.

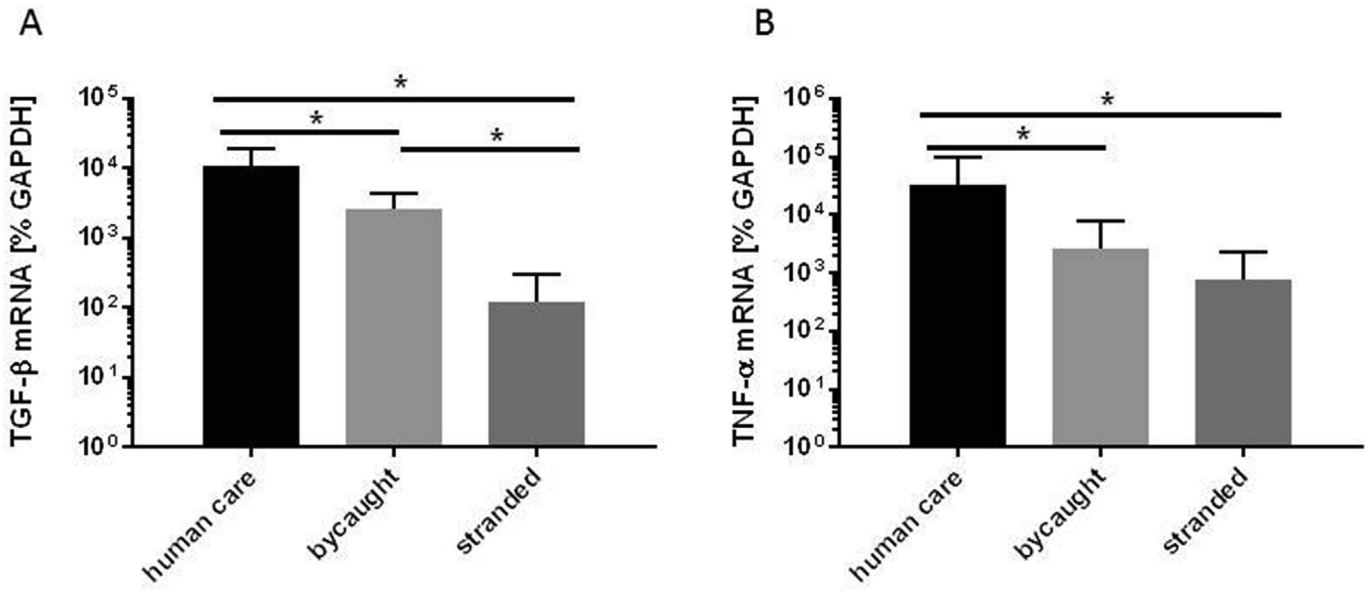

Fig. 2. Cytokine mRNA expression analyses of harbor porpoises blood samples: (A) transforming growth factor-β (TGF-β). (B) tumor necrosis factor-α (TNF-α). Column bars display median and maximum values. * = significant difference $p \leq 0.05$.

harbor porpoises (Beineke et al., 2010; De Guise et al., 1996; de Swart et al., 1993). In order to investigate lymphocyte responses in more detail, lymphocyte stimulation tests were performed, complemented by cytokine expression analyses. Mitogen-induced lymphocyte proliferation was significantly reduced in diseased stranded harbor porpoises. ConA-stimulated PBMC of healthy harbor porpoises in human care showed a significantly higher stimulation index (SI) than in stranded porpoises ($p = 0.045$; Fig. 1). Interestingly, bycaught porpoises showed no significant differences in lymphocyte proliferation towards animals in human care and stranded porpoises, respectively. Since only limited clinical and laboratory data could be obtained from bycaught whales before their release into wildlife, the reason for intermediate SI values in this group remains speculative. An increased infection pressure and reduced food supply in free-ranging porpoises compared to animals in human care have to be considered (Müller et al., 2013; Siebert et al., 2001). Moreover, elevated blood stress hormone levels (e.g. cortisol) during bycaught and handling have the ability to reduce lymphocyte proliferative capacities (De Guise et al., 1996; Noda et al., 2007). For PWM-stimulated lymphocytes no significant differences between the harbor porpoise groups were observed. Our data reveal reduced lymphocyte proliferation in severely diseased harbor porpoises when stimulated by the T cell mitogen Con A.

Cytokine gene expression analyses have been used to assess immune status and stress in cetaceans and pinnipeds (Beineke et al., 2007a; Fonfara et al., 2008; Müller et al., 2013) and to detect immune-relevant effects of xenobiotics in seals (Weirup et al., 2013; Lehnert et al., 2014, 2016). In the present study, TGF-β mRNA expression levels in whole blood were significantly higher in porpoises from human care compared to bycaught ($p = 0.004$) and stranded animals ($p = 0.005$; Fig. 2). In addition, there were significant differences between bycaught and stranded harbor porpoises, with bycaught animals showing significantly higher expression levels ($p = 0.004$; Fig. 2). Similarly, mRNA expression levels of TNF-α differed significantly between groups, with animals from human care showing significantly higher expression levels than bycaught ($p = 0.025$) and stranded harbor porpoises ($p = 0.030$; Fig. 2). According to the proposed mechanisms for reduced lymphocyte proliferative capacity, stress-induced

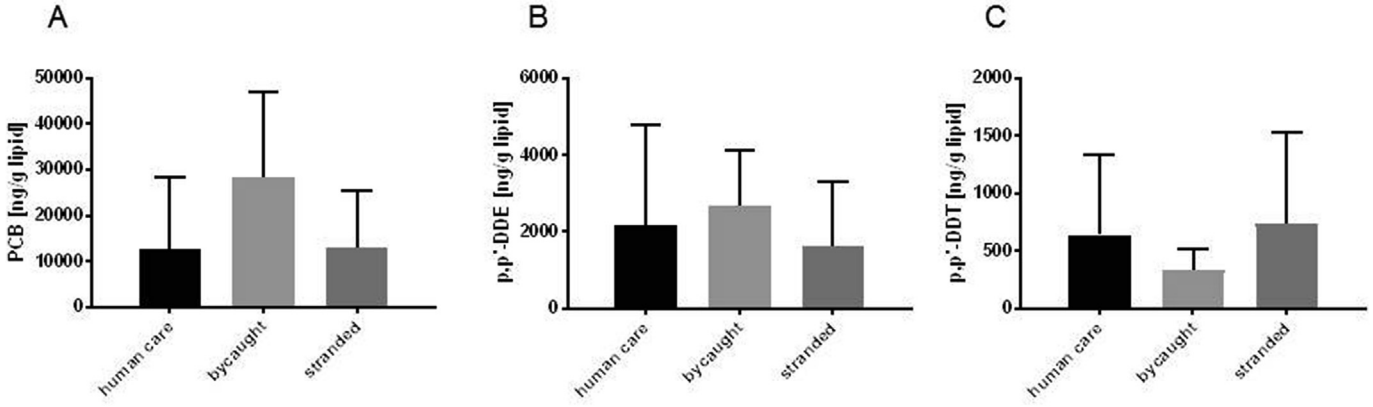

Fig. 3. Toxicological analyses of harbor porpoise blood samples: (A) polychlorinated biphenyls (PCB, sum of congeners 99, 138, 149, 153, 180, and 187); (B) p,p'-dichlorodiphenyldichloroethylene (DDE); (C) p,p'-dichlorodiphenyltrichloroethane (DDT). Column bars display median and maximum values. * = significant difference $p \leq 0.05$.

Table 4
Rank correlation analyses between contaminant levels in the blood of investigated harbor porpoises, mitogen-induced lymphocyte proliferation, and blood cytokine mRNA levels.

	PCB		*p,p'*-DDE		*p,p'*-DDT		ConA		PWM		IL-2		IL-4		IL-6		IL-10		TGF-β		TNF-α	
	p-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s
PCB	–	–	**0.001**	**0.811**	**0.013**	**0.586**	0.926	−0.025	0.567	0.150	0.104	0.408	0.159	−0.357	0.453	0.195	0.196	0.330	0.985	0.005	0.140	−0.373
p,p'-DDE	**0.001**	**0.811**	–	–	**0.001**	**0.720**	0.704	-0–099	0.848	0.050	0.846	−0.051	0.104	−0.408	0.606	0.135	0.289	0.273	0.544	0.158	0.094	−0.419
p,p'-DDT	**0.013**	**0.586**	**0.001**	**0.720**	–	–	0.397	−0.220	0.704	−0.099	0.846	−0.051	0.104	−0.408	0.204	0.301	0.422	0.209	0.666	−0.113	0.700	−0.101

PCB = polychlorinated biphenyls; DDE = dichlorodiphenyldichloroethylene; DDT dichlorodiphenyltrichloroethane; ConA = concanavalin A, PWM = pokeweed mitogen; IL = interleukin, TGF = transforming growth factor, TNF = tumor necrosis factor; r_s = rank correlation coefficient of Spearman; bold values display significant correlation.

suppression of cellular immunity as well as infectious or nutritional causes might also have led to diminished cytokine expression observed in bycaught whales. Correlation analyses between lymphocyte proliferation assay data and cytokine mRNA levels revealed that both TGF-β ($p = 0.028$; $r_s = 0.448$) and TNF-α levels ($p = 0.038$; $rs = 0.425$) significantly correlate with ConA-induced lymphocyte proliferation (Supplemental Fig. S1). Blood mRNA levels of other investigated cytokines (IL-2, IL-4, IL-6, IL-10) did not differ significantly between groups and did not correlate significantly with mitogen-induced lymphocyte proliferation values.

Fonfara et al. (2007b) observed changes in TGF-β and TNF-α mRNA expression in two harbor porpoises in human care and related the findings to an activated immune system. Blood cytokine expression in infectious diseases is a dynamic process, influenced by a complex interplay between different inflammatory mediators. Initially, acute infection leads to elevated blood levels of acute phase proteins and pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α (Wang et al., 2018). Short half-life of cytokines with rapid disappearance from the blood and elevated levels of cytokine inhibitors, such as soluble TNF receptors, account for cytokine kinetics during the disease course (Cabioglu et al., 2002; Ertel et al., 1995; Goldie et al., 1995). Reduced expression of both pro- and anti-inflammatory cytokines can be observed in human patients with fatal systemic infectious diseases (Cabioglu et al., 2002). Septic patients develop severe immune alteration for which the intensity and duration correlate with increased risk of nosocomial infections. Septic patients undergo a transition from systemic inflammatory response syndrome (SIRS) to compensatory anti-inflammatory response syndrome (CARS), which limit tissue damage but attenuate robust immune responses. Consequently disturbed lymphocyte function potentially aggravates bacteria overgrowth and increases the risk of sepsis mortality (Venet et al., 2012). Similarly, impaired T cell proliferation and effector function has been observed in a mouse model of severe sepsis (Carson et al., 2010). Chronic debilitating disease, as observed in animals S1, S2, and S4, can induce immunosuppression due to adrenal glucocorticoid secretion. Moreover chronic antigen exposure leads to disturbed T cell effector functions and impaired cytokine production, including TNF-α expression, by up-regulation of inhibitory molecules (e.g. programmed cell death protein-1). Exhausted immune cells also show a diminished proliferative ability and a failure of memory T cell responsiveness (Agnellini et al., 2007; Saeidi et al., 2018). Moreover, severe malnutrition and starvation is associated with diminished lymphocyte survival, T cell proliferation and cytokine responses in human patients and animal models (Alwarawrah et al., 2018; Cohen et al., 2017). Interpretation of cytokine expression and lymphocyte proliferation data together with additional immune parameters, such as acute phase proteins (e.g. C-reactive protein, haptoglobin), and blood hormone levels (e.g. cortisol, thyroid hormones) will give further insights in leukocyte interaction in free ranging harbor porpoise (Müller et al., 2013; Schnitzler et al., 2008). Moreover, development of novel molecular techniques, such as microarray-based gene expression analyses, will help to get a more holistic view on leukocyte function in marine mammals in future studies (Mancia et al., 2007; Raddatz et al., 2017).

5.3. Environmental contaminants accumulate in the blood of harbor porpoises but do not directly influence lymphocyte proliferation and cytokine expression

To determine whether the selected immune parameters are directly influenced by environmental contaminants, toxicological analyses of blood samples were performed. In this study PCB concentrations were highest in the investigated harbor porpoise blood samples, followed by DDE and DDT concentrations. In a previous

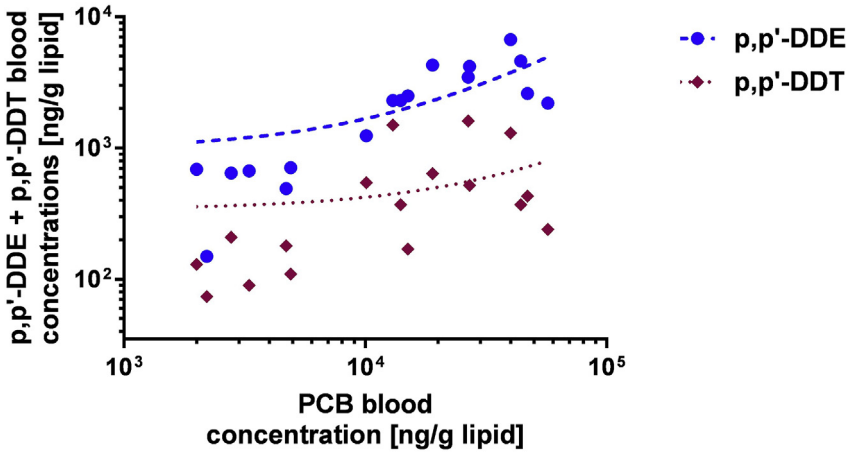

Fig. 4. Correlation between polychlorinated biphenyl (PCB), p,p'-dichlorodiphenyldichloroethylene (DDE), and p,p'-dichlorodiphenyltrichloroethane (DDT) concentrations in the blood of harbor porpoises.

study in harbor porpoise serum, the levels of sum PCBs were higher than sum DDTs followed by sum PBDEs (Weijs et al., 2009a). Toxicological analyses showed that harbor porpoises had contaminant loads in blood comparable to those from other studies around the North Sea and adjacent waters (Weijs et al., 2009a,b). The highest PCB levels in blood were found in bycaught harbor porpoises. However, none of the differences in levels between the different groups were statistically significant (Table 1; Fig. 3).

The harbor porpoises' high trophic feeding level and their limited capacity to metabolize DDE and some PCB congeners leads to a bioaccumulation of these contaminants (Houde et al., 2006; Weijs et al., 2009c). Accordingly, all measured contaminants were positively correlated, indicating co-accumulation of investigated xenobiotics (Table 4; Fig. 4).

Persistent organic pollutants (e.g. PCB) are associated with alterations of both the innate and adaptive immune system in marine mammals (Jepson et al., 2005), and are suspected to negatively affect health status in cetaceans (Ross, 2002; Weijs & Zaccaroni, 2016). Inhibited cellular immune response in free-ranging bottlenose dolphins (*Tursiops truncatus*) was associated with increased PCB and DDT levels (Lahvis et al., 1995) and PCB exposure has been shown to inhibit lymphocyte proliferation in harbor and grey seals (Mos et al., 2006; Dufresne et al., 2010). However, no significant correlations between toxicant levels (PCB, DDE, DDT) and stimulation indices (ConA, PWM) were observed in the present study. Similarly, in ringed seals from east Greenland no significant correlations were found between lymphocyte proliferation and any blood or blubber contaminant measured (Levin et al., 2016). While the analyzed/selected compounds showed no correlation with lymphocyte proliferation and gene transcription, this does not preclude that the contaminant mixture present in blood samples of the porpoises had deleterious health effects. Because new chemical compounds enter the global market constantly (Weijs and Zaccaroni, 2016), compounds that were not quantified in this study may be responsible for immune effects observed. Moreover, many toxicological effects of PCBs and other organochlorines have been attributed to their biologically reactive metabolites (Montaño et al., 2013), which were likely present and not identified here. For instance, halogenated phenolic compounds with a resemblance to thyroid hormones can reach high levels, especially in mammal blood, and have caused hormonal and neuronal effects in polar bears (Gutleb et al., 2010). Previous studies in cetaceans found several compounds not reported in this study, among others (methoxylated) polybrominated diphenyl ethers (PBDEs) and polychlorinated naphthalenes (Ross, 2006; Rayne et al., 2004). Methylmercury and heavy metal exposure are also suspected to negatively influence the immune system, thereby increasing disease susceptibility in harbor porpoises (Jepson et al., 1999; Siebert et al., 1999).

Cytokine transcription levels were not correlated with contaminant concentrations in blood, indicating that the immune endpoints measured in this study are not directly linked to exposure of these xenobiotics. While the cytokine biomarkers TNFα and TGFβ selected here are good indicators of inflammatory actions and infectious disease, their applicability for detecting direct effects of contaminant exposure at the molecular level may be limited. Recently adapted markers analyzing e.g. aryl hydrocarbon or thyroid hormone α receptor transcription (Lehnert et al., 2010) in marine mammals or transcriptomics studies to identify genes of interest modulated by pollutant burdens (Brown et al., 2017) are potential choices for future research. Critically, it has to be considered that confounding factors such as age, sex, different origins of wild animals, and impaired immune function due to infectious disease can obscure potential immunotoxic effects due to the small number of available animals. Moreover, capture and handling are stress factors which can impact innate and adaptive immune responses of free-ranging harbor porpoises (Müller et al., 2013). Also noise from shipping or offshore construction, and prey depletion through fisheries may have had additional debilitating and synergistic effects on the physiology of free-ranging porpoises causing immune suppression (Halvorsen et al., 2008). Multiple environmental stressors have been shown to have cumulative effects on lymphocyte proliferation in manatees (Walsh et al., 2005). In addition, dose-dependent effects and the duration of pollutant exposure is supposed to account for discrepancies among different experimental settings, which hinder definitive conclusions about immunotoxic effects in the present study (Brown et al., 2014; Daniel et al., 2001; Devos et al., 2004; Neale et al., 2005; Routti et al., 2010). Since standardized test conditions are often difficult to realize in field studies, further study of a larger animal cohort is clearly needed to confirm the observed lack of effect of contaminant concentrations on lymphocyte responsiveness and to assess possible indirect effects of environmental contaminants.

6. Conclusion

This is the first study investigating lymphocyte proliferation and blood cytokine expression in free-ranging harbor porpoises. Results

reveal an impaired function of peripheral blood leukocytes in severely diseased harbor porpoise, indicating immune exhaustion and increased disease susceptibility which might render the animals more susceptible to infectious diseases. Given the difficulties in assessing the health status and clinical prognosis of free ranging cetaceans, lymphocyte proliferation assays together with cytokine expression analyses can be used as adjuvant tools to determine immune function in wild harbor porpoises. The development of novel approaches in marine mammal immunology will help to objectify and better understand anthropogenic impacts upon cetaceans in the future.

Acknowledgements

This work was conducted and funded within the framework of the Environmental Research Plan of the German Ministry of Environment (Umweltbundesamt; F+E-Vorhaben 29965 221/0).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.01.079.

References

Agnellini, P., Wolint, P., Rehr, M., Cahenzli, J., Karrer, U., Oxenius, A., 2007. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. Proc. Natl. Acad. Sci. U.S.A. 104, 4565–4570.

Aguilar, A., Borrell, A., 2005. DDT and PCB reduction in the western Mediterranean from 1987 to 2002, as shown by levels in striped dolphins (*Stenella coeruleoalba*). Mar. Environ. Res. 59, 391–404.

Alwarawrah, Y., Kiernan, K., MacIver, N.J., 2018. Changes in nutritional status impact immune cell metabolism and function. Front. Immunol. 16 (9), 1055.

Beineke, A., Siebert, U., Van Elk, N., Baumgärtner, W., 2004. Development of a lymphocyte-transformation-assay for peripheral blood lymphocytes of the harbor porpoise and detection of cytokines using the reverse-transcription polymerase chain reaction. Vet. Immunol. Immunopathol. 98 (1), 59–68.

Beineke, A., Siebert, U., Müller, G., Baumgärtner, W., 2007a. Increased blood interleukin-10 mRNA levels in diseased free-ranging harbor porpoises (*Phocoena phocoena*). Vet. Immunol. Immunopathol. 115, 100–106.

Beineke, A., Siebert, U., Stott, J., Müller, G., Baumgärtner, W., 2007b. Phenotypical characterization of changes in thymus and spleen associated with lymphoid depletion in free-ranging harbor porpoises (*Phocoena phocoena*). Vet. Immunol. Immunopathol. 117 (3), 254–265.

Beineke, A., Siebert, U., Wohlsein, P., Baumgärtner, W., 2010. Immunology of whales and dolphins. Vet. Immunol. Immunopathol. 133 (2), 81–94.

Bilrha, H., Roy, R., Moreau, B., Belles-Isles, M., Dewailly, E., Ayotte, P., 2003. In vitro activation of cord blood mononuclear cells and cytokine production in a remote coastal population exposed to organochlorines and methyl mercury. Environ. Health Perspect. 111 (16), 1952–1957.

Brown, T.M., Ross, P.S., Reimer, K.J., Veldhoen, N., Dangerfield, N.J., Fisk, A.T., Helbing, C.C., 2014. PCB related effects thresholds as derived through gene transcript profiles in locally contaminated ringed seals (*Pusa hispida*). Environ. Sci. Technol. 48, 12952–12961.

Brown, T.M., Hammond, S.A., Behsaz, B., Veldhoen, N., Birol, I., Helbing, C.C., 2017. De novo assembly of the ringed seal (*Pusa hispida*) blubber transcriptome: a tool that enables identification of molecular health indicators associated with PCB exposure. Aquat. Toxicol. 185, 48–57.

Cabioglu, N., Bilgic, S., Deniz, G., Aktas, E., Seyhun, Y., Turna, A., et al., 2002. Decreased cytokine expression in peripheral blood leukocytes of patients with severe sepsis. Arch. Surg. 137 (9), 1037–1043.

Carson, W.F., Cavassani, K.A., Ito, T., Schaller, M., Ishii, M., Dou, Y., Kunkel, S.L., 2010. Impaired CD4+ T-cell proliferation and effector function correlates with repressive histone methylation events in a mouse model of severe sepsis. Eur. J. Immunol. 40 (4), 998–1010.

Cigliano, L., Nebbia, C., Rychen, G., Feidt, C., Girolami, F., Rossetti, C., Spagnuolo, M.S., 2016. Evaluation of serum markers of blood redox homeostasis and inflammation in PCB naturally contaminated heifers undergoing decontamination. Sci. Total Environ. 542 (Pt A), 653–664.

Cohen, S., Danzaki, K., MacIver, N.J., 2017. Nutritional effects on T-cell immunometabolism. Eur. J. Immunol. 47 (2), 225–235.

Daniel, V., Huber, W., Bauer, K., Suesal, C., Conradt, C., Opelz, G., 2001. Associations of blood levels of PCB, HCHS, and HCB with numbers of lymphocyte subpopulations, in vitro lymphocyte response, plasma cytokine levels, and immunoglobulin autoantibodies. Environ. Health Perspect. 109, 173–178.

De Guise, S., Bernier, J., Dufresne, M.M., Martineau, D., Béland, P., Fournier, M., 1996. Immune functions in beluga whales (*Delphinapterus leucas*): evaluation of mitogen-induced blastic transformation of lymphocytes from peripheral blood, spleen and thymus. Vet. Immunol. Immunopathol. 50 (1–2), 117–126.

de Swart, R.L., Kluten, R.M., Huizing, C.J., Vedder, L.J., Reijnders, P.J., Visser, I.K., et al., 1993. Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). Vet. Immunol. Immunopathol. 37 (3–4), 217–230.

de Swart, R., Ross, P., Vedder, L., Timmerman, H., Heisterkamp, S., Van Loveren, H., et al., 1994. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. Ambio 23 (2), 155–159.

Desforges, J.P.W., Sonne, C., Levin, M., Siebert, U., De Guise, S., Dietz, R., 2016. Immunotoxic effects of environmental pollutants in marine mammals. Environ. Int. 86, 126–139.

Desforges, J.P., Hall, A., McConnell, B., Rosing-Asvid, A., Barber, J.L., Brownlow, A., et al., 2018. Predicting global killer whale population collapse from PCB pollution. Science 361 (6409), 1373–1376.

Devos, S., Van Den Heuvel, R., Hooghe, R., Hooghe-Peters, E.L., 2004. Limited effect of selected organic pollutants on cytokine production by peripheral blood leukocytes. Eur. Cytokine Netw. 15, 145–151.

Dufresne, M.M., Frouin, H., Pillet, S., Lesage, V., De Guise, S., Fournier, M., 2010. Comparative sensitivity of harbour and grey seals to several environmental contaminants using in vitro exposure. Mar. Pollut. Bull. 60 (3), 344–349.

Ertel, W., Kremer, J.P., Kenney, J., Steckholzer, U., Jarrar, D., Trentz, O., Schildberg, F.W., 1995. Downregulation of proinflammatory cytokine release in whole blood from septic patients. Blood 85, 1341–1347.

Fonfara, S., Siebert, U., Prange, A., Colijn, F., 2007a. The impact of stress on cytokine and haptoglobin mRNA expression in blood samples from harbour porpoises (*Phocoena phocoena*). J. Mar. Biol. Assoc. U. K. 87 (01), 305–311.

Fonfara, S., Siebert, U., Prange, A., 2007b. Cytokines and acute phase proteins as markers for infection in harbor porpoises (*Phocoena phocoena*). Mar. Mamm. Sci. 23 (4), 931–942.

Fonfara, S., Kakuschke, A., Rosenberger, T., Siebert, U., Prange, A., 2008. Cytokine and acute phase protein expression in blood samples of harbour seal pups. Mar. Biol. 155 (3), 337–345.

Fournier, M., Cyr, D., Blakley, B., Boermans, H., Brousseau, P., 2000. Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics 1. Am. Zool. 40 (3), 412–420.

Gilles, A., Scheidat, M., Siebert, U., 2009. Seasonal distribution of harbour porpoises and possible interference of offshore wind farms in the German North Sea. Mar. Ecol. Prog. Ser. 383, 295–307.

Goldie, A.S., Fearon, K.C., Ross, J.A., Barclay, G.R., Jackson, R.E., Grant, I.S., Ramsay, G., Blyth, A.S., Howie, J.C., 1995. Natural cytokine antagonists and endogenous antiendotoxin core antibodies in sepsis syndrome. The Sepsis Intervention Group. J. Am. Med. Assoc. 274, 172–177.

Gutleb, A.C., Cenijn, P., Velzen, M.V., Lie, E., Ropstad, E., Skaare, J.U., Malmberg, T., Bergman, Å., Gabrielsen, G.W., Legler, J., 2010. In vitro assay shows that PCB metabolites completely saturate thyroid hormone transport capacity in blood of wild polar bears (*Ursus maritimus*). Environ. Sci. Technol. 44 (8), 3149–3154.

Hall, A.J., Hugunin, K., Deaville, R., Law, R.J., Allchin, C.R., Jepson, P.D., 2006. The risk of infection from polychlorinated biphenyl exposure in the harbour porpoise (*Phocoena phocoena*): a case-control approach. Environ. Health Perspect. 114, 704–722.

Halvorsen, K.M., Keith, E.O., 2008. Immunosuppression cascade in the Florida manatee (Trichechus manatus latirostris). Aquat. Mamm. 34 (4), 412–419.

Houde, M., Bujas, T.A., Small, J., Wells, R.S., Fair, P.A., Bossart, G.D., et al., 2006. Biomagnification of perfluoroalkyl compounds in the bottlenose dolphin (*Tursiops truncatus*) food web. Environ. Sci. Technol. 40 (13), 4138–4144.

Imbeault, P., Findlay, C.S., Robidoux, M.A., Haman, F., Blais, J.M., Tremblay, A., et al., 2012. Dysregulation of cytokine response in Canadian first nations communities: is there an association with persistent organic pollutant levels? PLoS One 7 (7), e39931.

Jepson, P.D., Law, R.J., 2016. Persistent pollutants, persistent threats. Science 352 (6292), 1388–1389.

Jepson, P.D., Bennett, P.M., Allchin, C.R., Law, R.J., Kuiken, T., Baker, J.R., et al., 1999. Investigating potential associations between chronic exposure to polychlorinated biphenyls and infectious disease mortality in harbour porpoises from England and Wales. Sci. Total Environ. 243, 339–348.

Jepson, P.D., Bennett, P.M., Deaville, R., Allchin, C.R., Baker, J.R., Law, R.J., 2005. Relationships between PCBs and health status in harbour porpoises (*Phocoena phocoena*) stranded in the United Kingdom. Environ. Toxicol. Chem. 24, 238–248.

Jepson, P.D., Deaville, R., Barber, J.L., Aguilar, À., Borrell, A., Murphy, S., et al., 2016. PCB pollution continues to impact populations of orcas and other dolphins in European waters. Sci. Rep. 6, 18573.

Kannan, K., Blakenship, A.L., Jones, P.D., Giesy, J.P., 2000. Toxicity reference values for the toxic effects of polychlorinated biphenyls to aquatic mammals. Hum. Ecol. Risk Assess. 6, 181–201.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L., Via, C.S., 1995. Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. Environ. Health Perspect. 103 (Suppl. 4), 67.

Law, R.J., Barry, J., Barber, J.L., Bersuder, P., Deaville, R., Reid, R.J., et al., 2012. Contaminants in cetaceans from UK waters: status as assessed within the cetacean strandings investigation programme from 1990 to 2008. Mar. Pollut. Bull. 64 (7), 1485–1494.

Lehnert, K., von Samson-Himmelstjerna, G., Schaudien, D., Bleidorn, C., Wohlsein, P., Siebert, U., 2010. Transmission of lungworms of harbour porpoises and harbour seals: molecular tools determine potential vertebrate intermediate hosts. Int. J. Parasitol. 40 (7), 845–853.

Lehnert, K., Müller, S., Weirup, L., Ronnenberg, K., Pawliczka, I., Rosenberger, T., Siebert, U., 2014. Molecular biomarkers in grey seals (*Halichoerus grypus*) to evaluate pollutant exposure, health and immune status. Mar. Pollut. Bull. 88 (1), 311–318.

Lehnert, K., Ronnenberg, K., Weijs, L., Covaci, A., Das, K., Hellwig, V., Siebert, U., 2016. Xenobiotic and immune-relevant molecular biomarkers in harbor seals as proxies for pollutant burden and effects. Arch. Environ. Contam. Toxicol. 70 (1), 106–120.

Lehnert, K., Desforges, J.P., Das, K., Siebert, U., 2018. Ecotoxicological biomarkers and accumulation of contaminants in pinnipeds. In: Marine Mammal Ecotoxicology. Academic Press, pp. 261–289.

Levin, M., Gebhard, E., Jasperse, L., Desforges, J.P., Dietz, R., Sonne, C., et al., 2016. Immunomodulatory effects of exposure to polychlorinated biphenyls and perfluoroalkyl acids in East Greenland ringed seals (*Pusa hispida*). Environ. Res. 151, 244–250.

Mancia, A., Lundqvist, M.L., Romano, T.A., Peden-Adams, M.M., Fair, P.A., Kindy, M.S., Ellis, B.C., Gattoni-Celli, S., McKillen, D.J., Trent, H.F., Chen, Y.A., Almeida, J.S., Gross, P.S., Chapman, R.W., Warr, G.W., 2007. A dolphin peripheral blood leukocyte cDNA microarray for studies of immune function and stress reactions. Dev. Comp. Immunol. 31, 520–529.

Montaño, M., Gutleb, A.C., Murk, A.J., 2013. Persistent toxic burdens of halogenated phenolic compounds in humans and wildlife. Environ. Sci. Technol. 47 (12), 6071–6081.

Mos, L., Morsey, B., Jeffries, S.J., Yunker, M.B., Raverty, S., Guise, S.D., Ross, P.S., 2006. Chemical and biological pollution contribute to the immunological profiles of free-ranging harbor seals. Environ. Toxicol. Chem. 25 (12), 3110–3117.

Müller, S., Lehnert, K., Seibel, H., Driver, J., Ronnenberg, K., Teilmann, J., et al., 2013. Evaluation of immune and stress status in harbour porpoises (*Phocoena phocoena*): can hormones and mRNA expression levels serve as indicators to assess stress? BMC Vet. Res. 9 (1), 145.

Neale, J.C., Kenny, T.P., Tjeerdema, R.S., Gershwin, M.E., 2005. PAH- and PCB-induced alterations of protein tyrosine kinase and cytokine gene transcription in harbor seal (Phoca vitulina) PBMC. Clin. Dev. Immunol. 12, 91–97.

Noda, K., Akiyoshi, H., Aoki, M., Shimada, T., Ohashi, F., 2007. Relationship between transportation stress and polymorphonuclear cell functions of bottlenose dolphins, Tursiops truncatus. J. Vet. Med. Sci. 69, 379–383.

Raddatz, B.B., Spitzbarth, I., Matheis, K.A., Kalkuhl, A., Deschl, U., Baumgärtner, W., Ulrich, R., 2017. Microarray-based gene expression analysis for veterinary pathologists: a review. Vet. Pathol. 54, 734–755.

Rayne, S., Ikonomou, M.G., Ross, P.S., Ellis, G.M., Barrett- Lennard, L.G., 2004. PBDEs, PBBs, and PCNs in three communities of freeranging killer whales (*Orcinus orca*) from the northeastern Pacific Ocean. Environ. Sci. Technol. 38, 4293–4299.

Ross, P.S., 2002. The role of immunotoxic environmental contaminants in facilitating the emergence of infectious diseases in marine mammals. Hum. Ecol. Risk Assess. 8 (2), 277–292.

Ross, P.S., 2006. Fireproof killer whales (*Orcinus orca*): flame-retardant chemicals and the conservation imperative in the charismatic icon of British Columbia, Canada. Can. J. Fish. Aquat. Sci. 63 (1), 224–234.

Ross, P.S., De Swart, R.L., Reijnders, P.J., Van Loveren, H., Vos, J.G., Osterhaus, A.D., 1995. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals fed herring from the Baltic Sea. Environ. Health Perspect. 103 (2), 162.

Routti, H., Arukwe, A., Jenssen, B.M., Letcher, R.J., Nyman, M., Bäckman, C., Gabrielsen, G.W., 2010. Comparative endocrine disruptive effects of contaminants in ringed seals (*Phoca hispida*) from Svalbard and the Baltic Sea. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 152, 306–312.

Saeidi, A., Zandi, K., Cheok, Y.Y., Saeidi, H., Wong, W.F., Lee, C.Y.Q., Cheong, H.C., Yong, Y.K., Larsson, M., Shankar, E.M., 2018. T-cell exhaustion in chronic infections: reversing the state of exhaustion and reinvigorating optimal protective immune responses. Front. Immunol. 9, 2569.

Savino, W., Dardenne, M., 2010. Nutritional imbalances and infections affect the thymus: consequences on T-cell-mediated immune responses. Proc. Nutr. Soc. 69 (4), 636–643.

Scheidat, M., Gilles, A., Kock, K.H., Siebert, U., 2008. Harbour porpoise *Phocoena phocoena* abundance in the southwestern Baltic Sea. Endanger. Species Res. 5 (2–3), 215–223.

Schnitzler, J.G., Siebert, U., Jepson, P.D., Beineke, A., Jauniaux, T., Bouquegneau, J.M., Das, K., 2008. Harbor porpoise thyroids: histologic investigations and potential interactions with environmental factors. J. Wildl. Dis. 44, 888–901.

Schwacke, L.H., Zolman, E.S., Balmer, B.C., De Guise, S., George, R.C., Hoguet, J., et al., 2011. Anaemia, hypothyroidism and immune suppression associated with polychlorinated biphenyl exposure in bottlenose dolphins (*Tursiops truncatus*). Proc. R. Soc. Lond. B Biol. Sci. rspb20110665.

Siebert, U., Joiris, C., Holsbeek, L., Benke, H., Failing, K., Frese, K., Petzinger, E., 1999. Potential relation between mercury concentrations and necropsy findings in cetaceans from German waters of the North and Baltic Seas. Mar. Pollut. Bull. 38, 285–295.

Siebert, U., Wünschmann, A., Weiss, R., Frank, H., Benke, H., Frese, K., 2001. Post-mortem findings in harbour porpoises (*Phocoena phocoena*) from the German North and baltic Seas. J. Comp. Pathol. 124, 102–114.

Sormo, E.G., JU, Skaare, Jussi, I., Jussi, M., Jenssen, B.M., 2003. Polychlorinated biphenyls and organochlorine pesticides in Baltic and Atlantic gray seal (*Halichoerus grypus*) pups. Environ. Toxicol. Chem. 22, 2789–2799.

Venet, F., Foray, A.P., Villars-Méchin, A., Malcus, C., Poitevin-Later, F., Lepape, A., Monneret, G., 2012. IL-7 restores lymphocyte functions in septic patients. J. Immunol. 189 (10), 5073–5081.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambre, C., Tarazona, J.V., et al., 2000. Health effects of endocrine disrupting chemicals on wildlife, with special reference to the European situation. Crit. Rev. Toxicol. 30, 71–133.

Walsh, C.J., Luer, C.A., Noyes, D.R., 2005. Effects of environmental stressors on lymphocyte proliferation in Florida manatees, Trichechus manatus latirostris. Vet. Immunol. Immunopathol. 103 (3–4), 247–256.

Wang, L., Zhao, H., Wang, D., 2018. Inflammatory cytokine expression in patients with sepsis at an intensive care unit. Exp. Ther. Med. 16, 2126–2131.

Weijs, L., Zaccaroni, A., 2016. Toxicology of marine mammals: new developments and opportunities. Arch. Environ. Contam. Toxicol. 70 (1), 1–8.

Weijs, L., Dirtu, A.C., Das, K., Gheorghe, A., Reijnders, P.J., Neels, H., et al., 2009a. Inter-species differences for polychlorinated biphenyls and polybrominated diphenyl ethers in marine top predators from the Southern North Sea: Part 1. Accumulation patterns in harbour seals and harbour porpoises. Environ. Pollut. 157 (2), 437–444.

Weijs, L., Das, K., Siebert, U., van Elk, N., Jauniaux, T., Neels, H., et al., 2009b. Concentrations of chlorinated and brominated contaminants and their metabolites in serum of harbour seals and harbour porpoises. Environ. Int. 35 (6), 842–850.

Weijs, L., Losada, S., Das, K., Roosens, L., Reijnders, P.J., Santos, J.F., et al., 2009c. Biomagnification of naturally-produced methoxylated polybrominated diphenyl ethers (MeO-PBDEs) in harbour seals and harbour porpoises from the Southern North Sea. Environ. Int. 35 (6), 893–899.

Weijs, L., van Elk, C., Das, K., Blust, R., Covaci, A., 2010. Persistent organic pollutants and methoxylated PBDEs in harbour porpoises from the North Sea from 1990 until 2008: young wildlife at risk? Sci. Total Environ. 409, 228–237.

Weirup, L., Müller, S., Ronnenberg, K., Rosenberger, T., Siebert, U., Lehnert, K., 2013. Immune-relevant and new xenobiotic molecular biomarkers to assess anthropogenic stress in seals. Mar. Environ. Res. 92, 43–51.

Wünschmann, A., Siebert, U., Frese, K., Lockyer, C., Heide-Jørgensen, M.P., Müller, G., Baumgärtner, W., 2001. Evidence of infectious diseases in harbour porpoises (*Phocoena phocoena*) hunted in the waters of Greenland and by-caught in the German North Sea and Baltic Sea. Vet. Rec. 148, 715–720.